# CHARACTERIZATION OF HISTONE DEACETYLASE 4 AND HISTONE DEACETYLASE 5 IN COCAINE-RELATED BEHAVIORS

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences
The University of Texas Southwestern Medical Center at Dallas
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center
Dallas, Texas
May, 2016

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**Dedication and Acknowledgements**

I am in absolute gratitude for so much and to so many. I was never entirely sure, confident or capable that I would make it to this point in my career and if I have, it is only because of an amazing group of family, friends, mentors, co-workers, kind individuals and the unconditional love of my boys, Yoda and Bruce Wayne!

My biggest motivation, best friend and role model is my Mom, Paulina. To her, I dedicate every second of my scientific career and this work because she was the inspiration behind it all. I remember being a child playing in her lab decorating it with post-it notes and pretending to know what to do with a pipette. Those days were the beginning of an absolute love affair with biological research and I have her to credit for the inspiration and support to do research, love biology and pursue a career in this field. My rock, my strength and my brawn always resided in my brother, Remy. Thank you for being my safe-place and the voice of reason through the years. To my brother Bruno, thank you for the smiles, the travels and the love. To my Dad, I still strive to make you proud- and that is an amazing motivator when times are rough. Thank you to the rest of my family whom have been supportive of me, encouraged me to keep pursuing my aspirations, and showered me with love and hugs and so much yummy food every chance I got to go home. You all make life meaningful and wonderful.

To my chosen family, the many friends I have made throughout the years and as a consequence of pursuing this degreet I could not have survived without your support and friendship. I am especially grateful to Dr. Melissa Bawa, Dr. Dhruba Deb and Dr. Chi-Lun Chang for being the kind of friend that breaks the mold and reminds you that kindness and a friend-in-crime is all one really needs to make it through the bad days of horrible experiments and bad data. I would also like to acknowledge Dr. Erin Larson and Dr. Dan Guzman. These “Selfians” taught me every bit of self-administration knowledge they could and in the process we built an amazing friendship. I would also like to thank my friends outside of grad school as they have made my life in the US an absolute fun ride. To Lindsey, Michelle, Karla, Ben and a few others I am sure I am forgetting to mention: Thank you for the awesomeness you are and bring to my life. I keep you close and look forward to the next time.

I am confident the people I have shared the most adult life with are the super talented squad of intellectuals known as the "Cowanites" (former and present). I still think I've tricked you all into welcoming me into the think tank that is our lab! And although I am grateful that you've let me pretend to be one of you, super smarty-pants club members, I am most appreciative of the amazing human quality you all have. I want to thank you for every time you've held my hand- figuratively and/or literally, completed my experiments, explained an assay, shared your ice or your buffers, setup my transfers, checked on my animals, brought sweets, surprised me with chocolate and empathized with my (many) tears. The hardest part about moving on is realizing that I leave you and a part of me will always hold on to our journal clubs and happy hours (Harpoon?). You are the most talented, scientifically-savvy individuals I have ever had the privilege of working with and I can't wait to see you all advance your careers and become even better versions of yourselves. I hope life and my own professional growth allow me to continue to grow with you so that we may always collaborate! And hang-out! :)

I say this often but I cannot overemphasize how every bit of what I have done has been because of the support of those around me. To my technical mentor, Makoto Taniguchi, my thesis and graduate degree is your work. Everything I have generated has been the result of your patient training. I came into lab knowing how to run a pre-cast western blot and how to handle rats. And now, I can say I know a thing or two more than that and this is thanks to you. Makoto, developing projects together and having your support and encouragement has meant the world to me. I cannot thank you enough for taking me under your wing and helping me grow. You are officially allowed to move on but please know I will look forward to continuing to work together on any projects we may think to pursue!

I have been fortunate in acquiring an outstanding mentor for my doctoral work. Commander-in-chief, this work is all ultimately truly yours and I am honored you took me in and tried your best to teach me how to think, breathe and live science. I think I've turned out all right although I could use more of your contagious enthusiasm moving forward. Thank you Dr. Cowan for being more than a graduate mentor to me- you have truly stepped above and beyond to be a career mentor, a life mentor and an expert diffuser of panics and existential crises. I am equal parts embarrassed, proud and appreciative to admit that

you've seen me through my worst moments and helped me through them too. I hope I have succeeded in making you proud as a member of your lab and that we may remain collaborators through the years. I can tell you with certainty that I have so much more to learn from you.

I would like to thank my thesis committee, Dr. David Self, Dr. Adrian Rothenfluh and Dr. Kim Huber. I have to admit I have grown fond of data discussion with you in spite of my public speaking anxiety. You have all been incredibly helpful and willing to discuss and craft this project with my mentor and me. The development of a self-administration project would have not been feasible without the support of Dr. Self. More personally, thank you for being invested in my career beyond my thesis. Your input and advice is something I value and consider as I move forward.

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The University of Texas Southwestern Medical Center at Dallas, 2016

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In recent years a focus on epigenetic mechanisms as mediators of cocaine-related behavioral, structural and functional plasticity has developed. One family of epigenetic molecules that may underlie cocaine behavioral and functional changes is the histone deacetylase family that acts to mediate transcriptional repression. The class IIa subgroup of histone deacetylases is poised as an intracellular signal detector and effector by virtue of their ability to shuttle subcellularly in a dynamic and activity-dependent manner primarily

driven by phosphorylation status of the protein at multiple residues. The overlying goal of this thesis was to two-faceted: to characterize the regulation of two class IIa members, HDAC4 and HDAC5, by cocaine-mediated signaling and to characterize the role of HDAC4 and HDAC5 in cocaine-associated behavioral plasticity.

We report the regulation of phosphorylation and localization of HDAC4 and HDAC5 is in opposition. HDAC5 is dephosphorylated and accumulated in the nuclear compartment in response to cocaine, dopamine dependent signaling and cAMP activity. Meanwhile, we observe that HDAC4 is weakly dephosphorylated by cAMP activity in culture but weakly phosphorylated in vivo. These findings encouraged the analysis of function of these highly homologous class members. We assessed the function of HDAC4 and HDAC5 in the nucleus accumbens, a critical region for reward, by over-expressing wildtype and nuclear variants by targeted viral-mediated gene transfer. We report an attenuation of cocaine reward learning by nuclear HDAC5 but not wildtype or HDAC4 over-expression. We further analyzed the role of HDAC5 in self-administration behavior and report an effect of nuclear HDAC5, but not wildtype, on models of reinstatement of seeking, a preclinical model of relapse. These effects were observed in the absence of an effect on intake, sensitivity or motivation to self-administer. Because HDAC4 and HDAC5 bind nuclear transcriptional regulators to exert transcriptional repression of target genes we analyzed the dependence of nuclear HDAC5 on interacting with MEF2, a primary binding partners, and report that this interaction is likely required for modulating reinstatement of seeking but dispensable for cocaine reward. Taken together, these findings highlight the role of nuclear HDAC5 but not HDAC4 to limit cocaine reward and aspects of cocaine addiction-like behavior.

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**Prior Publications**

Taniguchi, M., **M. B. Carreira**, L. N. Smith, B. C. Zirlin, R. L. Neve and C. W. Cowan (2012). "Histone deacetylase 5 limits cocaine reward through cAMP-induced nuclear import." Neuron **73**(1): 108-120.

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**Abbreviations**

AAV- Adeno-associated virus

AC- adenylate cyclase

AC- anterior commisure

AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AP5- D-(-)-2-amino-5-phosphonopentanoic acid, NMDA antagonist

BCL6- B-cell CLL/lymphoma 6 co-repressor

BDNF- Brain Derived Neurotrophic Factor

$Ca^{2+}$- Calcium

CaMK- Calcium/calmodulin kinase

cAMP- Cyclic adenosine monophosphate

Cdk5- Cyclin dependent kinase 5

ChIP-seq- Chromatin Immuno-precipitation and massive parallel DNA sequencing

CPP- Conditioned place preference

CREB- cAMP response element-binding protein

CtBP- C-terminal binding protein

D1- Dopamine type 1

D1R- Dopamine type 1 receptor

D2- Dopamine type 2

D2R- Dopamine type 2 receptor

DA- Dopamine

DAT- Dopamine transporter

DNA- Deoxyribonucleic acid

DR- Dose Response

DS- Dorsal Striatum

eGFP- enhanced Green fluorescent protein

Egr- Early growth response protein

FR- Fixed ratio

GABA- Gamma-aminobutyric acid

GFP- Green fluorescent protein

$G_i$- G-protein coupled receptor to

GLT1- Glutamate transporter 1

$G_o$- G-protein coupled receptor to

H2A- Histone H2A

H2B- HIstone H2B

H3- Histone H3

H4- Histone H4

HAT- Histone acetyltransferase

HDAC- Histone deacetylase

HP1-Heterochromatin protein 2

HSV- Herpes simplex virus

ICSS- Intracranial self-stimulation

IVSA- Intravenous self-administration

KCl- Potassium chloride

KLF6- Kruppel-like factor 6

KO; Knockout

MBD- MEF2 binding domain

MEF2- Myocyte enhancer factor 2

mg/kg- milligram/kilogram

mGluR1- Metabotropic glutamate receptor 1

mGLUR2/3- Metabotropic glutamate receptor 2/3

N-CoR- Nuclear receptor co-repressor

NaBt- Sodium butyrate

NAc- Nucleus accumbens

NAC1- Nucleus accumbens-associated protein 1

NES- Nuclear Export Sequence

NFAT3- Nuclear factor of activated T-cells 3

NIDA- National Institute of Drug Abuse

NLS- Nuclear Localization Sequence

NMDA- N-methyl-D-aspartate

NMDAR- N-methyl-D-aspartate receptor

NRSF/REST- Neuron restrictive silencer factor/RE-1 silencing transcription factor

PhB- Phenylbutyrate

PKA- Protein kinase A

PKC- Protein kinase C

PKD- Protein kinase D

PP2A- Protein phosphatase 2A

PPARγ- peroxisome proliferator-activated receptor

PR- Progressive Ratio

PRK2- Protein kinase C-related kinase 2

RNA-seq- RNA-sequencing

ROS- Reactive Oxygen Species

SAHA- Suberoylanilide hydroxamic acid

SAMHSA- Substance Abuse and Mental Health Services Administration

SARE- Synaptic Activity Response Element

SIK1- Salt Inducible Kinase 1

SMRT

SRF- Serum response factor

THC-Tetrahydrocannabinol

TSA- Trichostatin A

UNODC- United Nations Office on Drugs and Crime

VS- Ventral Striatum

VTA- Ventral Tegmental Area

WTSS - Whole transcriptome shotgun sequencing

## Chapter 1: Introduction

### Cocaine abuse and preclinical models

Addiction and substance abuse are devastating, chronic conditions that result in a significant strain on the individual as well as on global healthcare costs. Estimates place the worldwide prevalence of cocaine use of up to 3% of the adult population (ages 18-64, UNODC, World Drug Report). The total costs of drug abuse and addiction in the United States due to use of tobacco, alcohol and illegal drugs are estimated at $524 billion a year. Of this staggering cost, Illicit drug use alone in the United Stated accounts for $181 billion in health care, productivity loss, crime, incarceration and drug enforcement (NIDA). Despite severe societal and health-related adverse consequences, however, addicted individuals continue to seek and take substances of abuse and only 10.4% of abusers receive treatment. Cocaine dependence accounts for about 11.3% of total admissions to publicly funded substance abuse treatment programs (2008, SAMHSA National Survey on Drug Use and Health). However, across the board relapse rates for drugs of abuse are estimated to occur in 40 to 60% of treated patients- a staggering reality that highlights the need for treatment development McLellan, Lewis et al. (2000). This observation highlights the importance of studying not only the effects of cocaine but models of seeking and relapse that may yield therapeutic strategies aimed at curbing relapse rates.

From a conceptual standpoint addiction can be dissected within a framework of stages that is based upon both clinical and preclinical observations of the disease. These stages include: 1) the impulse/drive to seek and take cocaine, often referred to as the binge and intoxication phase, 2) the development of a negative affective state, and 3) a

preoccupation/anticipation/craving of drugs (Koob and Volkow 2010). These stages represent the complex interaction between the impulsive nature of substance abuse and the compulsive drive hypothesized to underlie the cyclical, relapsing nature of this disease (Koob 1992, Koob 2009). One prominent theory for the development of addiction involves the idea that a shift occurs from positive reinforcement associated with drug use to negative reinforcement driving motivated behavior to seek and take drug. The impulse/drive to seek and take drugs – coined as the binge and intoxication phase by Koob and Volkow (Koob and Volkow 2010) – is heavily dependent on the ability of drugs of abuse to activate signaling in the mesolimbic dopamine system (Koob 1992, Koob 1992, Nestler and Malenka 2004). The rewarding nature of drugs of abuse is hypothesized to drive the intake by positive reinforcement. The initial ability of drugs of abuse to drive reward and reinforcing-mediating neurotransmission is followed by the development of negative affective states. A number of circuit plasticity events are hypothesized to underlie this stage. First, dopamine systems become hypofunctional during withdrawal from drugs of abuse (Melis, Spiga et al. 2005). In parallel, systems subserving aversive and anxiety states are engaged following chronic drug experience (Koob 2008). Together, these changes are thought to underlie the negative affect that promotes consumption in a negatively reinforcing manner (Koob and Volkow 2010). Finally, the preoccupation/anticipation stage is defined by the experience of craving in addicted individuals. Behavioral assays can model seeking by presenting stimuli (drug, cues, context) associated with the drug-taking experience or by producing stressors (e.g. acute and discrete stressors, such as electric shock, or protracted abstinence from drug availability (Koob and Volkow 2010). In preclinical models of 'craving', seeking is highly

dependent on glutamatergic neurotransmission from prefrontal cortices and amygdala structures (Everitt, Dickinson et al. 2001, McFarland and Kalivas 2001, Weiss and Koob 2001, Kalivas 2008). Furthermore, in extended access IVSA models, as well as in human addicts, a decrease of dopamine D2 type receptor in prefrontal cortex is observed (Volkow, Wang et al. 1997, Martinez, Broft et al. 2004, Martinez, Gil et al. 2005, Briand, Flagel et al. 2008, Briand, Flagel et al. 2008). These data support a converging hypothesis for these two neurotransmitter systems to mediate addiction states. The conceptualization of addiction stages provide a useful framework for research in that they allow for the analyses of discrete behavioral phenomena and facilitate the characterization of mechanisms that can regulate discrete stages of addiction. They also highlight the possible heterogeneity of the disease itself in that it is not merely a set of symptoms but rather involves a complex program or cycle with discrete symptoms or manifestations at each stage. This idea is an important one to consider in light of the fact that clinical therapies are aimed primarily at reducing or substituting intake and only partially, and in more recent years, at promoting prolonged abstinence or curbing relapse (McLellan, Lewis et al. 2000). Current therapeutic treatments include behavioral interventions (NIDA drugabuse.gov) and pharmacological substitution therapies for the use of drugs of abuse, e.g. methadone in the case of heroin, but remain limited in the case of psychostimulants such as cocaine, i.e. partial antagonists for dopamine transmission or indirect modulation via other transmitter systems (SSRIs) (Kalivas 2007).

From the preclinical standpoint many behavioral models have been developed to characterize the complex physiology and pathophysiology associated with drugs of abuse and, although debated, these generally serve as preclinical correlates to the stages of the

disease (Lu, Grimm et al. 2004, Lu, Grimm et al. 2004, Self 2004, Anderson and Pierce 2005, Koob and Volkow 2010, Steketee and Kalivas 2011). Prominent models in the literature include the locomotor sensitization assay, conditioned place preference and intravenous self-administration. Each of these approaches highlights unique aspects of the disease that are detailed below.

Locomotor sensitization is a behavioral assay that measures activity in response to drugs of abuse. Acutely, drugs of abuse promote locomotion. Over repeated exposures, however, the amount of locomotion produced by the same drug dose is enhanced. This phenomenon is termed sensitization of the locomotive response as the behavioral output has 'sensitized' or increased in response to the chronic exposure to the drug. Sensitization to drugs of abuse occurs in response to cocaine, amphetamine, morphine, ethanol, nicotine and THC (Joyce and Iversen 1979, Robinson and Becker 1986, Benwell and Balfour 1992, Cunningham, Niehus et al. 1992, Post, Weiss et al. 1992, Post, Weiss et al. 1992, Cadoni, Pisanu et al. 2001) and often cross sensitizes (Lett 1989, Vezina, Giovino et al. 1989, Vezina and Stewart 1990, Itzhak and Martin 1999, Cadoni, Pisanu et al. 2001). Upon repeated presentations, drugs of abuse generate greater locomotor responses to the same drug dose in a context-dependent or -independent manner (Vezina, Giovino et al. 1989, Steketee and Kalivas 2011). The development of this response is described as the initiation of sensitization. This is followed by expression of the sensitized locomotor response, a phenomena that is long-lasting and often context-dependent. The expression phase constitutes the expression of this sensitized response following varying periods of withdrawal from the drug experience. In practical terms, animals are sensitized and allowed to remain undisturbed for days, week or months after which the response to a

drug administration is monitored. Under these conditions, maintenance of the sensitized response is observed (Kalivas, Pierce et al. 1998, Hope, Simmons et al. 2006). This observation constitutes a unique aspect of this assay. Its long-term persistence can be detected in some cases after many months following the initial sensitizing regimen. Many studies indicated that the sensitized response is specifically to the re-experience of the drug in the context despite a prolonged absence of both the drug and context (Vezina, Giovino et al. 1989, Henry and White 1995, Steketee and Kalivas 2011). Because of the robust, long-term nature of the behavioral plasticity evoked by substances of abuse in this model it is often conceptualized as a model that allows the characterization of functional and/or structural changes in brain function that may underlie the chronicity or long-term behavioral maladaptations observed in addicted patients (Steketee and Kalivas 2011).

Conditioned place preference (CPP) is a Pavlovian model of learning during which animals learn to associate a unique environment with discrete, often complex, cues to the experience of a rewarding substance or experience, such as drugs of abuse. This model uses either a two-chambered or three-chambered apparatus to expose animals, in a restricted manner, to the presence or experience of the reward in one context and the absence of the reward in the other context. Following conditioning, animals are given free access to all the chambers of the conditioning apparatus. Most conditioned animals will spend more time in the context where they received the reward, likely reflecting a learned association between the rewarding stimuli (e.g. cocaine) and both contextual and discrete cues. Importantly, the expression of conditioned place preference occurs in a drug-free state, and is an indirect measure of drug reward. Conceptually, this model is thought to reflect the rewarding nature of drugs of abuse and not the reinforcing properties. This distinction is critical when

considering the interpretation of findings and comes from the understanding that reward is defined as the appetitive nature of a stimulus, whereas reinforcement is defined as the ability of an experience or stimulus to increase the probability of eliciting a specific behavioral outcome (Mackintosh 1974) (Bardo and Bevins 2000). Interestingly, CPP is amenable to extinction under repeated exposure to the reward-paired context in the absence of drug (Mueller and Stewart 2000, Parker and McDonald 2000, Wang, Luo et al. 2000, Sanchez, Bailie et al. 2003) and can be reinstated by drug re-experience or stress (Wang, Luo et al. 2000, Sanchez, Bailie et al. 2003).

Intravenous self-administration (IVSA) is an operant behavioral paradigm during which animals are provided access to chambers designed to provide a reward, i.e. drug of abuse, in response to the animal's operant action in the box. This assay offers high face validity to the human disease in that it allows animals to self administer the drug of interest and become dependent without administration of cocaine by the experimenter. Furthermore, this behavioral model allows experimenters to assay the relative reinforcing properties of a reward - a key characteristic of drugs of abuse (Self 2004, Thomsen and Caine 2006, Koob and Volkow 2010). Animals are implanted with an intrajugular catheter that connects to a back port. The back port is connected to a syringe pump that is under the control of the operant chamber. The box contains two levers- one active and one inactive. Only responses in the active lever result in an infusion of drug (or presentation of reward). Over repeated exposures and in response to reinforcing rewards, most animals will continue to press the active lever for the reward. This model has been thoroughly developed and iterations of the same have been described to model distinct aspects or stages of addiction. Especially relevant to this body of work is the use of short-access

sessions that result in stable intake across sessions under stable lever press criteria (fixed ratio, FR) and in response to varying doses of drug. This allows experimenters to assess the pharmacological sensitivity to drugs of abuse by varying the unit dose provided in each infusion and analyzing the behavioral response- ie, infusions. This analysis generates a dose response (DR) function (Self 2004, Thomsen and Caine 2006). The use of progressive ratio (PR) schedules of reinforcement, under which the requirement for lever presses increases across subsequent drug infusions, allows experimenters to assess the motivation to self-administer in dependent animals (Depoortere, Li et al. 1993, Richardson and Roberts 1996). This measure is thought to reflect processes driven by negative affective states experienced in the absence of the drug and which are hypothesized to underlie craving and seeking (Koob and Volkow 2010). In practical terms the number of lever presses required for an infusion is exponentially increased over the session until animals fail to self-administer within a predetermined timeframe. In most cases, animals are given up to 60 minutes to earn an infusion (Depoortere, Li et al. 1993, Richardson and Roberts 1996). The use of the FR, DR and PR schedules of reinforcement provide measures of cocaine taking. Other IVSA-based studies take advantage of the observation that animals trained to self-administer for drugs will seek for reward in the absence of the reward. These assays measure non-reinforced lever presses and are therefore considered a measure of seeking and reinstatement behaviors, a model of relapse (Shalev, Grimm et al. 2002, Shaham, Shalev et al. 2003, Anderson and Pierce 2005, Steketee and Kalivas 2011). Reinstatement of seeking can occur in response to re-exposure to the context (de Wit and Stewart 1981), re-exposure to drug associated cues (Meil and See 1996, Crombag, Grimm et al. 2002, Crombag and Shaham 2002), priming doses of drug (an interoceptive cue) (de

Wit and Stewart 1981, Shalev, Grimm et al. 2002), or an acute stressor (Shaham and Stewart 1995, Shaham and Stewart 1995, Shaham, Erb et al. 2000). In summary, the IVSA paradigm allows for a complete and thorough characterization of drug reinforcement and behavioral plasticity that has strong face validity for multiple aspects of human drug addiction and relapse.

**Circuitry of addiction (and known "dogma")**

Drugs of abuse act within the brain reward circuit. This circuit is complex and not fully understood, but it involves a number of limbic and cortical structures that include the ventral tegmental area (VTA), ventral striatum (VS) or nucleus accumbens (NAc), dorsal striatum (DS), amygdala, hippocampus and prefrontal cortex. Within this circuit, the NAc is considered the center of reward and is the sight of convergence of both excitatory, glutamatergic and modulatory, dopaminergic afferents. In its majority the NAc consists of medium spiny neurons; they account for up to 95% of the cell population of the NAc with the other ~5% of cells being accounted for by cholinergic and GABAergic interneurons (Meredith, Baldo et al. 2008, Russo, Dietz et al. 2010, Ikemoto, Yang et al. 2015). The NAc itself subdivides anatomically into the core and shell; a distinction that aligns with discrete functional roles in cocaine-related behaviors (Self 2004, Anderson and Pierce 2005, Hyman, Malenka et al. 2006, Meredith, Baldo et al. 2008). Dopaminergic neurons from the VTA project to the NAc, and to a lesser extent to the prefrontal cortex and hippocampus; while cortical structures, such as the amygdala, hippocampus and prefrontal cortex, all project glutamatergic excitatory afferents into the NAc (Koob and Volkow 2010, Kravitz, Tomasi et al. 2015, Volkow and Morales 2015).

All drugs of abuse enhance synaptic dopamine levels in the NAc (Self 2004, Anderson and Pierce 2005, Volkow and Morales 2015). It is at this site of enhanced dopamine that convergent glutamatergic signaling gets integrated in downstream signaling cascades via complex molecular events (Haydon, Blendy et al. 2009, Girault 2012, Cahill, Pascoli et al. 2014, Cahill, Salery et al. 2014). These convergent signaling cascades act in a multitude of ways. Important for the content of this thesis is the observation that dopaminergic signaling acts primarily to modulate cyclic AMP (cAMP) (Self, Genova et al. 1998) activity downstream of either $G_i$- or $G_o$- protein coupled molecular events (Self, Terwilliger et al. 1994, Self, McClenahan et al. 1995). Glutamatergic signaling, meanwhile, is complex in nature and acts to facilitate excitatory, $Ca^{2+}$ -dependent intracellular processes (Jonas and Burnashev 1995, Cortes-Mendoza, Diaz de Leon-Guerrero et al. 2013). This integration is possible not only by co-innervation of both dopaminergic and glutamatergic afferents, but also in part due to the physical formation of tripartite synapses that receives at the head of the spine glutamatergic innervation and on the neck modulatory dopaminergic innervation (Araque, Parpura et al. 1999, Hyman, Malenka et al. 2006, Cahill, Salery et al. 2014) . Although primarily driven by dopamine and glutamate this circuit is also subject to complex modulation by other monoamine systems (Howell and Negus 2014) that are not addressed within the scope of this work. Importantly, the release of dopamine in the NAc is thought to underlie reward and promote goal-directed behaviors. Dopamine is necessary and sufficient for reward and has therefore merited thorough study and characterization (Ikemoto, McBride et al. 1997, Ikemoto and Panksepp 1999, Self 2004, Anderson and Pierce 2005, Hyman, Malenka et al. 2006, Thomas, Kalivas et al. 2008).

**Dopamine in cocaine addiction**

The role of dopaminergic signaling has been extensively characterized in cocaine behaviors. Agonists for D1 or D2 receptors both support IVSA when administered systemically (Wise, Murray et al. 1990, Self and Stein 1992, Caine and Koob 1993, Maldonado, Robledo et al. 1993, Ikemoto, Glazier et al. 1997, Self 2004), but interestingly, co-delivery of D1- and D2-class agonists are required for intra-NAc self-administration (Ikemoto, Glazier et al. 1997). Agonism at either D1 or D2 enhances cocaine self-administration when delivered in either core or shell, but not when co-administered during cocaine self-administration (Bachtell, Whisler et al. 2005). Consistent with an essential role for dopamine-dependent signaling, D1 receptor KO mice do not self-administer cocaine (Caine, Thomsen et al. 2007). Furthermore, cocaine-insensitive DAT transgenic mice that cannot bind cocaine, and therefore do not have increased dopamine release, do not show cocaine reward in the CPP assay (Cornish and Kalivas 2000, Chen, Tilley et al. 2006). In the context of reinstatement, dopamine infusions in the NAc reinstate cocaine seeking (Cornish and Kalivas 2000) and only D2 agonists induce (Wise, Murray et al. 1990, Self and Stein 1992) and promote cocaine-primed reinstatement (Self, Barnhart et al. 1996, Khroyan, Barrett-Larimore et al. 2000, Spealman, Khroyan et al. 2000, Alleweireldt, Weber et al. 2002) whereas D1 agonists fail to induce reinstatement and attenuates cocaine-prime dependent reinstatement when pre-administer systemically (Self, Barnhart et al. 1996). Interestingly, antagonists of either D1 or D2 in the shell seem to limit prime-induced reinstatement (Anderson, Bari et al. 2003, Bachtell, Whisler et al. 2005). The apparent paradox of D1 agonism and antagonism both limiting cocaine-primed seeking supports the idea of a permissive role of D1 in reinstating paradigms (Self 2004). The finding that D2

agonism is sufficient and required to reinstate cocaine seeking in preclinical models holds some translational validity in that craving is associated in human addicts to D2 receptor activity in striatum (Volkow, Wang et al. 1997, Martinez, Broft et al. 2004, Martinez, Gil et al. 2005, Martinez, Narendran et al. 2007). These findings propose a central role of dopamine in driving addiction and cocaine-related behaviors.

**Glutamate in cocaine addiction**

The role of glutamate in cocaine-related behaviors is also extensively characterized. While dopamine in the NAc is driven by dopamine neurons of the VTA, glutamatergic innervation of the MSNs of the NAc comes from multiple cortical structures including the prefrontal cortex, hippocampus and amygdala. A role for glutamate in cocaine-related behaviors is supported by findings demonstrating that cocaine, contingent and non-contingent, reduces extracellular glutamate in the NAc core (Pierce, Bell et al. 1996, McFarland and Kalivas 2001, Kalivas, Volkow et al. 2005, Kalivas and Volkow 2005, Miguens, Crespo et al. 2008, Kalivas 2009, Knackstedt and Kalivas 2009). This effect is likely mediated by reductions of the cystine-glutamate exchanger (Madayag, Lobner et al. 2007, Baker, Madayag et al. 2008, Knackstedt and Kalivas 2009, Knackstedt, LaRowe et al. 2009), glutamate transporters (Knackstedt and Kalivas 2009, Pendyam, Mohan et al. 2009), and reduced metabotropic glutamate receptor, mGLUR2/3, tone (Moran, McFarland et al. 2005) all of which contribute to a hypoglutamatergic tone and homeostatic compensatory changes in glutamate transporter (GLT-1) and postsynaptic AMPA receptor subunit composition (Boudreau and Wolf 2005, Kourrich, Rothwell et al. 2007, Conrad, Tseng et al. 2008). Cocaine reinstating conditions such as cues, stress or drugs increase glutamate

release in the NAc core (McFarland, Lapish et al. 2003, McFarland, Davidge et al. 2004, Miguens, Del Olmo et al. 2008) and increased glutamate from PFC to NAc is required for cocaine seeking (Capriles, Rodaros et al. 2003, McFarland, Davidge et al. 2004). AMPA glutamate receptor antagonists in NAc core prevent cocaine-prime reinstatement (Cornish and Kalivas 2000) while AMPA agonists, but not NMDA, induce seeking (Cornish, Duffy et al. 1999, Di Ciano and Everitt 2001, Park, Bari et al. 2002). Conversely, agonist stimulation of mGlur2/3, a manipulation that limits glutamate release, inhibits cocaine seeking (Baptista, Martin-Fardon et al. 2004, Peters and Kalivas 2006). Similar effects have been reported with activation of the cystine-glutamate exchanger by N-acetylcystine; a manipulation that restores extracellular glutamate levels and prevents cocaine reinstatement (Baker, McFarland et al. 2003, Moussawi, Pacchioni et al. 2009). Most importantly, therapeutics aimed at regulating exclusively dopamine or glutamate neurotransmission in the addicted population has failed to provide relief from the characteristic craving and relapse cycle of addiction (Kalivas 2007, Howell and Negus 2014). The lack of effective therapeutic warrants further investigation and characterization of mechanisms that may underlie aspects of this complex disease and provide novel therapeutic strategies.

**The epigenetic landscape and its role in cocaine addiction**

Identification of mechanisms that mediate the transition from substance use to abuse and addiction are a key goal of this field of study as they may elucidate aberrant plasticity and provide novel targets for therapies (Kalivas 2004, Hyman, Malenka et al. 2006, Renthal and Nestler 2008, Dietz, Dietz et al. 2009, Nestler 2009, Maze, Covington et

al. 2010, Robison and Nestler 2011). One such mechanism involves epigenetic modifications that are defined as changes to chromatin structure resulting in either increased or decreased gene transcription (Kenny 2014). Epigenetic modifications are not direct modifications to genetic information but rather modifications that change the overall accessibility of genes such that transcription may be altered. Epigenetic modification includes post-translational modifications of histone tails. Histones are proteins that form in an octamer generally containing two subunits of each of four histone types- H2A, H2B, H3, and H4. Along with DNA these octamers form chromatin structures in the nucleus and serve to condense DNA and regulate gene transcription. While histone modifications may occur on a number of residues in the long N-terminal tail of the histone proteins the focus of this thesis will be on mechanisms that modify lysine residues dynamically by regulation of acetylation and deacetylation. Addition of acetyl groups on lysine residues of histone tails relaxes the local chromatin structure by neutralizing the positive charge of lysine and promotes transcription of surrounding genes by decreasing affinity of DNA to histones and allowing for access of transcriptional machinery. Conversely, removal of acetyl groups by histone deacetylases results in a tightening of the DNA and histone structure promoting the condensation of DNA and transcriptional repression (Bertos, Wang et al. 2001, Martin and Zhang 2007). It is hypothesized that these modifications may then result in key alterations to cellular function which mediate addiction related behavioral, functional and/or structural plasticity (Kumar, Choi et al. 2005, Renthal, Maze et al. 2007, Renthal, Kumar et al. 2009, LaPlant, Vialou et al. 2010, Wang, Lv et al. 2010).

Published studies have looked at the regulation of acetylation and deacetylation in preclinical models of cocaine abuse and addiction. These studies are summarized in table

1-1. Most strinkingly, aceylation of either H3 or H4 or both has been reported to increase in response to cocaine, primarily chronic experience and specifically in NAc shell but not core (Wang, Lv et al. 2010). Other studies report NAc specific increases in acetylation of H3 in the presence of sodium butyrate (NaBt) and CPP extinction (Malvaez, Sanchis-Segura et al. 2010) or acetylation of H4 in the absence of the class I HDAC3 basally or in response to acute cocaine (Malvaez, McQuown et al. 2013). Finally, increases in phospho-acetylated H3 have been described in striatum in response to cocaine (Renthal, Maze et al. 2007) or the combined administration of the D1 agonist, SKF82958, and the HDAC inhibitor, NaBt (Schroeder, Penta et al. 2008). These findings highlight the dymanic responsivity of acetylation marks to cocaine experience and propose that these changes may promote plasticity associated to drugs of abuse. All studies report increases of specific lysine residues or global acetylation increases suggesting that cocaine may act, directly or indirectly, to either promote HAT activity or limit HDAC activity. One important observation is that most of these studies have focused on the effect of cocaine on acetylation and little is known about the epigenetic landscape during other stages of addiction or in response to other cocaine-related behavioral assays.

Interestingly, the use of HDAC inhibitors, which favor increases in acetylation in NAc, have been shown to have complex effects on cocaine-related behaviors (Wang, Lv et al. 2010, Malvaez, McQuown et al. 2013, Rogge, Singh et al. 2013, Rogge and Wood 2013). These findings are summarized in table 1-2. Systemic HDAC inhibitors, trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and sodium butyrate (NaBT) increase CPP (Kumar, Choi et al. 2005, Hui, Wang et al. 2010, Wang, Lv et al. 2010, Raybuck, McCleery et al. 2013). Similarly, intra-NAc administration of SAHA enhances CPP (Renthal, Maze et al.

2007). Paradoxically, other behavioral paradigms have not mirrored the effects reported of HDAC inhibitors on CPP behaviors. Reports describing cocaine locomotion have shown to be enhanced by systemic NaBT (Kumar, Choi et al. 2005) but systemic TSA (Romieu, Host et al. 2008, Malvaez, Mhillaj et al. 2011) decreases locomotor sensitization. TSA administered systemically has been shown to decrease fixed ratio intake and progressive ratio, a measure of motivation in dependent animals (Romieu, Host et al. 2008, Host, Anglard et al. 2010). In contrast, direct administration of TSA or SAHA in the shell increases dose response and progressive ratio while having no effect on measures of IVSA cocaine intake intra-core (Wang, Lv et al. 2010). Perhaps most relevant to the human disease condition are studies of seeking or reinstatement. To this end, systemic TSA and phenylbutyrate (PhB) have been shown to reduce primed reinstatement (Romieu, Deschatrettes et al. 2011). Similarly, extinction of CPP has been accelerated, while CPP primed reinstatement attenuated by systemic NaBt (Malvaez, Sanchis-Segura et al. 2010) or the HDAC3 selective inhibitor RGFP966 (Malvaez, McQuown et al. 2013). One explanation for the complex phenotypic response to HDAC inhibitors may be a result of differences in administration protocols, specific inhibitors tested and/or doses that do not all produce the same level of HDAC inhibition and do not have the same specificity at the level of class or individual HDAC. Most importantly, the studies outlined above report the observation that the use of HDAC inhibitors decreases reinstatement behaviors. Although limited to a few studies, these findings support the use of HDAC inhibitors as a strategy to limit relapse-like behaviors in preclinical models and relapse in the clinical setting. Controlled studies testing HDAC inhibitor efficacy in curbing addiction and drug abuse associated effects are lacking. One provocative study analyzed the role of TSA on alcohol

induced reactive oxygen species (ROS) generation in a human cell line and reported a neuroprotective effect suggesting that TSA administration could limit neurodegeneration associated to alcohol use disorders (AUDs) (Agudelo, Gandhi et al. 2011). Studies up to date encourage a thorough characterization of HDAC function in cocaine behaviors that may help describe the function and relative therapeutic utility of manipulating specific HDAC function for cocaine addiction.

**HDACs and the class IIa's**

So far, there are 18 mammalian histone deacetylases identified which are further categorized in families based on structure, function, size and homology. The families include the class I, II, III and IV. Class I HDACs are nuclearly localized and ubiquitously expressed. These include HDAC1, 2, 3 and 8. Class II HDACs are further subdivided into IIa and IIb. The class IIa's are highly expressed in heart and brain and include the members HDAC4, 5, 7 and 9. Class IIb's include HDAC6 and 10 (Bertos, Wang et al. 2001, Martin and Zhang 2007, Parra 2015). Meanwhile class III HDACs, also named sirtuins, constitute a structurally and functionally distinct class of deacetylase in that they require the cofactor NAD and have distinct catalytic mechanisms and sequences (Haigis and Guarente 2006). Finally the more recently classified class IV subfamily containing HDAC11 described to share homology with both HDAC classes I and II, high conservation across species and for which a role is undescribed (Yang and Seto 2008).

Studies described in this thesis focus on the class IIa's because of their unique structure and regulation. Class IIa's contain highly homologous C-terminal domains that contain the deacetylase domain common to all HDACs. Unlike all the other member families

however the class IIa's contain an extended N-terminal domain that confers these members numerous binding domains for either transcription factors or co-repressor complexes. The N-terminal domain is also subject to complex post-translational modifications that regulate their binding affinity to a number of binding partners (Bertos, Wang et al. 2001, Martin and Zhang 2007, Parra 2015). Furthermore, this class is unique in that it features a C-terminal localized nuclear export signal (NES) and an N-terminal localized nuclear localization signal (NLS) (Wang, Bertos et al. 1999, Grozinger and Schreiber 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2000, Youn, Grozinger et al. 2000, Nishino, Miyazaki et al. 2008). These sequences, along with posttranslational modifications of specific residues, regulate class IIa function by virtue of subcelullar compartment localization and binding partner affinity, respectively (Lu, McKinsey et al. 2000, Lu, McKinsey et al. 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2000, Bertos, Wang et al. 2001, Martin and Zhang 2007, Backs, Backs et al. 2008).

**Class IIa's function in brain**

The role of class IIa's has been broadly characterized using global or transgenic genetic approaches. Knockout in mouse models of either HDAC4 or HDAC7 are not viable through adulthood, while HDAC5 and HDAC9 global KOs are and have been further characterized. HDAC4 global KO results in premature bone calcification, postnatal lethality (Vega, Matsuda et al. 2004), and neuronal cell death (Majdzadeh, Wang et al. 2008). Using conditional deletion, a forebrain specific role for HDAC4 has been described in learning and memory and synaptic plasticity (Kim, Akhtar et al. 2012, Sando, Gounko et al. 2012). Conversely, overexpression of HDAC4 has been shown to promote depression, but not

anxiety-like behavior in hippocampus (Sarkar, Chachra et al. 2014) and limit cocaine reward (Kumar, Choi et al. 2005) and motivation (Wang, Lv et al. 2010)) in NAc. HDAC5 global knockouts have uncovered a role for HDAC5 in axonal regeneration (Cho and Cavalli 2012), cardiac stress responses (Zhang, McKinsey et al. 2002, Chang, McKinsey et al. 2004), spatial memory formation (Agis-Balboa, Pavelka et al. 2013), and cocaine-behavioral responses (Renthal, Maze et al. 2007, Taniguchi, Carreira et al. 2012). HDAC7 KO leads to embryonic lethality (E11) due to a failure to form tight junctions in the cardiovascular system (Chang, Young et al. 2006) while HDAC9 phenocopies HDAC5's role in limiting cardiac hypertrophy (Chang, McKinsey et al. 2004). HDAC7 and HDAC9 have been primarily characterized outside of the nervous system and very little is known of their functions in brain. Sugo (Sugo, Oshiro et al. 2010) reported a role for HDAC9 as a negative regulator of dendritic growth of cerebellar postnatal cortical development while Lang (Lang, Alrahbeni et al. 2012) reported a hemizygous deletion in a small proportion of schizophrenia patients suggesting a potential functional role in this disease pathology. Bang (Bang, Kwon et al. 2015) reported a role for HDAC7 as a negative regulator of dopamine neuron differentiation from neural precursor cells *in vitro*. Finally, a role for neuronal survival or protection has been described for HDAC4 (under low NMDAR activity) (Chen, Wang et al. 2014), HDAC5 (NMDA dependent) (Wei, Lu et al. 2015), and HDAC7 (low KCl induced apoptosis, deacetylase independent) (Ma and D'Mello 2011). These reports highlight the dynamic functional range of thes class IIa HDACs and encourage cell-type and region-specific analyses of their role in multiple cellular processes and disease states.

**Class IIa's and localization**

The class IIa HDACs are unique in that they shuttle between cytoplasm and nucleus in an activity-dependent manner (Miska, Karlsson et al. 1999). Elevations of intracellular, nuclear calcium result in CamK superfamily-dependent binding (Youn, Grozinger et al. 2000, Chawla, Vanhoutte et al. 2003, Linseman, Cornejo et al. 2003, Belfield, Whittaker et al. 2006, Backs, Backs et al. 2008, Schlumm, Mauceri et al. 2013) and phosphorylation of at least two CamK-family regulated serine residues, S259 and S498 of HDAC5 (Lu, McKinsey et al. 2000, Lu, McKinsey et al. 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2000, Backs, Backs et al. 2008) and a PKA- or CdK5- responsive serine residue in the NLS, S279 of HDAC5 (Ha, Kim et al. 2010, Taniguchi, Carreira et al. 2012). Phosphorylation promotes the subsequent binding to 14-3-3 proteins and localization to the cell cytoplasm (Wang, Bertos et al. 1999, Grozinger and Schreiber 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2001, Nishino, Miyazaki et al. 2008). While primarily described as CamK targets, the class IIa's have also been described as targets for PKD (Collins, Adriaanse et al. 2015), PKC (Vega, Harrison et al. 2004), PRK2 (protein-kinase C-related kinase-2) (Harrison, Huynh et al. 2010), SIK1 (Dietrich, Takemori et al. 2012, Finsterwald, Carrard et al. 2013), PKA (Ha, Kim et al. 2010) and CdK5 (Taniguchi, Carreira et al. 2012) phosphorylation. Dephosphorylation of these sites by phosphatases or by antagonizing $Ca^{2+}$-dependent phosphorylation, eg. increased cAMP activity, reduces class IIa HDAC binding to 14-3-3, increases nuclear accumulation, and in many cases, promotes transcriptional repression (Wang, Bertos et al. 1999, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2001, Chawla, Vanhoutte et al. 2003, Belfield, Whittaker et al. 2006). Mechanisms to promote nuclear export independent of the phosphorylation status of these proteins have also been reported (Soriano, Chawla et al. 2013).

The class IIas often act as signal transducers by virtue of their ability to, in an activity or signaling dependent manner, accumulate across subcelullar compartments. In cortex, HDAC4, but not HDAC5, is redistributed to the nucleus of neurons in response to stroke, an effect associated with increased dendritic, axonal and myelin densities, suggesting neuronal remodeling (Kassis, Shehadah et al. 2015). PKA-dependent signaling promotes HDAC4 nuclear accumulation in hippocampus neurons and this shuttling promotes neuronal death in a MEF2D and KLF6 dependent manner (Salma and McDermott 2012). *In culture*d cortical neurons, oxidative stress promotes HDAC4 nuclear accumulation and inhibition of the peroxisome proliferator-activated receptor gamma (PPARy) to antagonize cell survival (Yang, Qin et al. 2011). Similarly, under low-potassium conditions or excitotoxic glutamate conditions HDAC4 is rapidly shuttled into the nucleus of cerebellar granule neurons to promote apoptosis (Bolger and Yao 2005). Similar regulation and role for HDAC5 is described by Linseman and colleagues (Linseman, Bartley et al. 2003). HDAC5 has also been shown to be phosphorylated by SIK1 either downstream of BDNF signaling to induce phosphorylation and export of HDAC5 in developing cortical cultures (Finsterwald, Carrard et al. 2013) or in response to cocaine experience to promote phosphorylation and cytoplasmic translocation in striatal cells (Dietrich, Takemori et al. 2012). Cocaine self-administration is reported to promote phosphorylation of HDAC5 and cytoplasmic localization (Renthal, Maze et al. 2007, Host, Dietrich et al. 2011). Even more interesting is the observation that HDAC4 can be exported from the nucleus of cultured hippocampal neurons by spontaneous electrical activity while HDAC5 required NMDA or L-type calcium channel activity (Chawla, Vanhoutte et al. 2003) . Consistently, excitotoxic NMDA administration to cultured cortical neurons rapidly phosphorylates and translocates

HDAC5 to the cytoplasm to promote cell apoptosis (Wei, Lu et al. 2015). Conversely, AP5 treatment (NMDAR antagonist) induces nuclear accumulation of HDAC4 (and enhanced expression of HDAC4, HDAC5 and HDAC9) to promote cell survival in low NMDAR conditions (Chen, Wang et al. 2014). In the case of HDAC9, nuclear to cytoplasmic translocation has been reported in response to spontaneous firing *in culture*d mouse cortical neurons to promote dendritic development (Sugo, Oshiro et al. 2010). These observations confirm that the localization of these HDACs is crucial for their function and provide another level of regulation of the function/activity of the class IIa's. It is interesting to note that the class IIa's have been described to play distinct cell-type specific functions that in many cases are phenotypically opposite (cell survival or death). Is it also worth noting that examples of sucebullar localization regulation exist in the literature in response to excitatory glutamatergic signaling and downstream of cAMP activity, a cascade recruited by dopamine and other monoamines (Cahill, Pascoli et al. 2014, Cahill, Salery et al. 2014). These observations underscore the functional versatility of this family of epigenetic modifiers, the importance of studying their regulation under cell-type specific conditions, and their unique ability to integrate signaling systems, glutamatergic and dopaminergic, known to modulate cocaine-associated behavioral, functional and structural plasticity.

**IIa's and binding partners**

Class IIa HDACs do not appear to directly bind to DNA in the nucleus, but instead appear to associate indirectly with genomic DNA. However, few nuclear binding partners have been identified for the class IIa HDACs. It is interesting to point out that the class IIa's have been described to associate with many co-repressor complexes but not many

transcription factors have been described for this subfamily of HDACs. Binding partners described in the literature of the class IIa include the C-terminal binding protein (CtBP) (Bertos, Wang et al. 2001, Dressel, Bailey et al. 2001, Zhang, McKinsey et al. 2001), nuclear receptor co-repressor (N-CoR) (Bertos, Wang et al. 2001, Fischle, Dequiedt et al. 2002), BCL-6 interacting co-repressor (BCoR) (Huynh, Fischle et al. 2000, Bertos, Wang et al. 2001), Silencing mediator for retinoid or thyroid-hormone receptors (SMRT)/HDAC3 and the class I HDACs (Fischle, Dequiedt et al. 2001, Fischle, Dequiedt et al. 2002), NAC1 (Korutla, Wang et al. 2005), heterochromatin P1 (HP1) (Zhang, McKinsey et al. 2002) and neuron restrictive silencer factor/repressor element-1 silencing transcription factor (NRSF/REST) (Nakagawa, Kuwahara et al. 2006). All of these binding partners have defined co-repressor roles in multiple organ systems but functional characterization in nervous system and/or in cocaine-associated plasticity remain scarce. Interestingly, many of these co-repressors are expressed in the central nervous system. CtBP isoforms 1 and 2, for instance, are expressed in striatum (Hubler, Rankovic et al. 2012) while N-CoR and SMRT are ubiquitously expressed in brain (van der Laan, Lachize et al. 2005). One recent study has outlined a functional co-association of SMRT and the class IIa's co-shuttling promotes class IIa translocation to the cytoplasm independent of phosphorylation sites (Soriano, Chawla et al. 2013).

Transcription factors described as class IIa binding partners have been characterized primarily outside of central nervous system and include the nuclear factor of activated T cells (NF-AT3C) (Dai, Xu et al. 2005), Runx2/3 (Vega, Matsuda et al. 2004), GATA1/2 (Ozawa, Towatari et al. 2001, Watamoto, Towatari et al. 2003), FOXP3 (Li and Greene 2007), Nkx2-5 (Song, Backs et al. 2006), serum response factor (SRF) (Davis, Gupta

et al. 2003), BCL6 (Lemercier, Brocard et al. 2002). Of these a few have relevant roles on striatal and/or cocaine transcriptional plasticity. NFATc dependent transcription is induced by D1 dopamine receptor stimulation in striatal cultures and cocaine exposure via nuclear translocation of NFATc4 (Groth, Weick et al. 2008) (Groth RD 2008). SRF is described as playing a role in synapse-specific and activity-dependent neuronal plasticity (Nikitin and Kozyrev 2007, Iacono, Altafini et al. 2013, Foxworthy and Medina 2015), regulating axonal growth (Lu and Ramanan 2011, Li, Sathyamurthy et al. 2014), and long-term consolidation downstream of NMDA signaling (Platenik, Kuramoto et al. 2000). Furthermore, a unique synaptic activity-responsive element (SARE) sequence that contains binding motifs for SRF, MEF2 and CREB mediates the activity dependent upregulation of Arc (Rodriguez-Tornos, San Aniceto et al. 2013, Fukuchi, Kanesaki et al. 2015, Fukuchi, Nakashima et al. 2015). Interestingly, loss of SRF in dopaminoceptive (D1) neurons results in hyperlocomotion and a loss of cocaine-mediated induction of early growth response (Egr) transcripts (Parkitna, Bilbao et al. 2010). The most well-described target is the transcription factor myocyte enhancer factor 2 (MEF2) (Lemercier, Verdel et al. 2000, Lu, McKinsey et al. 2000, Lu, McKinsey et al. 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2000, Belfield, Whittaker et al. 2006). The MEF2 family of transcription factors plays a key role regulating synapse number in neurons through its activity-dependent elimination of glutamatergic synapses (Flavell, Cowan et al. 2006, Barbosa, Kim et al. 2008, Pfeiffer, Zang et al. 2010). Importantly, reductions of MEF2 in the NAc *in vivo* result in increased dendritic spine density of medium spiny neurons in the NAc. In addition, expression of constitutively-active MEF2 in the NAc dramatically reduces cocaine-induced spine density increases, suggesting that the negative regulation of MEF2 *in vivo* by cocaine-

dependent processes may be important for structural plasticity following cocaine experiences. Consistent with this idea, repeated cocaine administration increases inhibitory phosphorylation of MEF2 (S408) and cocaine triggers nuclear accumulation of HDAC5 in the adult striatum (Pulipparacharuvil, Renthal et al. 2008, Taniguchi, Carreira et al. 2012). *In cultured* striatal neurons, cAMP elevation triggers nuclear accumulation of HDAC5 and strong co-localization with MEF2 proteins in the nucleus (Taniguchi, Carreira et al. 2012). However, the role of cocaine-induced spine density increases is poorly understood. While initially proposed to underlie behavioral adaptations to repeated cocaine administration, more recent findings have challenged this notion using various genetic and molecular manipulations *in vivo* (Pulipparacharuvil, Renthal et al. 2008, LaPlant, Vialou et al. 2010, Dietz, Sun et al. 2012, Ma, Huang et al. 2012). Interestingly, psychostimulants trigger an increase in NAc spine density in both contingent and experimenter-administered chronic cocaine conditions (Robinson and Kolb 1999, Robinson, Gorny et al. 2001, Robinson, Gorny et al. 2002), suggesting that it may be playing an important role in supporting, or suppressing, the development of behavioral plasticity following cocaine administration. Consistent with a potential role for HDAC5-dependent attenuation of MEF2 activity *in vivo* following cocaine, two recent studies have demonstrated that cAMP-dependent signaling attenuates MEF2 activity in neurons (Belfield, Whittaker et al. 2006, Pulipparacharuvil, Renthal et al. 2008). In addition, we recently reported that cocaine and cAMP trigger delayed nuclear import of HDAC5 in striatal neurons *in culture* and *in vivo*, and showed that nuclear accumulation of HDAC5 in the NAc functions to limit the development of cocaine conditioned place preference (Taniguchi, Carreira et al. 2012).

**Known regulation and function of the class IIa's in cocaine**

Consistent with a proposed role for epigenetic regulation in mediating substance-abuse dependent plasticity, several studies (Kumar, Choi et al. 2005, Renthal, Maze et al. 2007, Wang, Lv et al. 2010) have implicated the class IIa histone deacetylases (HDACs) as playing a key role in the nucleus accumbens (NAc), a key striatal site of action for cocaine, in limiting cocaine reward. The class IIa HDACs contains four family members (HDAC4, 5, 7 and 9); two of which have been partially characterized in cocaine related behaviors. Renthal and colleagues (Renthal, Maze et al. 2007) reported that mice lacking HDAC5 are hypersensitive to cocaine conditioned place preference after prior exposure to repeated cocaine. This effect in the HDAC5 KO mice was rescued by viral-mediated expression of HDAC5 in the NAc. In addition, they found that overexpression of HDAC5 in the NAc of wild-type mice reduced cocaine reward, suggesting a key role in cocaine-induced behavioral plasticity *in vivo*. Similar to what has been reported for HDAC5, Kumar and colleagues (Kumar, Choi et al. 2005) reported that the overexpression of HDAC4 in the NAc of wild-type mice reduced cocaine reward, suggesting not only a key role in cocaine-induced behavioral plasticity *in vivo* for the class IIa HDACs but also that HDAC5 and 4 may largely be functionally redundant. Further studies have characterized the role of HDAC4 in cocaine IVSA. In an elegant body of work, Wang et al (Wang, Lv et al. 2010) demonstrated that NAc viral mediated overexpression of HDAC4 in the shell but not the core significantly decreases the descending limb of the dose response suggesting a sensitization to cocaine and a significant attenuation of progressive ratio suggesting a decrease in overall motivation to self-administer despite comparable levels of stable intake under fixed ratio

conditions. In both cases HDAC4 and 5 require the DAC domain to either limit DR/PR (Wang, Lv et al. 2010) and CPP (Renthal, Maze et al. 2007), respectively. This observation suggests that their function may be dependent on their ability to deacetylate either histone tails or transcriptional binding partners. Taken together, these studies highlight a role for the class IIa members HDAC4 and HDAC5 in cocaine. Importantly they generate a number of interesting questions regarding the regulation of localization of these HDACs in response to cocaine and cocaine-related behaviors.

**Goal of Thesis Research**

A role for epigenetic modulation of reward related circuitry is clearly established in the literature. Broad manipulations of epigenetic factors, however, have failed to modulate cocaine-related behaviors in predictable and therapeutically relevant ways. Therefore, a clear characterization and understanding of the role of specific molecules may help shed light on not only the apparently opposing observed effects of HDACs in animal models but also illustrate potential molecular targets for therapeutic development. A few studies on the histone deacetylases and their role in cocaine-related behavior have been described. The class IIa's HDAC4 and HDAC5, specifically, have been shown to play a role in cocaine reward. The goal of this thesis was 1) to characterize the regulation of HDAC4 and HDAC5 localization by cocaine experience and 2) test the role of driving HDAC4 or HDAC5's nuclear function on cocaine-related behaviors. Finally, we analyzed the dependence of HDAC5 on its most well characterized transcription factor-binding partner, MEF2, in cocaine-related behaviors.

Table 1-1. Modification of acetylation marks by cocaine experience and related behaviors

| Modification | Region | Administration | Effect | Reference |
|---|---|---|---|---|
| AcH3 | Shell | Acute IVSA | – | Wang et al 2010 |
| | | Chronic IVSA | ↑ | |
| | | Acute Yoke IVSA | – | |
| | | Chronic Yoke IVSA | ↑ | |
| | Core | Acute IVSA | – | Wang et al 2010 |
| | | Chronic IVSA | – | |
| | | Acute Yoke IVSA | – | |
| | | Chronic Yoke IVSA | – | |
| | NAc | NaBt + CPP extinction | ↑ | Malvaez et al 2010 |
| AcH4 | Shell | Acute IVSA | – | Wang et al 2010 |
| | | Chronic IVSA | ↑ | |
| | | Acute Yoke IVSA | – | |
| | | Chronic Yoke IVSA | ↑ | |
| | Core | Acute IVSA | – | Wang et al 2010 |
| | | Chronic IVSA | – | |
| | | Acute Yoke IVSA | – | |
| | | Chronic Yoke IVSA | – | |
| | NAc | HDAC3 cKO | ↑ | Rogge et al 2013 |
| | | Acute cocaine i.p. in HDAC3 cKO | ↑ | |
| pAcH3 | Striatum | Cocaine i.p. | ↑ | Kumar et al 2005 |

Table 1-2. Effect on cocaine-related behaviors of HDAC5 inhibitors

| Inhibitor | Region | Administration | Effect | Reference |
|---|---|---|---|---|
| TSA | | Systemic (i.p.) | ↑ CPP<br>↓ Prime reinstatement | Kumar et al 2005<br>Romieu et al 2011 |
| | | Systemic (i.v.) | ↓FR1 intake<br>↓FR1 intake<br>↓Progressive Ratio<br>↓ Locomotor sensitization | Host et al 2010<br>Romieu et al 2008 |
| | NAc Shell | Systemic (i.p.) | ↑ AcH3<br>↑AcH4 | Wang et al 2010 |
| | | Intra-NAc | −FR5 intake<br>↑ Dose Response<br>↑Progressive Ratio | |
| | NAc core | Systemic (i.p.) | ↑ AcH3<br>↑AcH4 | |
| | | Intra-NAc | −FR5 intake<br>− Dose Response<br>− Progressive Ratio | |
| SAHA | NAc Shell | Systemic (i.p.) | ↑ AcH3<br>↑AcH4 | Wang et al 2010 |
| | | Intra-NAc | −FR5 intake<br>↑ Dose Response<br>↑Progressive Ratio | |
| | NAc core | Systemic (i.p.) | ↑ AcH3<br>↑AcH4 | |
| | | Intra-NAc | ↑ CPP | Renthal et al 2007 |
| PhB | | Systemic (i.p.) | ↓Prime reinstatement | Romieu et al 2011 |
| NaBt | | Systemic (i.p.) | ↑ Cocaine locomotion<br>↑CPP<br>↑ AcH3<br>↑CPP extinction<br>↓CPP prime reinstatement | Kumar et al 2005<br>Raybuck et al 2013<br>Hui B et al 2010<br>Malvaez et al 2010 |
| RGFP966 (HDAC3 selective) | | Systemic (i.p.) | ↑ CPP extinction<br>↓CPP prime reinstatement | Malvaez et al 2013 |

Table 1-3. Class IIa (and other HDACs) and cocaine related behaviors

| | Region | Administration | Effect | Reference |
|---|---|---|---|---|
| HDAC4 | NAc | | ↓CPP | Kumar et al 2005 |
| | Shell | | ↓Dose Response<br>↓Progressive Ratio | Wang et al 2010 |
| | Core | | –Dose Response<br>– Progressive Ratio | |
| HDAC4 ΔDAC | | | | |
| | Shell | | –Dose Response<br>– Progressive Ratio | |
| | Core | | –Dose Response<br>– Progressive Ratio | |
| HDAC5 | NAc | | ↓CPP | Renthal et al 2007 |
| | | TSA (systemic, i.p.) | –CPP | |
| HDAC5 ΔDAC | | | –CPP | |
| HDAC5 ΔMBD | | | ↓CPP | |
| HDAC5 KO | germline | | –CPP | |
| | | Chronic coc (i.p.) | ↑CPP | |
| HDAC7 | | | None reported | |
| HDAC9 | | | None reported | |
| Other HDACs: | | | | |
| HDAC1 | NAc | | ↓Locomotor Sensitization | Kennedy et al 2013 |
| HDAC2 | NAc | | – Locomotor Sensitization | |
| HDAC3 cKO | NAc | | – Locomotor Sensitization<br>↑CPP | Rogge et al 2013 |

Chapter 2: Histone Deacetylase 5 Limits Cocaine Reward through cAMP-Induced Nuclear Import

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Author Contributions

MT, MBC and CWC designed experiments. MT, MBC, LNS and BCZ conducted experiments. Specifically, MBC generated and or significantly contributed to figures 2-1, 2-2, 2-3, 2-4 and 2-5. RLN prepared Herpes Simplex Viruses used in this study. MT, CWC and MBC prepared this manuscript.

Originally published and modified from: Taniguchi, M., **M. B. Carreira**, L. N. Smith, B. C. Zirlin, R. L. Neve and C. W. Cowan (2012). "Histone deacetylase 5 limits cocaine reward through cAMP-induced nuclear import." Neuron **73**(1): 108-120.

**Summary**

Chromatin remodeling by histone deacetylases (HDACs) is a key mechanism regulating behavioral adaptations to cocaine use. We report here that cocaine and cyclic adenosine monophosphate (cAMP) signaling induce the transient nuclear accumulation of HDAC5 in rodent striatum. We show that cAMP-stimulated nuclear import of HDAC5 requires a signaling mechanism that involves transient, protein phosphatase 2A (PP2A)-dependent dephosphorylation of a Cdk5 site (S279) found within the HDAC5 nuclear localization sequence. Dephosphorylation of HDAC5 increases its nuclear accumulation, by accelerating its nuclear import rate and reducing its nuclear export rate. Importantly, we show that dephosphorylation of HDAC5 S279 in the nucleus accumbens suppresses the development, but not expression, of cocaine reward behavior *in vivo*. Together, our findings reveal a molecular mechanism by which cocaine regulates HDAC5 function to antagonize the rewarding impact of cocaine, likely by putting a brake on drug-stimulated gene expression that supports drug-induced behavioral changes.

**Introduction**

Histone deacetylation by chromatin-modifying enzymes plays a critical role in shaping transcriptional responses to experience. Drug addiction is thought to represent a long-lasting, maladaptive change in the function of the brain reward circuitry, and drug-induced transcriptional responses contribute to behavioral adaptations relevant to addiction (McClung and Nestler 2003, Kalivas 2004, Hyman, Malenka et al. 2006). Several recent studies have reported an important role for histone deacetylase (HDAC) activity in the regulation of cocaine-induced behaviors in rodent models of addiction (Kumar, Choi et

al. 2005, Renthal, Maze et al. 2007, Renthal, Kumar et al. 2009, Sanchis-Segura, Lopez-Atalaya et al. 2009, Hui, Wang et al. 2010, Wang, Lv et al. 2010). However, how cocaine regulates HDAC function in brain reward circuitry, and whether regulation is important for its ability to modulate addiction-related behavioral responses, is poorly understood.

The class IIa HDACs emerged recently as important modulators of cocaine-induced behavioral responses *in vivo* (Kumar, Choi et al. 2005, Renthal, Maze et al. 2007, Wang, Lv et al. 2010). The class IIa HDACs (HDAC4, 5, 7, and 9) are unique among the HDAC family proteins in that they shuttle between the nucleus and the cytoplasm in cells (McKinsey, Zhang et al. 2000, Bertos, Wang et al. 2001, McKinsey, Zhang et al. 2001, Belfield, Whittaker et al. 2006). Nucleocytoplasmic shuttling is governed bya basic residue-rich nuclear localization sequence (NLS) located within the N-terminal half of the proteins and a nuclear export sequence (NES) located within the C-terminal region (McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2001). Numerous studies have reported that CaMK superfamily proteins, in response to an intracellular calcium rise, increase phosphorylation at two conserved sites, S259 and S498, which serve to (1) increase binding of HDAC5 to the 14-3-3 cytoplasmic-anchoring proteins, (2) disrupt binding between HDAC5 and myocyte enhancer factor 2 (MEF2) transcription factors in the nucleus, and (3) promote cytoplasmic localization of HDAC5 (McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2001, Chawla, Vanhoutte et al. 2003, Vega, Matsuda et al. 2004, Belfield, Whittaker et al. 2006, Sucharov, Langer et al. 2006)

HDAC5 in the nucleus accumbens (NAc) was shown recently to reduce the rewarding impact of cocaine and inhibit cocaine experience-dependent reward sensitivity (Renthal, Maze et al. 2007), suggesting that it plays an active role in the nucleus to repress

gene expression that promotes cocaine reward behavior. One of the only known HDAC5-interacting proteins in the nucleus is the MEF2 family of transcription factors, and HDAC5 is known to antagonize MEF2-dependent transcription (Lu, McKinsey et al. 2000). Consistently, expression of active MEF2 in the NAc enhances cocaine reward behavior (Pulipparacharuvil, Renthal et al. 2008), which is opposite to the effects of HDAC5 expression in the NAc (Renthal, Maze et al. 2007). Activation of D1 class dopamine receptors (D1-DARs), or elevation of cyclic adenosine monophosphate (cAMP) levels, reduces basal and calcium-stimulated MEF2 activity in striatal or hippocampal neurons (Belfield, Whittaker et al. 2006, Pulipparacharuvil, Renthal et al. 2008), which motivated us to explore the possibility that cocaine and cAMP signaling might regulate HDAC5's nuclear localization and/or function in the striatum *in vivo*.

In the present study, we uncover a signaling mechanism by which cocaine and cAMP signaling promote transient nuclear accumulation of HDAC5 through dephosphorylation-dependent regulation of NLS function in striatal neurons *in vitro* and *in vivo*, and demonstrate that this regulatory process is essential for the ability of HDAC5 to limit cocaine reward in the NAc *in vivo*. Taken together with previous work, our findings reveal that transient and dynamic regulation of this epigenetic factor plays an important role in limiting the rewarding impact of cocaine after repeated drug exposure.

## Results

### cAMP Signaling Promotes Nuclear Import of HDAC5 in Striatal Neurons

To test whether cAMP signaling regulates striatal HDAC5, we transiently transfected a plasmid expressing HDAC5-EGFP fusion protein into cultured primary striatal neurons,

and then analyzed the basal and cAMP-stimulated steady-state subcellular distribution. Under basal culture conditions, we observed that a majority of HDAC5 is localized in the cytoplasm or is evenly distributed between the nucleus and cytoplasm (Figures 2-1A and 2-1B). However, elevation of cAMP levels with the adenylyl cyclase activator, forskolin (10 μM), induced the rapid nuclear import of HDAC5 (Figures 2-1A and 2-1B) where it accumulated in a predominantly punctate pattern (Figure 2-1A). The cAMP-induced steady-state nuclear accumulation of HDAC5 occurred over a time course of ~1–2 hr in striatal neurons (Figure 2-1B, bottom). Interestingly, the nuclear HDAC5 puncta colocalized with endogenous MEF2 proteins (data not shown), suggesting that the nuclear HDAC5 is associated with transcriptional complexes on genomic DNA and that previously noted cAMP-dependent suppression of MEF2 activity is likely mediated by HDAC5 (Belfield, Whittaker et al. 2006, Pulipparacharuvil, Renthal et al. 2008).

**Identification of a Conserved HDAC5 Phosphorylation Site Within the NLS**

We speculated that cAMP signaling might regulate nuclear accumulation by regulating HDAC5 phosphorylation. By in silico analysis of the HDAC5 primary amino acid sequence, we identified a highly conserved serine (S279) that was a candidate substrate for protein kinase A (PKA) or cyclin-dependent kinase 5 (Cdk5), both of which are implicated in drug addiction-related behavioral adaptations (Self, Genova et al. 1998, Bibb, Chen et al. 2001, Benavides, Quinn et al. 2007, Pulipparacharuvil, Renthal et al. 2008). Because S279 resides within the HDAC5 NLS, which is characterized by a high density of basic residues (Figure 2-1C, noted by asterisks), we speculated that phosphorylation at this site may modulate nucleocytoplasmic localization of HDAC5. The HDAC5 S279 site (and surrounding

residues) was highly conserved from fish to humans (Figure 2-1C) and in both HDAC4 and HDAC9. Tandem mass spectrometry analysis of flag-epitope tagged HDAC5 *in cultured* cells revealed a singly phosphorylated peptide (SSPLLR: 278–283 amino acids) (data not shown). Therefore, we generated a phosphorylation site-specific antibody against HDAC5 S279 to study its regulation by cAMP signaling. The P-S279 peptide antibody recognizes wild-type (WT) HDAC5, but not a mutant form that cannot be phosphorylated at this site (HDAC5 S279A) (Figure 2-1D). It also recognizes endogenous P-HDAC5 after immunoprecipitation (IP) of total HDAC5 from cultured striatal neurons or adult striatal tissues, but not from anti-HDAC5 IPs using HDAC5 knockout (KO) mouse lysates (data not shown) (Chang et al., 2004), indicating that endogenous HDAC5 is basally phosphorylated at S279 in striatum *in vitro* and *in vivo*.

To determine whether Cdk5 or PKA can phosphorylate HDAC5 S279, we incubated full-length, dephosphorylated HDAC5 with recombinant Cdk5/p25 or PKA *in vitro* and found that either kinase can phosphorylate S279 *in vitro* (Figures 2-3A and 2-3B). However, when we incubated striatal neurons with specific kinase inhibitors for either Cdk5, PKA or p38 MapK (all potential kinases predicted for S279), we observed dramatically reduced P-S279 levels in the presence of Cdk5 inhibitors (Figures 2-2A and 2-3C) but observed no change in P-S279 in the presence of PKA or p38 MapK inhibitors (Figure 2-3C). Together, these findings indicate that whereas PKA is able to phosphorylate HDAC5 *in vitro*, it is not required for endogenous HDAC5 P-S279 in striatal neurons.

**cAMP Signaling Dephosphorylates S279 through a PP2A-Dependent Mechanism**

To test whether cAMP signaling regulates HDAC5 P-S279 levels, we cultured primary striatal neurons and elevated cAMP levels with forskolin (10 μM) or a nonselective phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 200 μM), for various periods of time. We observed a rapid and robust dephosphorylation of HDAC5 S279 within 20 min of forskolin or IBMX treatment (Figures 2-2B and 2-3D), an effect that was stable for at least 3 hr. In addition, forskolin induced robust dephosphorylation of endogenous HDAC5 S279 *in cultured* primary cortical neurons, COS7 cells (Figures 2-3E and 2-3F), as well as with overexpressed HDAC5-EGFP in HEK293T cells (data not shown). These findings suggest that cAMP-stimulated dephosphorylation of HDAC5 S279 is a conserved mechanism across multiple cell types, including nonneuronal cells.

We next sought to identify the molecular mechanisms by which cAMP signaling stimulates HDAC5 dephosphorylation of P-S279. Elevation of cAMP levels increases the activity of the protein phosphatase 2A (PP2A) in striatal neurons (Ahn, McAvoy et al. 2007, Ceglia, Kim et al. 2010). Consistent with this pathway, we found that okadaic acid, a potent inhibitor for PP2A and partial inhibitor of PP1, blocked cAMP-induced dephosphorylation of P-S279 in striatal neurons (Figure 2-4A), whereas the PP1-specific inhibitor, tautomycetin, had no effect (Figure 2-5). In addition we observed that purified PP2A was sufficient to dephosphorylate endogenous HDAC5 P-S279 *in vitro* (Figure 2-4B). Together, these data reveal that PP2A activity is necessary and sufficient for cAMP-stimulated dephosphorylation of HDAC5 S279 in striatal neurons.

**Dephosphorylation of S279 Is Required for Nuclear Import of HDAC5**

To test the role of PP2A activity on nucleocytoplasmic localization of HDAC5, striatal neurons were treated with okadaic acid or tautomycetin in the presence or absence of forskolin treatment. Okadaic acid treatment increased basal HDAC5 localization in the cytoplasm, and it blocked the cAMP-induced nuclear import of WT HDAC5-EGFP (Figure 2-6A). In contrast, tautomycetin altered neither basal nor cAMP-induced localization of WT HDAC5-EGFP (data not shown), indicating that PP2A activity is required for cAMP-induced nuclear accumulation.

To test whether the PP2A-dependent dephosphorylation of HDAC5 S279, specifically, was required for the cAMP-induced nuclear import of HDAC5, we generated a phosphomimetic mutant at this site by changing S279 to a negatively charged residue, glutamic acid (E), and then analyzed the subcellular localization pattern of the HDAC5 before and after elevation of cAMP *in cultured* striatal neurons. Compared to WT HDAC5, we observed that most of the HDAC5 S279E protein localized in the cytoplasm under unstimulated conditions (Figure 2-6B). However, unlike WT HDAC5, the HDAC5 S279E mutant failed to relocalize to the nucleus by 3 hr after forskolin treatment (Figure 2-6B, middle). The S279E mutant did not simply disrupt the NLS function because treatment with the Crm1-mediated nuclear export inhibitor, leptomycin B (LMB) (Harrison et al., 2004 and Vega et al., 2004), led to near-complete nuclear accumulation of both WT and S279E HDAC5 at similar rates (data not shown and figure 2-7A). These findings indicate that dephosphorylation of HDAC5 S279 is necessary for cAMP-induced nuclear accumulation.

To test whether dephosphorylation of S279 is sufficient to promote nuclear localization, we expressed in striatal neurons the nonphosphorylatable HDAC5 S279A

mutant. Under basal conditions localization of the HDAC5 S279A mutant was similar to WT HDAC5 (Figure 2-6B, right), indicating that dephosphorylation of S279 alone is not sufficient to confer nuclear localization of HDAC5. Similar to WT HDAC5, forskolin stimulated nuclear accumulation of HDAC5 S279A, which indicates that dephosphorylation of S279 is necessary, but not sufficient, for cAMP-induced nuclear accumulation of HDAC5. Similar basal subcellular distribution and responses to cAMP were observed with HDAC5 proteins lacking EGFP fusion protein (data not shown).

CaMK or PKD-dependent phosphorylation of HDAC5 P-S259 and P-S498 confers cytoplasmic localization of HDAC5 in nonneuronal cells (McKinsey, Zhang et al. 2000), mediates binding to 14-3-3 cytoplasmic-anchoring proteins, and disrupts association with MEF2 transcription factors (McKinsey, Zhang et al. 2000, Vega, Harrison et al. 2004, Harrison, Huynh et al. 2010). Interestingly, forskolin treatment stimulated dephosphorylation of both S259 and S498 to a similar extent as S279 (Figure 2-6C), indicating that all three sites are negatively regulated by cAMP signaling. Consistent with previous studies (McKinsey et al., 2000a and Vega et al., 2004), we found that HDAC5 S259A or S259A/S498A mutants were distributed evenly between the cytoplasm and nucleus or were concentrated in the nucleus (Figure 2-6D, left, and Figure S4C), confirming a critical role for these phosphorylation sites in striatal neurons. However, we found that the HDAC5 S259A and S259A/S498A mutants had significantly reduced (60%) P-S279 levels (data not shown), confounding a straightforward interpretation of the S259A and the S259A/S498A effects on nuclear/cytoplasmic localization and suggesting that P-S279 is sensitive to the phosphorylation status of S259. Interestingly, forskolin treatment of striatal neurons stimulated strong nuclear accumulation of HDAC5 S259A or S259A/S498A

(Figure 2-6D), indicating that dephosphorylation of S259 and S498 alone cannot account for cAMP-induced nuclear import. To test the specific importance of P-S279 in this context, we generated compound HDAC5 mutants, S259A/S279E and S259A/S498A/S279E, and observed that the S279E mutation shifted the basal subcellular localization away from the nucleus in a pattern similar to WT HDAC5 (Figures 2-6D). Consistent with the single mutant (S279E, Figure 2-6B), forskolin-induced nuclear accumulation of HDAC5 was defective in either of the compound mutants, confirming an essential and independent function for dephosphorylation of HDAC5 S279 in cAMP-induced nuclear import.

**Dephosphorylation of S279 Increases the Nuclear Import Rate**

We next sought to understand how dephosphorylation of HDAC5 S279 promotes nuclear accumulation in striatal neurons. As mentioned earlier, blocking Crm1-dependent nuclear export with LMB results in strong nuclear accumulation of both WT and S279E HDAC5 proteins (data not shown), indicating that both of these proteins shuttle between the nucleus and cytoplasm under basal conditions. The steady-state, nucleocytoplasmic distribution of HDAC5 is determined by the balance of nuclear import and nuclear export kinetics. Therefore, the cAMP-induced accumulation of HDAC5 in the nucleus likely represents a change in the nuclear import rate, the nuclear export rate, or both. To evaluate these parameters, we used conditions where HDAC5 nuclear export was blocked (LMB) with or without simultaneous elevation of cAMP. Compared to the LMB-only condition, we observed a dramatic increase in the nuclear import rate of WT HDAC5 after forskolin treatment, resulting in near-complete disappearance from the cytoplasm by 20 min (Figure 2-7A); this condition showed similar kinetics to forskolin-induced dephosphorylation of

S279 (Figure 2-2B). In contrast, the import rate of HDAC5 S279E after forskolin plus LMB treatment is nearly indistinguishable from the rate of nuclear import of WT HDAC5 treated with vehicle plus LMB (Figure 2-7A), which indicates that dephosphorylation of S279 accelerates the nuclear import rate. We next tested potential effects of P-S279 on HDAC5 nuclear export by first incubating striatal neurons with LMB to force accumulation of WT or S279E HDAC5 into the nucleus (Figure 2-7B). Following washout of LMB we monitored the initial rate of nuclear export and observed that the HDAC5 S279E mutant disappeared from the nucleus more rapidly than WT HDAC5 (Figure 2-7B). Therefore, our findings suggest that cAMP increases the HDAC5 nuclear import rate and decreases the nuclear export rate by stimulating dephosphorylation of HDAC5 S279. In addition we observed that the HDAC5 S279E mutant coprecipitates with a cytoplasmic chaperone protein, 14-3-3, to a significantly greater extent than WT HDAC5 *in cultured* cells (Figure 2-7C), suggesting that P-S279 enhances the affinity of 14-3-3 and HDAC5, potentially enhancing cytoplasmic retention and nuclear export of HDAC5. However, the HDAC5 S259A/S498A/S279E mutant, despite its enhanced cytoplasmic localization, fails to coimmunoprecipitate with 14-3-3 (data not shown), indicating that the primary cytoplasmic localizing function of P-S279 is not likely due to its enhancement of 14-3-3 binding.

**Cocaine Stimulates Dephosphorylation of HDAC5 S279 and Nuclear Import**

Cocaine and dopamine signaling regulate cAMP levels in striatum. To test whether dopamine signaling regulates HDAC5 phosphorylation in striatum *in vivo*, we injected adult mice with a dopamine D1 class receptor agonist, SKF81297 (5 mg/kg), or a dopamine D2 class receptor agonist, quinpirole (5 mg/kg), and analyzed striatal HDAC5 P-S279 levels *in*

*vivo*. We found that SKF81297 administration stimulates significant dephosphorylation of HDAC5 S279 at 30 min and 3 hr after injections, whereas exposure to quinpirole stimulated a trend toward increased HDAC5 P-S279 levels (Figures 2-9A and 2-9B). We next injected adult C57BL/6 mice with cocaine (20 mg/kg) and analyzed both HDAC5 P-S279 levels and nuclear/cytoplasmic localization of endogenous HDAC5 in the striatum. We compared mice injected 7 days with saline (vehicle control), 7 days with cocaine (cocaine-experienced), or 6 days with saline and one cocaine injection on the seventh day (cocaine-naive), and analyzed HDAC5 P-S279 levels at 1, 4, and 24 hr after the last injection (Figure 2-8A). By first immunoprecipitating total HDAC5 we were able to measure HDAC5-specific P-S279 levels as confirmed in HDAC5 KO mice (data not shown). We observed a significant dephosphorylation of HDAC5 at 1 and 4 hr following the last injection in both the cocaine-naive and cocaine-experienced mice, but phosphorylation at S279 had returned to baseline levels by 24 hr after the last cocaine injection. We next analyzed the levels of P-S259 and P-S498 HDAC5 after cocaine, and similar to P-S279 regulation, we observed a significant reduction of all three sites (Figure 2-8B). Taken together, these findings reveal that cocaine stimulates the coordinated dephosphorylation of P-S259, P-S279, and P-S498 on HDAC5.

We next analyzed the effects of cocaine on nuclear/cytoplasmic distribution of endogenous striatal HDAC5 using a biochemical fractionation approach. Similar to the subcellular distribution of HDAC5 in primary striatal neurons *in culture* (e.g., Figure 2-1B), a majority of the striatal HDAC5 cofractionated with cytoplasmic proteins (Figure 2-8C). Following the same dosing paradigm detailed above, administration of cocaine to naive or cocaine-experienced mice resulted in a significant accumulation of HDAC5 in the nucleus at 4 hr after the last injection, and like the regulation of P-S279, nuclear accumulation was

transient and returned to saline control levels by 24 hr after the last injection (Figure 2-8D). Taken together, these results reveal that cocaine administration stimulates the rapid and transient dephosphorylation of HDAC5 and subsequent nuclear accumulation of endogenous HDAC5 *in vivo*.

**Dephosphorylation of HDAC5 S279 Antagonizes Cocaine Reward**

To test the importance of cocaine-induced dephosphorylation of HDAC5 S279 for the development of cocaine reward behavior, we utilized viral-mediated gene transfer to express full-length, HDAC5 WT or mutants (S279A or S279E) bilaterally in the NAc of WT, adult male mice (Figure 2-10A) prior to a cocaine-conditioned place preference (CPP) assay. This assay involved pairing one of two distinct chambers with either cocaine or saline injections for 2 consecutive days. Subsequently, the mice were given equal access to both chambers, and time spent in either the cocaine-paired or saline-paired chamber was measured. As expected, the control virus (GFP)-injected mice spent significantly more time in the cocaine-paired chamber (Figure 2-10C), indicating a clear positive preference for the context in which cocaine was experienced. Similar to a previous report (Renthal et al., 2007), the overexpression of WT HDAC5 reduced cocaine-associated place preference, but it did not reach significance. Mice expressing the HDAC5 S279E mutant protein had a cocaine place preference similar to the GFP-only control virus-injected mice, whereas mice expressing HDAC5 S279A dephosphorylation mutant showed significantly reduced cocaine place preference (Figure 2-10C; S279A, 81 s, versus S279E, 246 s). We observed similar expression levels of the HDAC5 WT, S279A, and S279E mutants in striatal neurons (Figure 2-10B), indicating that the results are not likely due to differences in HDAC5 protein

expression levels. As expected, we observed that mice injected with the lower dose of cocaine used in the CPP assay (5 mg/kg) showed a significant transient reduction of HDAC5 P-S279 levels (Figure 2-10D), although the magnitude and duration were somewhat attenuated when compared to the higher doses of cocaine (Figure 2-10D; data not shown). The absence of an effect by the HDAC5 S279E mutant is consistent with its localization in the cytoplasm in striatal neurons. These findings indicate that dephosphorylation of HDAC5 S279 in the NAc is required for HDAC5 to limit the rewarding impact of cocaine *in vivo*.

Because HDAC5 dephosphorylation was required for its ability to reduced cocaine reward behavior, we next asked whether HDAC5 dephosphorylation suppresses the development of cocaine CPP, which is the period where regulation of P-HDAC5 is observed (Figures 2-8 and 2-10D), or whether HDAC5 might be influencing the expression of CPP behavior during the test. To test this idea, we first performed cocaine versus saline context pairing prior to bilateral expression of HDAC5 S279A or GFP-only vector in the NAc and then tested for the expression of cocaine CPP. Unlike expression of HDAC5 S279A during the cocaine/context pairings (development of CPP), we observed no significant differences between vector and HDAC5 S279A treatments during the expression of cocaine CPP behavior on the test day (Figure 2-10E), indicating that dephosphorylation of HDAC5 S279 resists the development of cocaine reward behavior but does not reduce its expression.

Because HDAC5 dephosphorylation limits the development of cocaine reward, we next asked whether this mechanism might also regulate natural reward behavior, or whether the effect of HDAC5 is more specific for cocaine reward. To this end, we performed bilateral NAc injections of GFP-only control virus or the HDAC5 S279A virus and then

measured a natural reward behavior, sucrose preference. When sucrose preference was measured daily for 4 consecutive days, we observed no differences in 1% sucrose preference between mice expressing HDAC5 S279A mutant or GFP-only vector control (Figure 2-10F), suggesting that HDAC5 does not regulate natural reward behavior and may have a more specific role for substance abuse.

**Discussion**

Taken together, our findings reveal a molecular mechanism by which cocaine and cAMP signaling regulate HDAC5 nuclear accumulation to limit adaptations that increase the rewarding impact of cocaine (Figure 2-10). Our findings support the noted role for HDAC5 in limiting cocaine reward behavior (Renthal, Maze et al. 2007); however, our observations that cocaine induces transient, delayed dephosphorylation and nuclear import of HDAC5 to suppress cocaine reward are a significant departure from previous ideas of how cocaine regulates HDAC5 function *in vivo* (Renthal, Maze et al. 2007). We observed a significant regulation of HDAC5 phosphorylation and nuclear levels that strongly suggests that dynamic regulation of this epigenetic factor plays a crucial role in limiting the impact of cocaine reward *in vivo*.

Several studies have reported that cocaine exposure increases P-S259 HDAC5 levels by western blotting or immunohistochemistry (Renthal, Maze et al. 2007, Host, Dietrich et al. 2011, Dietrich, Takemori et al. 2012), but with the near-perfect conservation of amino acids spanning the P-S259 site in HDAC4, HDAC5, HDAC7, and HDAC9, it is important to note that the P-S259 antibody recognizes multiple class IIa HDAC proteins, not only HDAC5. In contrast to these reports, our study revealed a robust decrease in P-S259 and P-

S498 levels on HDAC5 (Figure 2-10B). Our analysis of the P-S279 HDAC5 site, which is also highly conserved in HDAC4 and HDAC9, revealed that total P-S279 immunoreactivity was not specific to HDAC5 (i.e., HDAC5 KO mouse tissues had significant residual P-S279 immunoreactivity). To achieve HDAC5-specific analysis of these conserved sites, we had to immunoprecipitate total HDAC5 protein prior to western blotting with the phosphorylation site-specific antibodies. In the future it will be important to determine whether the reported increases in P-S259 signal after cocaine exposure reflect specific regulation of HDAC5 or might instead represent regulation of other class IIa HDAC(s).

The binding of HDAC5 to 14-3-3 proteins is mediated by phosphorylation of S259 and S498 sites, and this association is thought to be important for HDAC5 cytoplasmic localization (McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2001, Chawla, Vanhoutte et al. 2003, Vega, Harrison et al. 2004, Sucharov, Langer et al. 2006). Similar to previous work, we observe that the HDAC5 S259A/S498A mutant protein is largely localized within the nucleus or evenly distributed between nucleus and cytoplasm. However, this mutant has significantly reduced P-S279 levels, which suggests that the increase in nuclear localization of this mutant may be due, at least in part, to reduced P-S279 levels. This conclusion is strengthened by the observation that combining the S279E phosphomimetic mutation with the S259A/S498A mutations results in increased cytoplasmic distribution of HDAC5 and resistance to cAMP-induced nuclear import. The S259A/S498A/S279E HDAC5 mutant does not bind to 14-3-3 (data not shown), which strongly suggests that P-S279 exerts its effect on HDAC5 nuclear import through a 14-3-3-independent mechanism. Several studies have reported that phosphorylation close to, or within, an NLS can mask a protein's interaction with other

proteins or inactivate its NLS function (Jans, Ackermann et al. 1991, Moll, Tebb et al. 1991). Due to the strong concentration of positively charged residues within the HDAC5 NLS, we speculate that the introduction of three negative charges by organic phosphate at S279 might neutralize the NLS charge or induce a conformational change that reduces association with nuclear import proteins.

During review of our manuscript, a study reported regulation of P-S279 HDAC5 by PKA in COS7 cells (Ha, Kim et al. 2010), and provided evidence that P-S279 promoted nuclear retention in these cells. Similar to this study, we had also found that purified PKA phosphorylates HDAC5 S279 *in vitro* (Figure 2-3A); however, we found that basal phosphorylation at this site, at least in striatal neurons, did not require PKA activity (Figure 2-3C). In addition our direct measurements of endogenous HDAC5 P-S279 levels revealed that forskolin treatment of COS7 cells, striatal neurons, cortical neurons, or acute, adult striatal slices actually decreased P-S279 HDAC5 levels (Figures 2-2B and 2-3; data not shown), which seems incompatible with the proposed role for P-S279 in the COS7 cells. We speculate that the expression of constitutively active PKA in COS7 cells may regulate additional HDAC5 sites that influence nuclear localization and require P-S279 or that overexpressed HDAC5-EGFP is regulated differently than endogenous HDAC5 in COS7 cells. Additional experiments will be required to help resolve the different conclusions drawn by these two studies, but in striatal neurons it seems clear that HDAC5 P-S279 does not promote nuclear accumulation, but quite the opposite.

Our observations about the role and regulation of HDAC5 P-S279 in cocaine-induced behavioral plasticity raise a number of interesting questions for future study. For example what is the nuclear function of HDAC5 that limits cocaine reward? Nestler and colleagues

(Renthal, Maze et al. 2007) reported that the enzymatic HDAC domain of HDAC5 is required for reducing cocaine reward, suggesting that the ultimate substrate is histone deacetylation and indirect suppression of HDAC5 target genes. Indeed, many hundreds of genes were aberrantly increased or decreased by cocaine in the HDAC5 KO mice at 24 hr after repeated cocaine injections. Because these were total HDAC5 KO mice, lacking HDAC5 expression throughout the lifetime of the animal, it is difficult to know whether these are direct effects of HDAC5 on the identified genes. Moreover, the time point analyzed (i.e., 24 hr) is during a phase when HDAC5 phosphorylation and nucleocytoplasmic localization are similar to saline control conditions. In the future it will be interesting to determine the target genes that are bound and regulated by HDAC5 after cocaine, particularly at those time points when enhanced HDAC5 nuclear function is observed following cocaine exposure. It is possible, and perhaps likely, that regulation of multiple HDAC5 gene targets contributes to the reduction of cocaine reward behavior, and dissecting out the relative contributions of each gene target will represent a major challenge going forward.

It is interesting to note that the HDAC5 S279A mutant suppressed cocaine reward to a greater extent than WT HDAC5 (Figure 2-10C). There are several possible explanations for this difference, including the following.

(1) The HDAC5 S279A mutant *in vivo* resides constitutively in the nucleus, whereas the WT HDAC5 is only transiently localized in nucleus upon cocaine exposure. In this case the levels of the P-S259/P-S498 would presumably be low such that P-S279 plays the dominant major role in subcellular localization (unlike the striatal cultures).

(2) The HDAC5 S279A mutant has reduced nuclear export kinetics compared to WT HDAC5, and as a result, resides in the nucleus for a longer time after cocaine exposure. We

found that P-S279 HDAC5 increases nuclear export kinetics (Figure 2-7B). If HDAC5 S279A cannot be rephosphorylated following nuclear import, then HDAC5 S279A may remain in the nucleus and exert longer-lasting effects following cocaine exposure.

(3) HDAC5 P-S279 may regulate not only its nuclear/cytoplasmic localization, as documented in our study, but might also regulate its function as a transcriptional corepressor in the nucleus. As such, the HDAC5 S279A mutant may be a more effective corepressor via unknown mechanisms. Due to technical limitations, we were unable to visualize the subcellular distribution of the HDAC5 mutants *in vivo*. Nevertheless, our findings in this study reveal an important role for dephosphorylation of P-S279 HDAC5 in the regulation of cocaine reward behavior.

Our findings in striatal cultured neurons revealed a high degree of colocalization of HDAC5-EGFP with endogenous MEF2A and MEF2D, two of the well-studied transcription factor proteins that interact with HDAC5, suggesting MEF2 as a possible mediator of HDAC5 function in reducing cocaine reward sensitivity after repeated cocaine experience. Consistent with this idea, we reported recently that expression of constitutively active MEF2 in the NAc enhances cocaine reward behavior (Pulipparacharuvil, Renthal et al. 2008), which is opposite of the effect of HDAC5 expression in this region. In the future, it will be important to determine whether HDAC5 exerts its effects on cocaine reward through binding to MEF2 proteins, or whether the critical nuclear target of HDAC5 in the mediation of cocaine reward may be one or more previously undescribed transcription factors. The identification of HDAC5 target genes after cocaine exposure may help determine whether MEF2 and HDAC5 bidirectionally regulate cocaine reward through a

common pathway or whether these proteins regulate cocaine behavior through distinct transcriptional mechanisms *in vivo*.

Similar to our observed regulation of HDAC5 P-S279, previous studies in striatal neurons have reported that cAMP signaling increases PP2A activity (Ahn, McAvoy et al. 2007), which then dephosphorylates the Cdk5 substrates, Wave1 (Ceglia, Kim et al. 2010) and dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) (Bibb, Snyder et al. 1999, Nishi, Bibb et al. 2000). Acute cocaine does not alter the levels or activity of Cdk5 or levels of p35 in striatum (Takahashi, Ohshima et al. 2005, Kim, Chen et al. 2006), suggesting that the decrease in P-S279 is due to increased phosphatase activity rather than decreased Cdk5 activity. Interestingly, cocaine and cAMP signaling have been shown to induce transient DARPP-32 nuclear accumulation via dephosphorylation in striatal neurons (Stipanovich, Valjent et al. 2008). Similar to our findings with HDAC5, nuclear accumulation of DARPP-32 attenuates cocaine reward behavior, which is proposed to involve epigenetic gene regulation (Stipanovich, Valjent et al. 2008). Together, these findings indicate that whereas cocaine induces rewarding effects, the striatum stimulates negative feedback processes, such as enhanced HDAC5 and DARPP-32 nuclear levels, to attenuate the reward impact of future cocaine exposures. As such, these proteins may represent critical intrinsic mechanisms for counteracting the maladaptive changes in reward circuit function, and understanding these negative feedback processes may reveal new avenues for the treatment of drug addiction.

Taken together, our findings reveal that cocaine regulates the transient nuclear accumulation of HDAC5, and this likely occurs through a molecular mechanism involving PP2A phosphatase-dependent dephosphorylation of HDAC5 at three critical

phosphoserines: S259, S279, and S498. The removal of phosphate from these sites likely increases the NLS function and decreases binding to 14-3-3 proteins, and promotes the repression of HDAC5 target genes in the nucleus. Importantly, our findings reveal that dephosphorylation of S279 HDAC5 is critical for its ability to limit the development of cocaine reward-related behavioral adaptations, but not natural reward behavior. Because cocaine-experienced HDAC5 KO mice have enhanced place preference to cocaine, and this effect is rescued by NAc expression of WT HDAC5 (Renthal, Maze et al. 2007), our combined findings suggest that HDAC5 provides a delayed braking mechanism on gene expression programs that support the development, but not expression, of cocaine reward behaviors. As such, deficits in this process may contribute to the development of maladaptive behaviors associated with addiction following repeated drug use in humans.

**Materials and Methods**

Plasmid: Herpes simplex virus (HSV)-flag-human HDAC5 plasmid was provided by Dr. Eric Nestler, and using PCR, we generated a nontagged version of HDAC5 for subcloning. The PCR fragment was subcloned into Bluescript vector, and the insert region was confirmed by DNA sequencing. The nontagged HDAC5 was then digested with XbaI and subcloned into HSV vector. Serine to alanine (S259A, S279A, S498A) and serine to glutamate (S279E) mutants of HDAC5 were generated by QuickChange Site-Directed Mutagenesis Kit.

Mice: All C57BL/6 mice (Charles River) used in this study were adult males tested between 10 and 12 weeks old. They were housed on a 12 hr light-dark cycle with access to food and

water ad libitum. All procedures were in accordance with the Institutional Animal Care and Use (IACUC) guidelines.

Generation of P-S279 HDAC5 Antibodies: Rabbits were injected with a synthesized P-HDAC5 encompassing HDAC5 amino acids 274–285, where position S279 was phosphorylated (Covance). The injected peptide was conjugated to Keyhole Limpet Hemocyanin via an N-terminal cysteine residue. Anti-P-S279 antibodies were affinity purified with peptide-conjugated Sepharose beads (SulfoLink; Pierce).

Dissociated Striatal Cultures: Embryonic striatal neurons (E18/19) were cultured from Long-Evans rats (Charles River) as described previously (Cowan, Shao et al. 2005, Pulipparacharuvil, Renthal et al. 2008). For *in vitro* experiments, primary embryonic (E18) striatal cultures were generated from Long Evans rats (Charles River Labs) as previously described (Pulipparacharuvil et al., 2008). Crude striatal cells were plated at $8.0x10^6$ /10 cm PDL (Sigma)-coated plate, $1.3x10^6$/well on PDL coated 6 well plates, or $2.4x10^6$ on PDL and Laminin coated glass coverslips in a 24-well plate in DMEM (Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), penicillin (50µg/ml)-streptomycin (50 units/ml; Sigma) and L-glutamine (4mM; Sigma) and incubated at 37C/5% CO2. 24 hours later, media was changed to supplemented Neurobasal (2% B27 (v/v); Invitrogen, penicillin (50µg/ml)-streptomycin (50 units/ml; Sigma) and L-glutamine (4mM; Sigma)).

Immunocytochemistry: Striatal neurons (E18 rat) were plated at 100,000 cells/well (24-well plate; Corning), grown on PDL/Laminin-coated glass coverslips for 8 days, and then

transfected using calcium phosphate method (Pulipparacharuvil, Renthal et al. 2008). Two days later, cells were stimulated with indicated agents. Neurons were fixed in 4% paraformaldehyde/2% sucrose in 1X PBS for 20 min at room temperature, permeabilized, and stained with indicated primary and secondary antibodies (see Supplemental Experimental Procedures). The localization of HDAC5 was categorized as cytoplasmic, nuclear, or both (evenly distributed across nucleus and cytoplasm) for each neuron under experimenter-blind conditions.

Sample Preparation from Cocaine-Injected Mice: C57BL/6 mice (Charles River) were injected once per day (intraperitoneally [i.p.]) with saline or cocaine (5 or 20 mg/kg) before rapid isolation of brain tissues at indicated times after injection. HDAC5 was immunoprecipitated from diluted total striatal lysates and analyzed by standard western blot analysis with indicated antibodies (see Supplemental Experimental Procedures for dilutions and sources). Cytosolic and nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology) according to the manufacturer's instructions.

Mass Spectrometry Analysis: HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) FBS, penicillin-streptomycin (1X; Sigma-Aldrich), and L-glutamine (4 mM; Sigma-Aldrich). HEK293T cells were transfected with HSV-flag-hHDAC5 using calcium phosphate and harvested 2 days after transfection. Flag-HDAC5 was prepared from HEK293T cell extracts in RIPA buffer (50 mM Tris [pH 7.4], 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholates, 10 mM NaF, 10 nM

okadaic acid, and complete protease inhibitor cocktail tablet [1X; Roche]) by IP with anti-flag antibody (M2)-conjugated beads. The protein was separated by SDS-PAGE and stained with Coomassie brilliant blue. The HDAC5 band was excised from the gel, washed, and then digested with trypsin. The tryptic digests were analyzed with an EC-MS/MS system.

*In vitro* PKA and Cdk5 Kinase Assay: Flag-HDAC5 was prepared from transfected HEK293T cell extracts by IP with anti-flag antibody (M2)-conjugated beads in RIPA buffer. For the PKA phosphorylation, immunoprecipitated beads were washed and suspended in PKA phosphorylation buffer (50 mM PIPES [pH 7.3], 10 mM MgCl2, 1 mM DTT, 0.1 mg/ml BSA, and protease inhibitor) and incubated with or without recombinant PKA catalytic subunit (Sigma-Aldrich) or alkaline phosphatase (Roche) in the presence of 1 mM ATP at 30°C. For the Cdk5 phosphorylation assay, the immunoprecipitated flag-HDAC5 on the beads was washed and resuspended in alkaline phosphatase buffer (Roche) and incubated with alkaline phosphatase at 37°C for 2 hr. Dephosphorylated beads were washed with RIPA buffer three times and Cdk5 kinase assay buffer (10 mM MOPS [pH 7.2], 10 mM MgCl2, 1 mM EDTA) three times, and the immunoprecipitated HDAC5 was incubated with or without Cdk5-p25 (Sigma-Aldrich) in the presence of 1 mM ATP at 30°C. After boiling SDS sample buffer to elute from the beads, the incubated IP samples were subjected to western blotting analysis.

*In vitro* PP2A Dephosphorylation Assay: Immunoprecipitated HDAC5 from striatal neurons in RIPA buffer was washed with dephosphorylation buffer (50 mM Tris-HCl [pH 8.5], 20 mM MgCl2, 1 mM DTT, protease inhibitor cocktail [1X; Roche]) five times and incubated

with or without 2.5 U of purified PP2A (Promega) at 30°C for 60 min. Proteins were subjected to western blotting analysis.

Viral-Mediated Gene Transfer: Expression plasmids for HDAC5 WT, S279A, and S279E mutants in HSV vector were packaged into high-titer viral particles as described previously (Barrot, Olivier et al. 2002). Stereotactic surgery was performed on mice under general anesthesia with a ketamine/xylazine cocktail (10 mg/kg:1 mg/kg). Coordinates to target the NAc (shell and core) were +1.6 mm anterior, +1.5 mm lateral, and −4.4 mm ventral from bregma (relative to dura) at a 10° angle. Virus was delivered bilaterally using Hamilton syringes at a rate of 0.1 μl/min for a total of 0.5 μl. Viral placements were confirmed by GFP signal, which was coexpressed in each virus.

Cocaine conditioned place preference: Mice were conditioned to cocaine using an unbiased accelerated paradigm to accommodate the timing of transient HSV expression (Barrot, Olivier et al. 2002, Renthal, Maze et al. 2007). Additional details can be found in the Supplemental Experimental Procedures.

Sucrose Preference: Single housed mice were provided tap water in two identical double-ball-bearing sipper-style bottles for 2 days followed by 2 days of 1% (w/v) sucrose solution to allow for acclimation and to avoid undesired effects of neophobia (Green, Alibhai et al. 2006). The next day mice underwent stereotactic injections of control or HDAC5 virus into the NAc (bilaterally, as described above for CPP assays). Forty-eight hours after viral injection, mice were again given two bottles: one containing water, and the other

containing 1% sucrose solution. The consumption of water versus sucrose was measured after 24, 48, 72, and 96 hr of access to the bottles to determine preference for sucrose (Renthal, Maze et al. 2007). Bottle positions of water and sucrose were swapped each day of testing to avoid potential drinking side bias.

Statistics: One-way, two-way, or repeated-measures ANOVAs with Tukey's multiple comparison post hoc tests were used to analyze the following: western blotting, phosphorylation level of S279, nuclear/cytoplasmic ratio of HDAC5 with cocaine exposure, CPP, sucrose preference, and rates of nuclear export and import of HDAC5. Student's t tests were used to analyze HDAC5-EGFP localization, western blotting for phosphorylation level of S279 for samples treated with roscovitine, forskolin, okadaic acid, tautomycetin, and for the *in vitro* dephosphorylation assay with PP2A, cocaine-treated samples compared to saline controls, and averaged sucrose preference data.

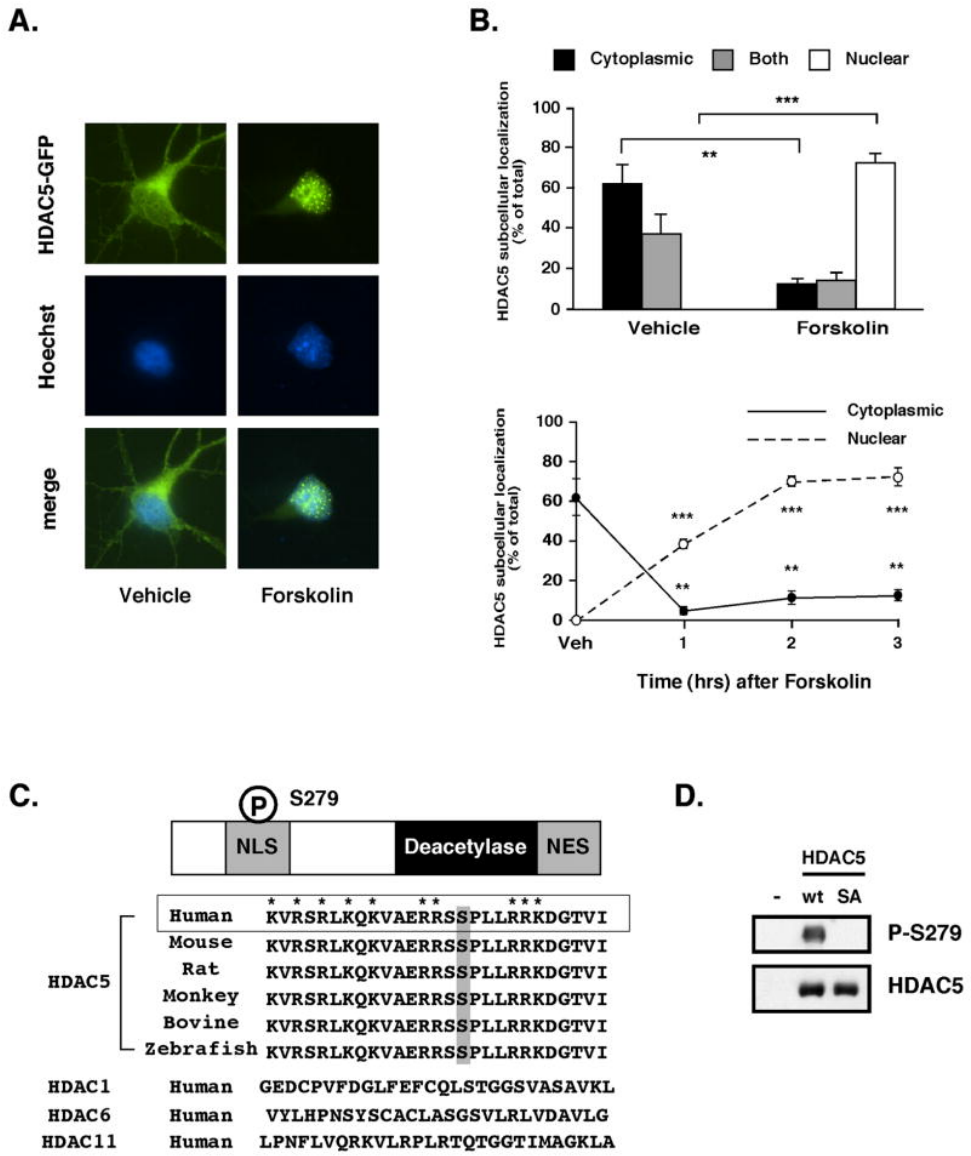


Figure 2-1. Elevation of cAMP Induces Nuclear Import of HDAC5 in Striatal Neurons. AB. Striatal neurons transfected with hHDAC5-EGFP were treated with either vehicle or forskolin (10 μM).

A. Representative image showing nuclear import of HDAC5-EGFP after forskolin treatment for 3 hrs. Sections were counterstained with Hoechst.

B. (top) The localization of HDAC5 was categorized as cytoplasmic, nuclear, or both for individual cells by an experimenter blind to treatment. The percentage for each category was calculated from the total number of transfected neurons counted in each condition, and the average across experiments is shown (mean +/− SEM, ** and *** indicate $p < 0.01$ and $p < 0.001$, Student's t-test; n = 3 wells/condition, ~96 transfected cells were counted in each well). (Bottom) Kinetics of nuclear import of HDAC5 after forskolin treatment (mean +/− SEM, ** and *** indicate $p < 0.01$ and $p < 0.001$ compared to vehicle condition, Student's t-test; n = 3 wells/condition, ~108 transfected cells were counted in each well).

C. The molecular structure of HDAC5. HDAC5 is depicted (upper portion), and is composed of a nuclear localization signal (NLS), a histone deacetylase domain and a nuclear export signal (NES). The Cdk5 phosphorylation site (S279) is shown within the NLS region. The lower portion depicts the amino acid sequence surrounding the S279 site for HDAC5 compared to other species and non-class IIa HDACs. * indicates positively charged amino acids.

D. Characterization of P-S279 HDAC5 antibodies. HEK-293T cells were transfected with GFP-tagged wild type (WT) and S279A (SA) HDAC5 expression plasmids. Phosphorylation site-specific immunoreactivity was detected only in the lysate containing WT HDAC5 and not in lysate containing the S279A phosphorylation mutant. Total expression levels of transfected hHDAC5-EGFP were confirmed using anti-GFP antibody.

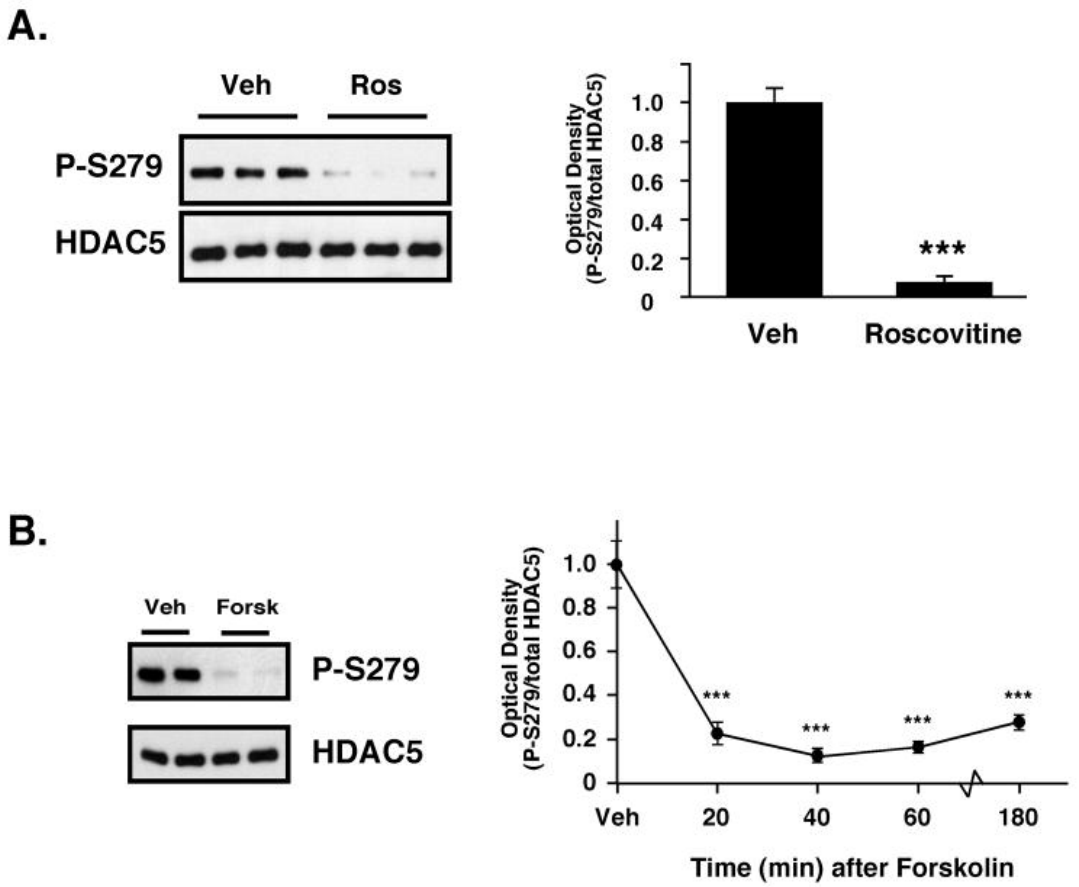


Figure 2-2. cAMP Signaling Promotes Dephosphorylation of S279, a Cdk5 Phosphorylation Site of HDAC5.
A. Cultured striatal neurons were treated with vehicle or the Cdk5 inhibitor roscovitine (50 μM) for 3 hrs. IPed HDAC5 from cultured striatal neurons using anti-HDAC5 antibody was immunoblotted for P-S279 and HDAC5 (mean +/− SEM, *** indicates $p < 0.001$, Student's t-test, n = 3/condition).
B. Cultured striatal neurons were treated with forskolin (10 μM) and lysed 20, 40, 60 or 180 min afterwards. IPed HDAC5 was analyzed by immunoblotting with anti-P-S279 and anti-HDAC5 antibodies. Representative blots show treatment for 180 min. (mean +/− SEM, *** indicates $p < 0.001$ compared to vehicle, Student's t-test; n = 4/condition, two independent experiments).

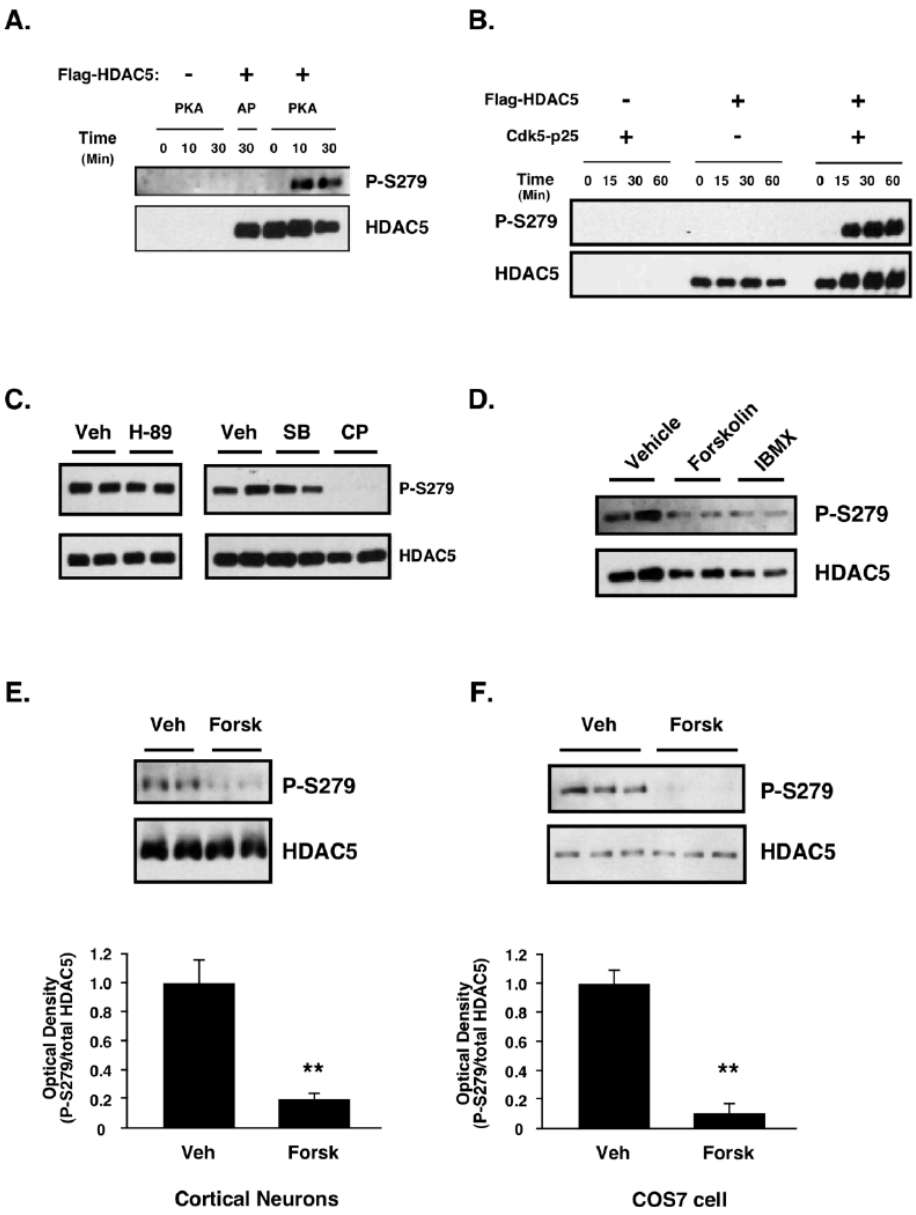


Figure 2-3. cAMP Signaling Induces Dephosphorylation of HDAC5 S279, a Cdk5 Phosphorylation Site.
A and B. PKA and Cdk5 phosphorylate HDAC5 at S279 in vitro. Immunoprecipitated flag-HDAC5 from HEK-293T cells were incubated with or without recombinant PKA, Cdk5 or alkaline phosphatase and analyzed by immunoblotting with P-S279 and HDAC5 antibodies.
C. Pharmacological inhibition of Cdk5, but not PKA or p38 MapK, reduces endogenous phosphorylation of HDAC5 at S279. Cultured striatal neurons were treated with inhibitors for Cdk5 (CP681301 50 M), PKA (H89 20 M) and p38 MapK (SB203580 1 M). Immunoprecipitated HDAC5 was analyzed by western blotting with anti-HDAC5 and P-S279 antibodies.
D. cAMP signaling induces dephosphorylation of HDAC5 at S279. Cultured striatal neurons were treated with forskolin (10 M) and the nonselective phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 200 M). Immunoprecipitated HDAC5 was analyzed by western blotting with anti-HDAC5 and P-S279 antibodies.
E and F. Elevation of cAMP induces dephosphorylation of HDAC5 at S279 in cortical neurons and non-neuronal cells. Cultured cortical neurons and COS7 cells were treated with forskolin (10 M). Immunoprecipitated HDAC5 was analyzed by western blotting with anti-HDAC5 and P-S279 antibodies (mean +/- SEM, ** indicates $p < 0.01$, Student's t-test; n = 4 or 3 per condition for cortical neurons and COS7 cells respectively).

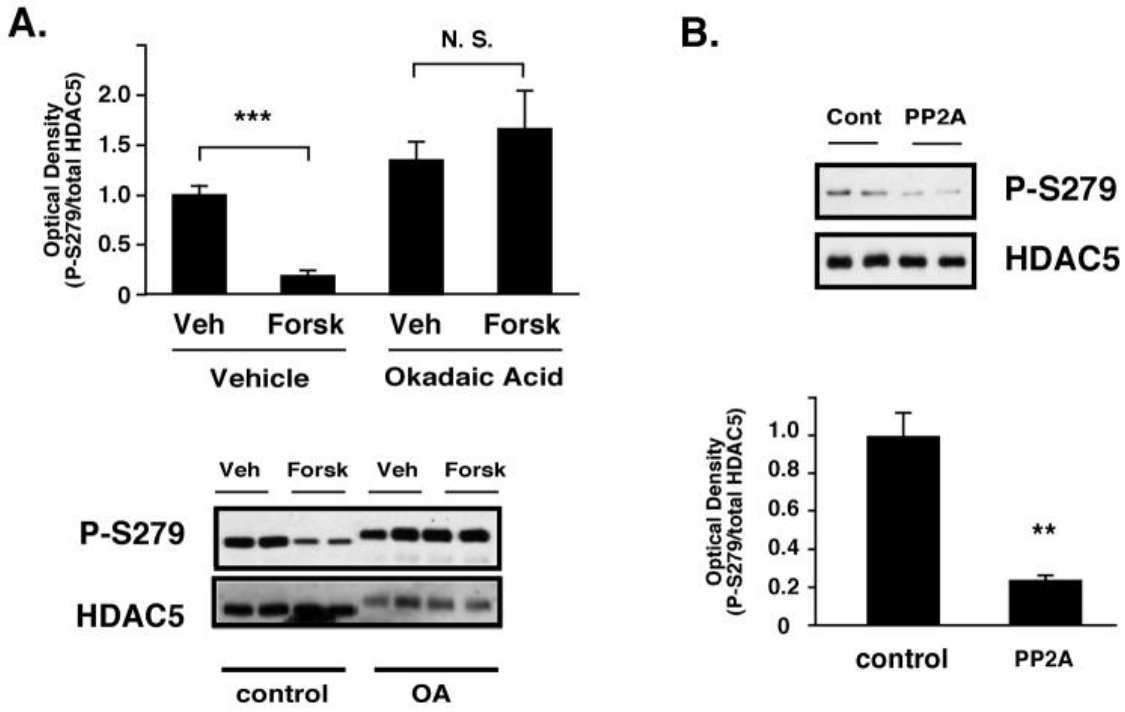


Figure 2-4. PP2A Activity is Required for cAMP-Induced Dephosphorylation of S279 HDAC5.
A. Okadaic acid blocks cAMP-induced dephosphorylation of HDAC5. Cultured striatal neurons were treated with forskolin (10 μM) in the presence or absence of okadaic acid (50 nM). IPed HDAC5 was analyzed by immunoblotting with P-S279 and anti-HDAC5 antibodies (mean +/− SEM, *** indicates $p<0.001$ compared to vehicle condition, Student's t-test; n = 6/condition from three independent experiments).
B. S279 was dephosphorylated by PP2A in vitro. IPed HDAC5 protein from striatal cultures was incubated in the absence or presence of PP2A (mean +/− SEM, ** indicates $p < 0.01$, Student's t-test; n = 3/condition from two independent experiments).

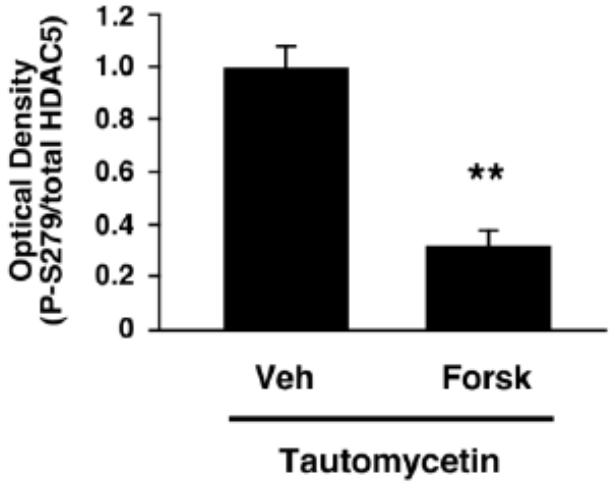


Figure 2-5. cAMP-Induced Dephosphorylation is Independent of Protein Phosphatase 1 (PP1) Activity. Dissociated striatal neurons were treated with forskolin (10 M) in the presence of protein phosphatase 1 inhibitor, tautomycetin (100 nM). Immunoprecipitated HDAC5 from striatal neurons was analyzed by western blotting using P-S279 and HDAC5 antibodes (mean +/- SEM, ** indicates $p < 0.01$, Student's t-test; n = 3 in each condition).

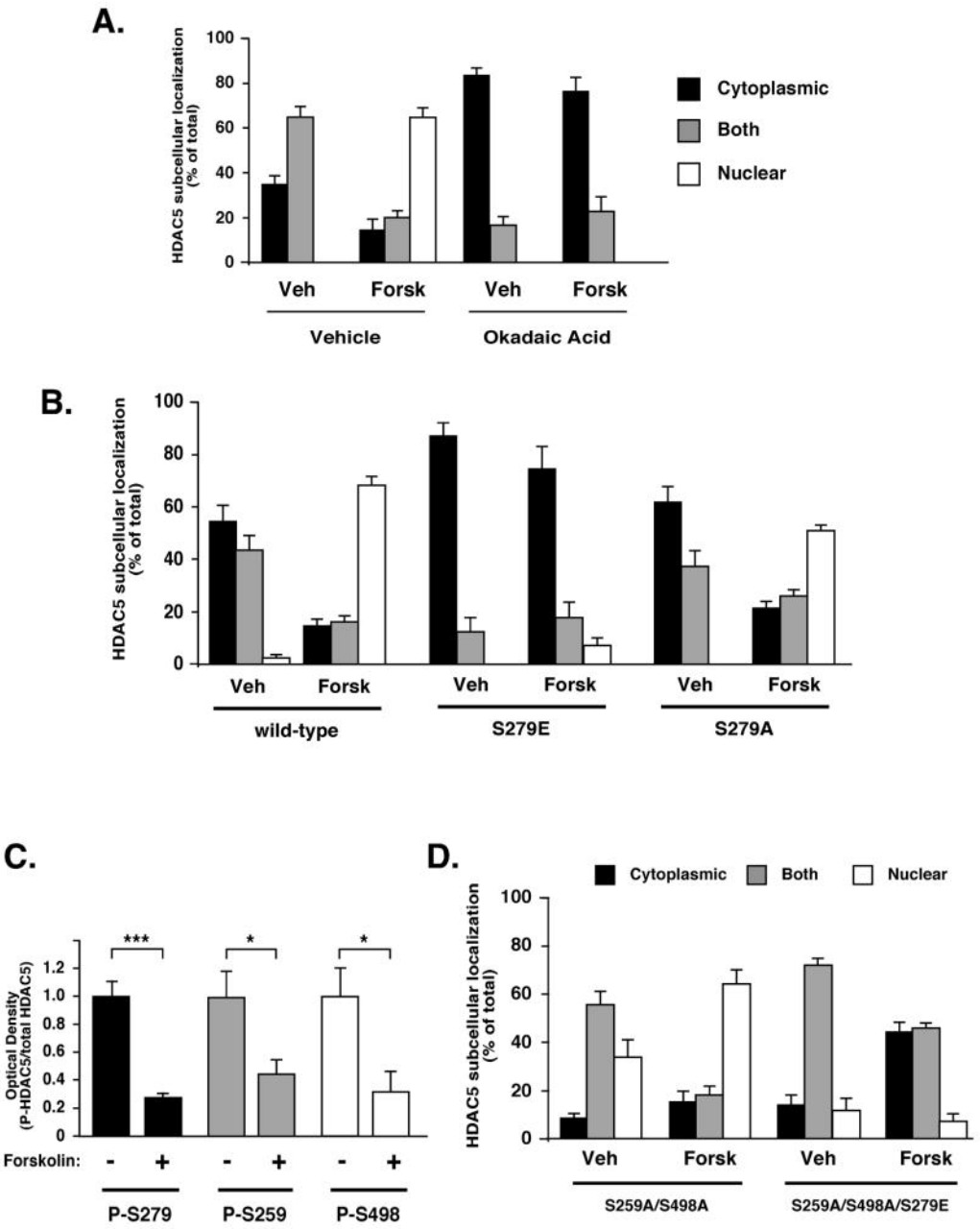


Figure 2-6. Dephosphorylation of S279 is necessary for cAMP-induced nuclear import of HDAC5.

A. Okadaic acid prevents cAMP-induced nuclear import of HDAC5. Transfected neurons were treated with vehicle or forskolin (10 μM) in the absence or presence of okadaic acid (50 nM) for 3 hrs (mean +/− SEM, n = 3 wells in each conditions, ~47 transfected neurons were counted in each well).

B. The phosphorylation mimic mutant S279E prevents cAMP-induced nuclear import of HDAC5. Transfected neuron with WT, phosphorylation mimetic mutant S279E and non-phosphorylation mutant S279A HDAC5-EGFP were treated with vehicle or forskolin (10 μM) for 3 hrs (mean +/− SEM, n = WT, S279E, and S279A, (9, 6 and 6) wells in each condition, ~83 transfected neurons were counted in each well).

C. cAMP signaling induces dephosphorylation of S279, S259 and S498. Cultured striatal neurons were treated with forskolin (10 μM) for 3 hrs. IPed HDAC5 was analyzed by immunoblotting with anti-P-S259 and P-S498 antibodies (mean +/− SEM, * indicates $p < 0.01$, Student's t-test; n = 6–8 in each condition from two independent experiments).

D. Dephosphorylation of S279 is required for cAMP-induced nuclear import of S259A/S498A mutant HDAC5. Transfected neurons with S259A/S498A mutant and S259A/S498A/S279E mutant HDAC5-EGFP were treated with vehicle or forskolin (10 μM) for 3 hrs (mean +/− SEM, n = 6 in each condition, ~81 transfected neurons were counted in each well).

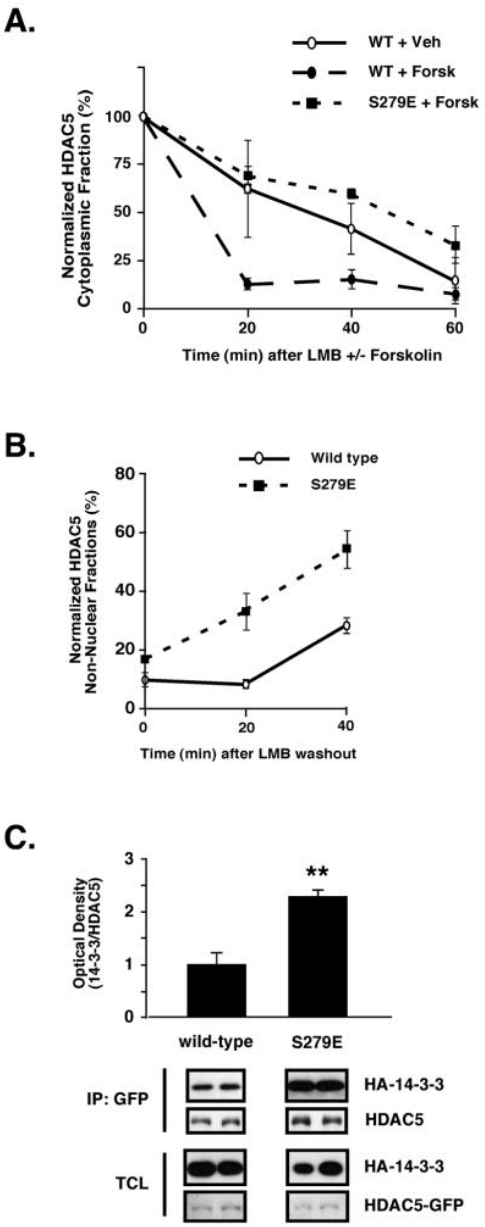


Figure 2-7. Phosphorylation of S279 controls nuclear import and export rate of HDAC5 and binding affinity to 14-3-3.

A. Phosphorylation of S279 decreases the nuclear import rate of HDAC5. Transfected neurons were treated with or without forskolin (10 μM) in the presence of Leptomycin B (10 ng/ml). The percentage of cytoplasmic HDAC5 protein in each time point was normalized to the percentage of cytoplasmic HDAC5 in the basal condition (ANOVA, $F2, 24 = 8.85$, $p<0.01$; Tukey's post hoc analysis, WT/vehicle versus WT/Forsk, $p<0.05$, WT/Forsk versus S279E/Forsk, $p<0.01$; n = 3 wells/condition and each time point, ~45 transfected neurons were counted in each well).

B. Phosphorylation of S279 accelerates nuclear export of HDAC5. Transfected neurons were treated with Leptomycin B (10 ng/ml) for 8 hrs. An average of the percent of nuclear exported HDAC5 (localized in cytoplasm or evenly distributed between nucleus and cytoplasm) to the total transfected neurons at two time points following washout of Leptomycin B is shown. There were significant effects of both HDAC5 mutant (ANOVA, $F1, 12 = 35.214$, $p<0.001$) and time after wash out (ANOVA, $F2, 12 = 27.0$, $p < 0.001$); n = 3 wells for each condition, average 50 transfected neurons were counted in each well.

C. S279E mutant has greater binding affinity to 14-3-3 than WT HDAC5. HEK-293T cells were co-transfected with GFP-tagged HDAC5 and HA-tagged 14-3-3 epsilon. Two days after transfection, HDAC5-EGFP protein was IPed using anti-GFP antibodies. IPs were analyzed by western blotting using HDAC5 and HA-tag antibodies (mean +/− SEM, ** indicates $p < 0.01$, Student's t-test; n = 3 in each condition).

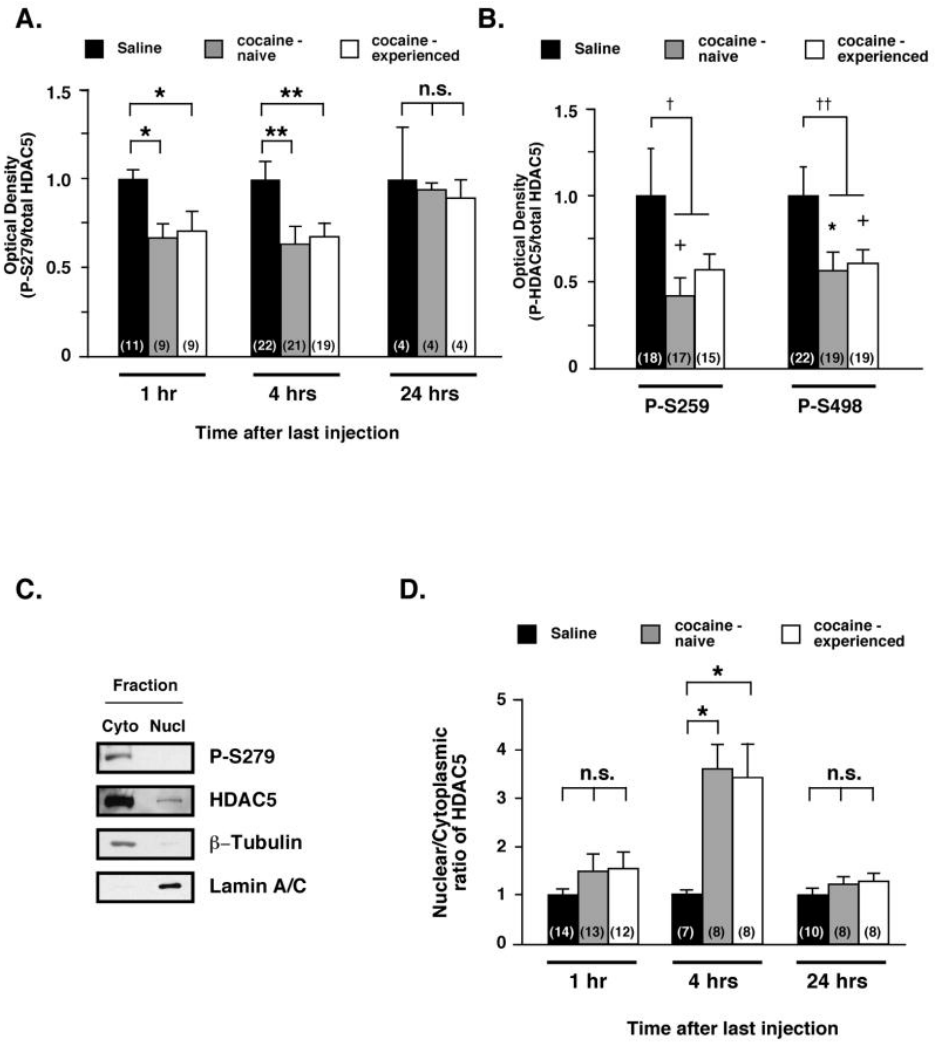


Figure 2-8. Cocaine Exposure Transiently Decreases Phosphorylation of HDAC5. Adult C57Bl/6 mice were injected with saline for 7 days (1 mL/kg, IP, 1x/day; control), with saline for 6 days followed by 1 day of cocaine (20 mg/kg; IP; cocaine-naïve) or with cocaine for 7 days (20 mg/kg; IP; cocaine-experienced).

A. Striatal tissue was dissected out 1, 4 or 24 hrs after the last injection and immunoprecipitated HDAC5 was subjected to western blotting using P-S279 and HDAC5 antibodies (mean +/− SEM, separate Univariate ANOVAs were performed for each time point: 1 hr, $F_{2, 26} = 5.481$, $p=0.01$, 4 hrs, $F_{2, 59} = 7.006$, $p<0.01$, and 24 hrs, $F_{2, 9} = 0.091$, $p>0.05$; significant Tukey's post hoc results compared to saline are indicated by * and **, $p<0.05$ and $p<0.01$, respectively; n = saline, cocaine-naïve or cocaine-experienced: 1 hr (11, 9 and 9), 4 hrs (22, 21 and 19), and 24 hrs (4, 4 and 4)).

B. Immunoprecipitated HDAC5 from striatal tissue at 4 hrs after the last injection was analyzed by western blotting using P-S259, P-S498 and HDAC5 antibodies (mean +/− SEM, separate Univariate ANOVAs were performed for P-S259 and P-S498; P-S259, $F_{2,47} = 2.894$, $p=0.065$, P-S498, $F_{2,57} = 4.297$, $p<0.05$, Tukey's post hoc results compared to saline are indicated by *($p<0.05$) and + ($p=0.06$); n = saline, cocaine naïve and cocaine experienced, P-S259 (18, 17 and 15), P-S498 (22, 19 and 19). Combined cocaine-treated groups were significantly different from saline control groups for both P-S259 and P-S498, Student's t-tests, † and ††, $p<0.05$ and $p<0.01$, respectively.

C. Striatal tissue was fractionated into cytoplasmic and nuclear fractions, and confirmed by blotting with β-tubulin and Lamin A/C, respectively.

D. Subcellular fractionation after cocaine exposure demonstrated transient nuclear accumulation of HDAC5 in striatum. Nuclear/cytoplasmic ratios of HDAC5, relative to saline, were calculated by comparing arbitrary units of nuclear and cytoplasmic HDAC5 (normalized to Lamin A/C in nuclear fraction and to β-tubulin in cytoplasmic fraction), (mean +/− SEM, Separate Univariate ANOVAs, 1 hr, $F_{2,36} = 1.283$, $p>0.05$, 4 hrs, $F_{2,20} = 7.446$, $p<0.01$, 24 hrs, $F_{2,23} = 2.005$, $p>0.05$; for Tukey's post hoc analysis * indicates $p<0.05$, n = saline, cocaine-naive and cocaine-experienced: 1 hr (14, 13 and 12), 4 hrs (7, 8 and 8), and 24 hrs (10, 8 and 8)).

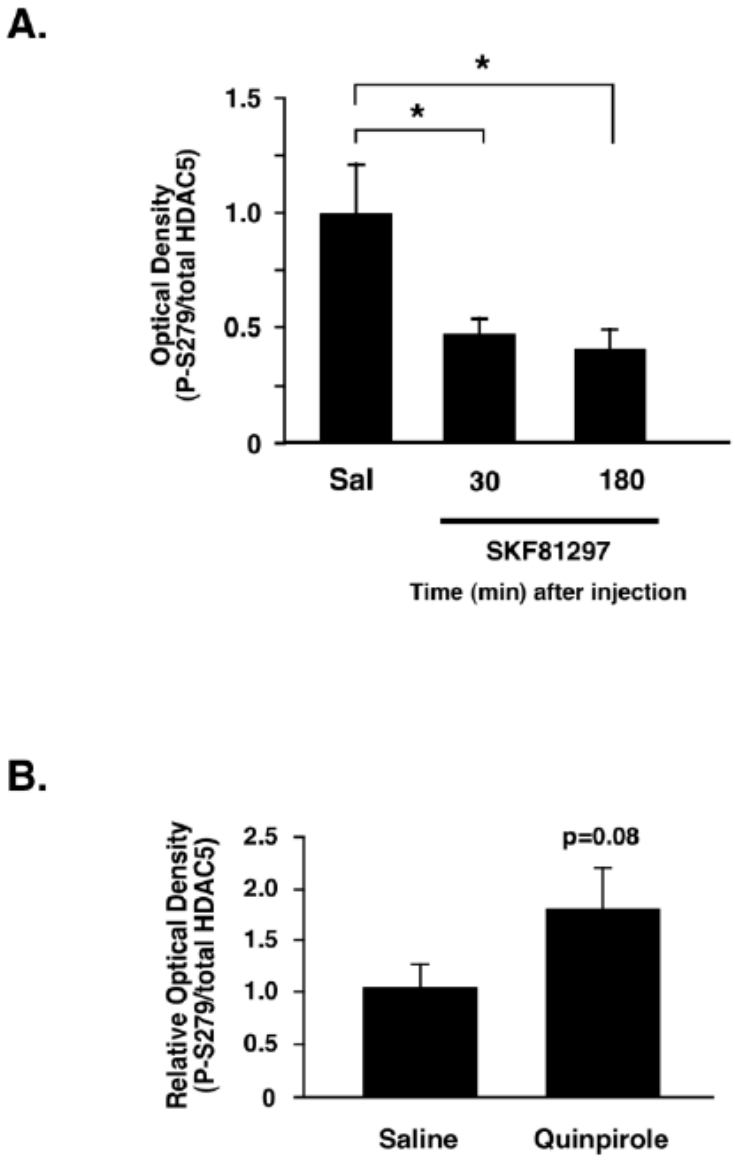


Figure 2-9. Dopamine D1 but not D2 receptor agonist exposure decreases phosphorylation of HDAC5.
A. Dopamine D1 signaling induces dephosphorylation of S279 HDAC5. Dopamine D1-type receptor agonist, SKF81297 (5 mg/ml), was IP injected into adult male C57Bl/6 mice. Immunoprecipitated HDAC5 from striatal tissues at 30 or 180 minutes after injection was analyzed by western blotting using P-S279 antibody (mean +/- SEM, * indicates $p < 0.05$ compare to saline condition, Student's t-test; n = 4-5 in each condition).
B. Dopamine D2 receptor signaling stimulates a trend toward increased HDAC5 P-S279. Adult C57Bl/6 mice were injected with D2-type dopamine receptor agonist, quinpirole (5 mg/ml). Three hours following injection, striatal tissue was dissected. Immunoprecipitated HDAC5 from striatum was analyzed with P-S279 and HDAC5 antibodies (mean +/- SEM, $p = 0.08$, Student's t-test; n = 6 and 5 in saline and quinpirole, respectively).

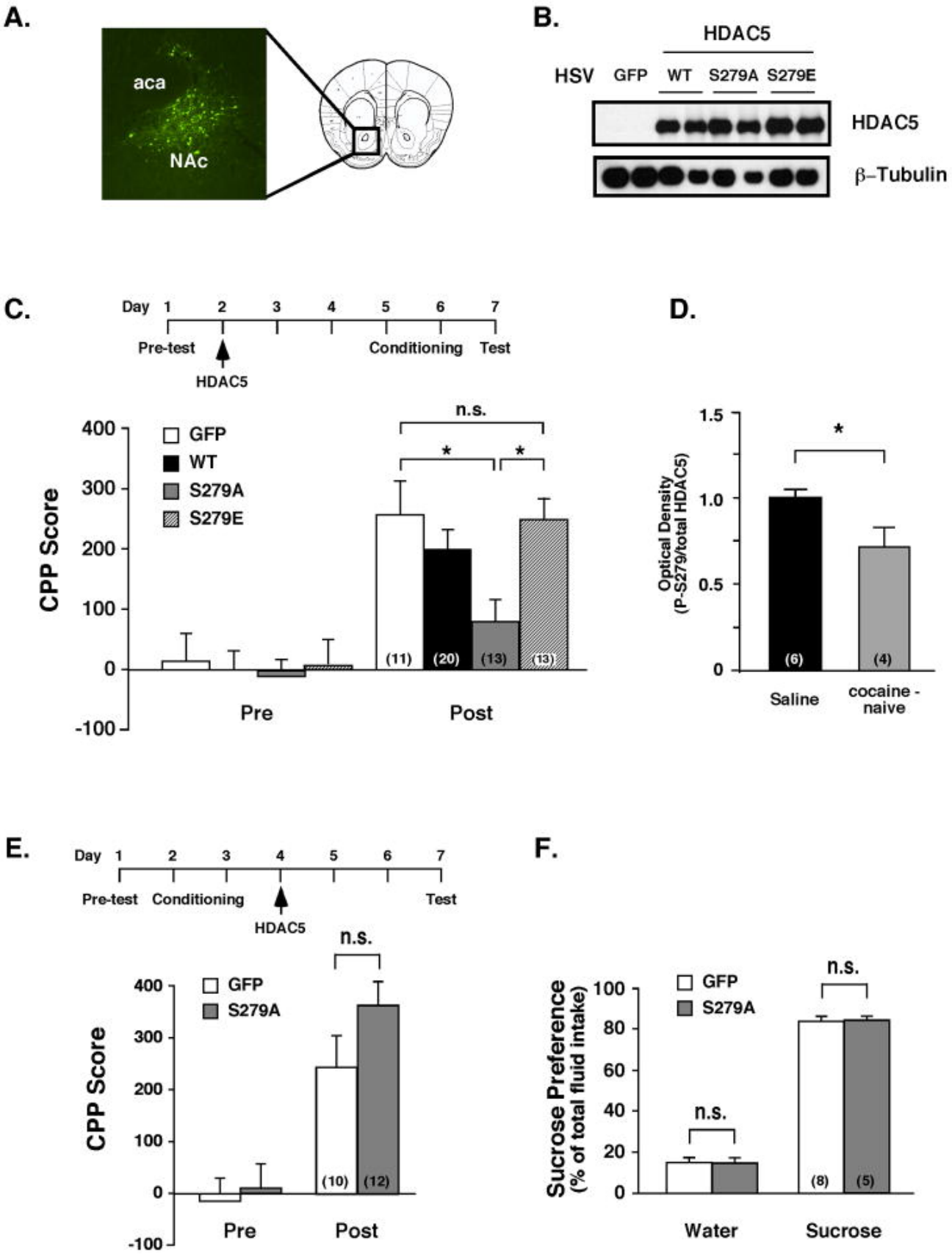


Figure 2-10. Dephosphorylation of HDAC5 S279 in the NAc limits the development of cocaine reward behavior. Legend on following page.

Figure 2-10. Dephosphorylation of HDAC5 S279 in the NAc limits the development of cocaine reward behavior. Continued

A. Representative image of HSV-mediated gene expression in the NAc. GFP expression was used to confirm virus injection placement.

B. Western blotting showing similar expression levels of WT HDAC5, S279A and S279E mutants in virus-infected cultured dissociated striatal neurons.

C. (top) Time course of CPP paradigm; (bottom) conditioned place preference (CPP) to cocaine (5 mg/kg) was attenuated by overexpression of the S279A mutant, but not by the S279E mutant ($F_{3, 53} = 3.797$, $p<0.05$; * indicates $p < 0.05$, Tukey post hoc analysis; n = 11, 20, 13 and 13 in GFP, wild type, S279A and S279E, respectively). CPP Score was defined as the time (sec) spent in the cocaine side minus the saline side for both pre- (Pre) and post-conditioning (Post) trials.

D. Immunoprecipitated HDAC5 from striatal tissue at two hrs after last cocaine (5 mg/kg) injection was analyzed by western blotting using P-S279 and HDAC5 antibodies (mean +/− SEM, * indicates $p < 0.05$, Student's t-test; n = 6 and 4 in each condition).

E. (top) Time course of CPP paradigm; (bottom) conditioned place preference (CPP) to cocaine (5 mg/kg) was not attenuated when the HDAC5 S279A mutant was overexpressed specifically during the expression of reward behavior. The stereotactic surgery to introduce HDAC5 S279 mutant in the NAc was performed at day 4 following conditioning (mean +/− SEM, n.s. indicates $p>0.05$, Student's t-test; n = 10 and 12 in each condition). CPP score was defined as the time (sec) spent in the cocaine side minus the saline side for both pre- (Pre) and post-conditioning (Post) trials.

F. Sucrose preference was not limited by overexpression of the HDAC5 S279A mutant. Mice injected with GFP or HDAC5 S279A mutant in the NAc showed indistinguishable preferences for either water or 1% sucrose (mean +/− SEM, n.s. indicates $p > 0.05$, Student's t-test; n = 8 and 5 in each conditions). Preference was defined as the percentage of water or 1% sucrose intake relative to total liquid consumption in each 24 hr period for 4 days.

# Chapter 3: Nuclear Histone Deacetylase 5 attenuates cue and prime-dependent reinstatement in an intravenous self-administration rat model and in the conditioned place preference assay

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## Author Contributions

MBC, MT, DWS and CWC designed experiments. MBC, MT and DG conducted experiments. DG and DWS provided tissue for figure 3-2. RLN prepared Herpes Simplex Viruses used in this study. MBC prepared this manuscript.

**Summary**

Epigenetic mechanisms represent a novel line of investigation in cocaine-related behaviors. One family of epigenetic molecules that may underlie cocaine behavioral and functional changes is the histone deacetylases, HDACs, which act to mediate transcriptional repression. One member, HDAC5, is regulated by experimenter-administer cocaine and plays a role in cocaine conditioned place preference. In this study, we report the regulation of localization of HDAC5 using the rat intravenous self-administration model and demonstrate a modest nuclear accumulation in ventral striatum of cocaine self-administering animals. We then analyzed the role of HDAC5 in self-administration behavior and report an effect of nuclear HDAC5 in limiting cue- and prime-dependent reinstatement of seeking in extinguished animals. These effects were observed in the absence of an effect on stable intake, dose response or progressive ratio. We next assessed the dependence of the effect of nuclear HDAC5 on its primary downstream binding partner, MEF2. Binding and inhibition of MEF2 by HDAC5 was abolished by generating a MEF2 binding domain deficient nuclear HDAC5, HDAC5 3SAΔMBD. HDAC5 3SAΔMBD is not able to repress any of the behaviors assayed in the rat IVSA paradigm despite pheno-copying the effect of nuclear HDAC5 (3SA) in the mouse conditioned place preference assay. Together, our findings propose nuclear HDAC5 function limits aspects of cocaine addiction-like behavior.

**Introduction**

Addiction and substance abuse are devastating conditions that result in a significant strain on the individual as well as on global healthcare costs. Despite adverse

consequences, however, addicted individuals continue to seek and take substances of abuse (Degenhardt and Hall 2012). Identification of mechanisms that mediate the transition from substance use to abuse and addiction are therefore a key goal of this field of study as they may elucidate aberrant plasticity associated with addiction and provide novel targets for therapies (Kalivas 2004, Nestler and Malenka 2004, Hyman, Malenka et al. 2006, Maze and Nestler 2011). One such mechanism involves epigenetic modifications that are defined as long-lasting changes to chromatin structure resulting in either increased or decreased gene transcription (Jiang, Langley et al. 2008). These modifications may then result in key alterations to cellular function which mediate addiction related behavioral and/or structural plasticity (Kumar, Choi et al. 2005, Renthal, Maze et al. 2007, Renthal, Kumar et al. 2009, LaPlant, Vialou et al. 2010, Maze, Covington et al. 2010, Wang, Lv et al. 2010, Malvaez, Mhillaj et al. 2011).

Consistent with a proposed role for epigenetic regulation in mediating substance-abuse dependent plasticity, several studies (Kumar, Choi et al. 2005, Renthal, Maze et al. 2007, Wang, Lv et al. 2010) have implicated the class IIa histone deacetylases (HDACs) as playing a key role in the nucleus accumbens (NAc), a key striatal site of action for cocaine, in limiting cocaine reward. The class IIa HDACs contains four family members (HDAC4, 5, 7 and 9). HDACs act in the nucleus to remove acetyl groups from histone tails resulting in a transcriptionally repressed state (Parra and Verdin 2010). The class IIa HDACs are unique in that they shuttle between cytoplasm and nucleus in an activity-dependent manner. Elevations of intracellular calcium result in $Ca^{2+}$-calmodulin-dependent kinase (CaMK) superfamily-dependent phosphorylation of two key residues, S259 and S498, subsequent binding to 14-3-3 proteins and localization in the cell cytoplasm (Wang, Bertos et al. 1999,

Grozinger and Schreiber 2000, Lu, McKinsey et al. 2000, Lu, McKinsey et al. 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2001, Backs, Backs et al. 2008, Nishino, Miyazaki et al. 2008). Dephosphorylation of these sites, conversely, reduces class IIa HDAC binding to 14-3-3, increases nuclear accumulation, and in many cases, promotes transcriptional repression (Chawla, Vanhoutte et al. 2003, Belfield, Whittaker et al. 2006, Taniguchi, Carreira et al. 2012). In the NAc, a key reward region, cocaine has been shown to antagonize $Ca^{2+}$-dependent and Cdk5-dependent phosphorylation of HDAC5 at three serine sites (S258, S279 and S498) in a cAMP- and PP2A-mediated signaling cascade. The dephosphorylation of HDAC5 in response to either acute or chronic cocaine experience results in its robust, but transient, nuclear accumulation(Taniguchi, Carreira et al. 2012).

Class IIa HDACs do not appear to directly bind to DNA in the nucleus, but instead appear to associate indirectly with genomic DNA (Jiang, Langley et al. 2008). However, few nuclear transcription factors have been identified as binding partners for the class IIa HDACs. One well-described target is the family of myocyte enhancer factor 2 (MEF2) transcription factors (Lu, McKinsey et al. 2000, Lu, McKinsey et al. 2000, Bertos, Wang et al. 2001, Belfield, Whittaker et al. 2006). The MEF2 transcription factors plays a key role regulating synapse number in neurons through its activity-dependent elimination of glutamatergic synapses (Flavell, Cowan et al. 2006, Barbosa, Kim et al. 2008, Pfeiffer, Zang et al. 2010). Importantly, reductions of MEF2 in the NAc *in vivo* result in increased dendritic spine density of medium spiny neurons in the NAc (Pulipparacharuvil, Renthal et al. 2008). In addition, expression of constitutively-active MEF2 in the NAc dramatically reduces cocaine-induced spine density increases, suggesting that the negative regulation of MEF2 *in vivo* by cocaine-dependent processes may be important for structural plasticity following

cocaine experiences. Likewise, overexpression of constitutively-active MEF2 in NAc promotes conditioned place preference behavior, an effect which suggests a novel role for the well-characterized spine plasticity events of cocaine in opposing, rather than promoting, behavioral plasticity (Pulipparacharuvil, Renthal et al. 2008).

Consistent with a potential role for HDAC5-dependent attenuation of MEF2 activity *in vivo* following cocaine, two recent studies have demonstrated that cAMP-dependent signaling attenuates MEF2 activity in neurons (Belfield, Whittaker et al. 2006, Pulipparacharuvil, Renthal et al. 2008). *In cultured* striatal neurons, cAMP elevation triggers nuclear accumulation of HDAC5 and strong co-localization with MEF2 proteins in the nucleus (Taniguchi, Carreira et al. 2012). In addition, we recently reported that cocaine and cAMP trigger delayed nuclear import of HDAC5 in striatal neurons *in culture* and *in vivo*, and we showed that nuclear accumulation of HDAC5 in the NAc functions to limit the development of cocaine conditioned place preference (Taniguchi, Carreira et al. 2012). Taken together, these findings suggest a mechanism by which HDAC5 acts upstream of MEF2 to limit MEF2-dependent transcription and cocaine-related behaviors.

In this study, we first asked whether self-administered cocaine recruits the same HDAC5 nuclear import mechanism observed with experimenter-administered cocaine. Next we tested whether enhanced nuclear HDAC5 modulates self-administered cocaine behaviors, and whether HDAC5's ability to regulate cocaine behaviors requires its association with MEFs transcription factors. The overarching hypothesis of this study is that self-administration of cocaine promotes nuclear accumulation of HDAC5 and that nuclear HDAC5 limits addiction-related behavioral phenotypes, possibly through regulation of MEF2 activity in the NAc.

## Results

### Cocaine intravenous self-administration induces weak nuclear accumulation of HDAC5 in ventral, but not dorsal, striatum

A previous study reported that following cocaine administration there is a transient (30 minutes) increase in pS259 signal (presumed to be from HDAC5) in NAc tissue homogenates (Renthal, Maze et al. 2007, Host, Dietrich et al. 2011, Dietrich, Takemori et al. 2012). We reported recently that cocaine administration regulates the levels of a novel Cdk5 site found within the nuclear localization signal (NLS) of HDAC5, serine 279 (S279). We showed that phosphorylated HDAC5 promotes 14-3-3 binding and cytoplasmic localization in a coordinated manner with the previously characterized CaMK sites, S259 and S498. In striatal culture neurons, the cAMP-induced dephosphorylation and nuclear accumulation depends upon protein phosphatase 2A (PP2A) activity, consistent with a known cAMP-dependent upregulation of PP2A activity in the striatum (Ahn, McAvoy et al. 2007, Ceglia, Kim et al. 2010). Upon experimenter-administered cocaine or elevation of cAMP signaling by systemic D1 receptor agonists (figure 2-9A), we observed significant dephosphorylation of P-S259, P-S279 and P-S498 sites and nuclear accumulation of HDAC5 in the striatum *in vivo*. This dephosphorylation and nuclear accumulation of HDAC5 was transient (1-4 hrs post-cocaine) since it returned to baseline levels by 24 hrs. The *in vivo* characterization in mouse was conducted by administering low to moderate cocaine doses via intraperitoneal (i.p.) injections. We observed a large reduction in P-HDAC5 levels in the striatum, and corresponding increase in nuclear HDAC5 accumulation, with 20mg/kg coc, but only a modest change at 5mg/kg coc (Taniguchi, Carreira et al. 2012). The observation

that lower doses seem to promote a more modest nuclear shift of HDAC5 indicates an expected dose dependence of the HDAC5 responses. These findings led us to question whether the dynamics and style of administration of cocaine influenced the response of HDAC5 phosphorylation and localization.

We aimed at testing the hypothesis that the dephosphorylation and nuclear accumulation of HDAC5 are mechanisms recruited by cocaine experience using the intravenous self-administration model – a model of contingent drug taking behaviors. To test this hypothesis we analyzed the subcellular distribution of HDAC5 following 10 days of fixed ratio 1 cocaine intake on a 1.0mg/kg dose/infusion. With this dose most animals achieve stable intake (defined as variability within 20% in the last three days of self-administration) and an average intake of ~25mg cocaine/ 3 hr session (figure 3-1A). Immediately following a 3 hr self-administration session samples were collected as crude dorsal and ventral striatum dissections performed on a 2-mm coronal section. Samples were then fractionated into cytoplasmic and nuclear fractions. Data from self-administering rats shows a trend toward an increase of HDAC5 in the nuclear fraction in the ventral striatum and no change in dorsal striatum (figure 3-1B). Although we observed a statistical trend for increased nuclear accumulation, we did not observe a significant reduction similar to our observations of a bolus i.p. cocaine injection in mice. This finding might be attributable to the levels and/or dynamics of cocaine administration or a species difference.

To test whether non-contingent cocaine injections in rats could produce a nuclear accumulation of HDAC5 in the striatum, we used a single acute dose of 30mg/kg cocaine delivered i.p. to rats habituated to the handling and injection procedures (figure 3-1C). We

chose to conduct this second experimental timeline to test the pharmacological effect of cocaine based on previous observations that the response to either acute or chronic experimenter-administered cocaine in mice resulted in comparable regulation of both phosphorylation and nuclear localization. Three hours after i.p. cocaine administration, the samples were collected as crude dorsal and ventral striatum dissections performed on a 2-mm coronal section. Samples were then fractionated into cytoplasmic and nuclear fractions, and we assessed the enrichement of HDAC5 by western blotting. We found that i.p. cocaine administration produced an increase in the nuclear-to-cytoplasmic ratio of HDAC5 in the ventral striatum and dorsal striatum of ~2-fold (figure 3-1D). These changes in the nuclear accumulation were not accompanied by, or attributed to, an increase in total HDAC5 protein levels following drug self-administration since analysis of total cell lysates from the NAc core of chronic saline, chronic, chronic yoked or acute yoked self-administering animals revealed no differences (Figure 3-2).

Taken together, we observe a modest nuclear accumulation of HDAC5 in response to contingent or non-contingent cocaine administration. We observed an interesting difference in the subcellular distribution of HDAC5 that was dependent on the mode of cocaine administration and whether we assessed dorsal or ventral striatum. An increase in nuclear HDAC5 was only observed in the ventral striatum, but not dorsal striatum, following chronic self-administration. Whereas, an increase in nuclear HDAC5 was detected in both dorsal and ventral striatum under experimenter-administered cocaine (i.p.). This effect on nuclear import of HDAC5 is previously reported in mice with experimenter delivered cocaine (Taniguchi, Carreira et al. 2012), suggesting that the recruitment of this

mechanism by cocaine is conserved from mouse to rat and from experimenter- to self-administration and might influence drug taking behaviors *in vivo*.

**Alanine substitutions at three serine residues on HDAC5 promote nuclear accumulation: generation, verification of expression and functional characterization of viral constructs**

Renthal et al (Renthal, Maze et al. 2007) reported that overexpression of HDAC5 in the NAc of wild-type mice reduced cocaine reward, suggesting a key role in cocaine-induced behavioral plasticity *in vivo*. In our initial studies, where we characterized the nuclear accumulation of HDAC5 in response to cocaine and the subsequent attenuation of cocaine reward behavior (CPP assay), we showed that viral-mediated expression of the dephosphorylated form of HDAC5 (S279A), but not the phospho-mimetic (S279E), significantly reduced cocaine CPP (figure 2-10). Taken together, these findings suggest that nuclear HDAC5 limits cocaine reward behavior. However, the role of nuclear HDAC5 in a cocaine self-administration model was unknown. We therefore set out to test whether overepression of WT or a nuclear-restricted mutant of HDAC5 in the adult NAc might reduce the self-administration of cocaine.

The HDAC5 S279A mutant is not basally accumulated in the nucleus, but it rather has a slower nuclear export rate once localized in the nucleus (see figure 2-6B) and this CdK5 site, S279, acts in concert with the $Ca^{2+}$/calmodulin kinase sites, S259 and S498 to regulate localization (see figure 2-6D). Since cocaine triggers coordinated dephosphorylation of HDAC5 S259, S279 and S498 and all three sites are important for nuclear accumulation, we decided to test whether the fully dephosphorylated form of

HDAC5 altered cocaine self-administration behaviors. HDAC5 "3SA" contains three point mutations at the CaM kinase and Cdk5 sites (S259A, S279A and S498A, Figure 3-3A and C). Following site-directed mutagenesis we sub-cloned HDAC5 WT and 3SA into adeno-associated virus (AAV) plasmids. To accommodate the size of this insert (~3.4 Kbp) we subcloned and replaced into the AAV backbone a short $CMV_{ie}$ promoter and a small SV40 polyA sequence (figure 3-3A). We next confirmed that the plasmids expressed full-length HDAC5 and that the 3 serine-to-alanine mutations did not affect the expression/stability of HDAC5. We transfected HEK-293T cells with the AAV-HDAC5 plasmids and harvested total protein lysates for western blotting. Both HDAC5 WT and 3SA expressed well under these conditions (figure 3-3B). Next we generated an HDAC5 3SA containing a deletion of amino acids 172-195 – corresponding to the essential region necessary for binding to MEF2 proteins (HDAC5 3SAΔ, figure 3-3C) (Lu, McKinsey et al. 2000, Rental, Maze et al. 2007).

We next confirmed *in vivo* viral-mediated overexpression of packaged AAV2 by stereotaxic deliver of AAV2 HDAC5 WT, HDAC5 3SA or HDAC5 3SAΔ into the medial NAc. Immunohistochemistry for HDAC5 of fixed sections revealed robust *in vivo* overexpression of HDAC5 (figure 3-3D). It is interesting to note that HDAC5 WT signal was clearly observed in both the cytoplasm and nucleus, whereas HDAC5 3SA or HDAC5 3SAΔ signal in many cases appeared in an intense, punctate nuclear pattern (figure 3-3E and data not shown).

The observation that HDAC5 3SA appears nuclear, and in nuclear puncta, prompted us to confirm that the dephosphorylated mutants were sufficient to promote the accumulation of overexpressed protein in the nuclear compartment. To test the subcelullar distribution, we cultured dissociated primary striatal neurons and transfected with

plasmids that expressed fusion proteins of HDAC5 and GFP. Two days post-transfection, the coverslips were fixed and processed for immunocytochemistry for GFP, HDAC5 and DAPI. Under blinded conditions, we scored transfected neurons for subcelullar localization of HDAC5 signal as enriched in the cytoplasm, nucleus or equal across both compartments. Our results indicate that the HDAC5 3SA and HDAC5 3SAΔ mutants are enriched in the nucleus, whereas the HDAC5 WT was concentrated in the cytoplasm or evenly in nucleus and cytoplasm (figure 3-3F).

We hypothesized that HDAC5 3SA would enhance binding to MEF2 and that HDAC5 3SAΔ would significantly reduce MEF2 binding. To test this idea, we co-transfected PC-12 cells with T7-tagged MEF2D and either GFP control, HDAC5 WT, HDAC5 3SA or HDAC5 3SAΔ. We then immunoprecipitated using anti-T7 antibodies and analyzed co-IP of HDAC5. We observed a non-significant reduction in the HDAC5 3SA overexpressing condition relative to WT overexpression. We also observed a significant reduction in the HDAC5 3SAΔ relative to HDAC5 WT overexpressing conditions (figure 3-4). Taken together, these findings confirm that HDAC5 3SAΔ at least partially reduces the direct association of HDAC5 to MEF2D.

We next tested whether over-expression of HDAC5 WT, 3SA and 3SAΔ contributed to repression of HDAC5's primary nuclear binding partners, i.e. MEF2 transcription factors. To this end, we co-transfected dissociated primary striatal cultures with a MEF2 activity reporter plasmid and HDAC5 vectors. The MEF2 reporter construct generates luciferase protein downstream of three tandem MEF2 response elements (3x MRE-luciferase). Using this assay, we looked at basal activity as well as depolarization (high KCl)-induced MEF2 activity. In control conditions, MEF2 activity is robustly induced by depolarization (i.e.

induced by 60 mM isotonic extracellular KCl). Similar to vector control, overexpression of HDAC5 WT and 3SAΔ showed a robust activation of MEF2 reporter activity in response to depolarization. In contrast, HDAC5 3SA suppressed both basal activity and depolarization-induced MEF2 activity (figure 3-5A). It is interesting to note that like WT over-expression, HDAC5 3SAΔ slightly reduces depolarization-induced MEF2 reporter activity, suggesting that this effect might be a non-specific effect. We also observe this effect in the presence of HDAC5 S279E, a mutant HDAC5 form that enhances cytoplasmic retention (data not shown). Collectively, these findings demonstrate that HDAC5 3SA uniquely inhibits MEF2 activity and that this ability is dependent on the MEF2 binding domain of HDAC5.

As a control for non-specific gain of function by HDAC5 3SA overexpression we looked at CREB dependent reporter activity using the CRE-luciferase assay. We generated and co-transfected dissociated primary striatal cultures with CREB reporter construct and HDAC5 vectors. In control conditions, CRE reporter activity is robustly induced by depolarization. Neurons co-expressing HDAC5 WT, HDAC5 3SA and HDAC5 3SAΔ all display robust activation of CREB reporter activity in response to depolarization (figure 3-5C). These findings support the conclusion that HDAC5 3SA enhances nuclear accumulation and drives inhibition of the HDAC5 target transcription factor MEF2 but does not appear to have gross non-specific gain of function on non-HDAC5 target transcriptional activity.

**Nuclear HDAC5 significantly attenuates cue- and prime-dependent reinstatement of cocaine seeking but does not modulate stable intake, sensitivity or motivation to self-administer cocaine**

To test the hypothesis that nuclear HDAC5 limits cocaine reward using a model for dependence we stereotaxically delivered adeno-associated virus (AAV) over-expressing either a GFP control, wild type HDAC5 (HDAC5 WT) or nuclear HDAC5 (HDAC5 3SA) into the medial nucleus accumbens (NAc). First, animals were trained on a fixed ratio schedule of intake. The fixed ratio begins with 1 lever press as the requirement for an infusion of drug, FR1, followed by an FR3 and finally an FR5. Following fixed ratio training animals were either allowed to withdraw for one week in their home cages and brought back to the chambers for extinction and reinstatement (figure 3-6A, bottom), or underwent a dose response schedule of reinforcement (DR) and a progressive ratio schedule (PR) followed by the same withdrawal, extinction and reinstatement schedule as the first group (figure 3-6A, top). Because these manipulations did not change the effects observed across designs these animals were combined into one data set. All groups acquire during short access daily session and stabilize their intake to similar levels (figure 3-6B). Next we assessed sensitivity to cocaine by analyzing responses to a randomized dose series. All groups generated an inverted u-shaped curve with comparable levels of infusions at each dose (figure 3-6C). Then animals were switched to a progressive ratio schedule to assess overall motivation to self-administer under increasing lever press requirements. We analyzed two doses under PR schedule of reinforcement, 0.25 and 0.75 mg/kg/infusion. Under these conditions all animals work to comparable levels and in a dose-dependent manner for cocaine infusions3-6D). On average, animals press the lever 170 and 336 times for a single infusion of 0.25 and 0.75 mg/kg/infusion, respectively before meeting criteria for breakpoint, defined as the last infusion requirement met in less than 60 minutes for an infusion (data not shown).

Following a week of withdrawal we asked about the ability of the context to induce cocaine seeking, or lever pressing in the absence of reward. All animals pressed to comparable levels in response to re-exposure to the operant chamber. We then extinguished lever-pressing behavior in the operant context over six days. All animals extinguish at comparable rates and to similar levels- indistinguishable from inactive lever presses (figure 3-7A). We next assayed reinstating stimuli designed to promote seeking under extinguished conditions. Within session, animals were first monitored during a 3-hour extinction session followed by an additional hour of non-contingent light cue administration, and on subsequent days a series of priming cocaine doses (0, 5 and 15 mg/kg) in randomized order. Importantly, we observe a significant increase in seeking in both the GFP and HDAC5 WT groups following cue presentations (figure 3-7B), 5 and 15mg/kg i.p. injections of cocaine (figure 3-7C). These effects were significantly attenuated by the overexpression of HDAC5 3SA (fig. 3-7B and C). Taken together, these findings propose a role for nuclear HDAC5 in limiting rodent reinstatement to cue and primes in the absence of an effect on measures of intake.

**The direct interaction of nuclear HDAC5 and MEF2 is dispensable to modulate cocaine intravenous self-administration and seeking**

To test the hypothesis that nuclear HDAC5 limits reinstatement via MEF2 binding and inhibition we stereotaxically delivered adeno-associated virus (AAV) over-expressing either a GFP control, wild type HDAC5 (HDAC5 WT), nuclear HDAC5 (HDAC5 3SA) and nuclear HDAC5 deficient for MEF2 binding (HDAC5 3SAΔ) into the medial nucleus accumbens (NAc). Unfortunately, following behavioral characterization we had difficulty

identifying over-expressed HDAC5 protein in the HDAC5 WT and HDAC5 3SA groups (figure 3-8). We attribute this observation to the viruses having 'expired' as they were generated and stored for a few years in the laboratory. In light of this limitation, we analyzed only the GFP control group and the HDAC5 3SAΔ over-expressing groups. In addition, a straightforward interpretation is also confounded by the use of a modified timeline that most importantly deviated from the previous study in two ways: First, by maintaining all animals on a fixed ratio 1 (FR1), instead of an FR5, schedule of reinforcement. Second, animals in the FR1 study where trained on increasing cocaine doses that, although generate stable intake behavior, exposes the animal overall to less cocaine during the FR training paradigm.

First, animals were trained on a fixed ratio schedule of intake. The fixed ratio was maintained as 1 lever press for an infusion (FR1) and used increasing cocaine doses every 3 sessions. Following fixed ratio training animals underwent a dose response schedule of reinforcement (DR) and a progressive ratio schedule (PR) followed by a week of withdrawal, six days of extinction and prime-dependent reinstatement (figure 3-9A). All groups acquire during short access daily session and stabilize their intake to similar levels (fig. 3-9B). Next we assessed sensitivity to cocaine by analyzing responses to a randomized dose series. All groups generated an inverted u-shaped curve with equal levels of infusions at each dose (figure 3-9C). Then animals were switched to a progressive ratio schedule to assess overall motivation to self-administer under increasing lever press requirements. We analyzed two doses under PR schedule of reinforcement, 0.3 and 1.0 mg/kg/infusion. Under these conditions all animals work to comparable levels for cocaine infusions. On

average, animals press the lever 58 and 95 times for a single infusion of 0.3 and 1.0 mg/kg/infusion, respectively before meeting criteria for breakpoint (data not shown).

We nest assessed the effect of HDAC5 3SAΔ on measures of cocaine seeking. Following a week of withdrawal we asked about the ability of the context to induce cocaine-seeking, lever pressing in the absence of reward. All animals pressed to comparable levels. We then extinguished lever-pressing behavior in the operant context over six days. All animals extinguish at comparable rates and to similar levels-indistinguishable from inactive lever presses (figure 3-10A). We next assayed reinstating stimuli designed to promote seeking. Within session, animals were first monitored during a 3-hour extinction session followed by an additional hour after priming cocaine doses (0, 5 and 15 mg/kg) in randomized order. We observed a significant increase in seeking in both the GFP and HDAC5 3SAΔ groups to comparable levels (fig. 3-10B). Taken together, these findings propose that HDAC5 3SAΔ does not modulate cocaine IVSA behavior. These findings are in contrast to those observed in the first study where HDAC5 3SA was effective at attenuating reinstatement behaviors. Taken together, these findings suggest that HDAC5 3SA attenuates at least prime-dependent reinstatement via a MEF2 direct interaction.

**Nuclear HDAC5 3SA attenuates cocaine conditioned place preference in a MEF2-independent manner**

We sought an alternative viral and behavioral strategy to test the role of nuclear HDAC5 and its interaction with the transcription factor MEF2 on cocaine-related behaviors. To this end we subcloned HDAC5 3SA and HDAC5 3SAΔ into p1005+ eGFP backbone (Figure 3-11A). Plasmids were then sent for packaging into herpes simplex virus (HSV) at

the MIT viral core (Rachael Neve). Prior to behavioral characterization we confirmed viral dependent gene transfer in striatal cultures. Striatal neurons were infected and allowed to express for 48 hours during which time cell health and GFP signal was monitored. Lysates were collected and processed for western blots. HDAC5 3SA and HDAC5 3SAΔ express to comparable levels *in vivo* (figure 3-11B).

To characterize the functional role of HSV-dependent overexpression of GFP, HDAC5 3SA and HDAC5 3SAΔ on cocaine-related behaviors within the transient and robust HSV timecourse, typically 6-7 days total (Barrot, Olivier et al. 2002), we chose a modified conditioned place preference assay (described in Taniguchi et al 2012). Briefly, Adult male mice were pre-tested (day 1) and assigned to groups in an unbiased manner (operationalized as average near zero preference for cocaine paired side). The following day (day 2) animals received stereotaxic delivery of HSV bilaterally into the NAc and allowed to recover for three days. On days 5 and 6 animals received AM saline conditioning and PM saline conditioning separated by at least 4 hours. 24 hours later animals were post-tested (day 7) during which time spent on all sides was recorded (figure 3-11C). The CPP score was calculated as the time spent on the cocaine-paired side minus the time spent in the saline paired side. We hypothesized based on the previous characterization of HDAC5 S279A on CPP and the effect of nuclear HDAC5 (3SA) in IVSA-based reinstatement model that HDAC5 3SA would limit cocaine reward in the CPP assay. Furthermore, because greater than 80% of HDAC5 targets contain MEF2 consensus sequences we hypothesized that nuclear HDAC5 would limit cocaine in a MEF2-dependent manner. Taken together, we made the *a priori* hypothesis that HDAC5 3SA, but not 3SAΔ, would limit development of cocaine reward. 5mg/kg i.p. cocaine significant induced CPP in all groups. Importantly,

HDAC5 3SA and 3SAΔ significantly limit CPP to 5 mg/kg cocaine relative to GFP controls (figure 3-11D). These findings suggest that HDAC5 3SA limits cocaine reward and does so independently of directly associating to and limiting transcription downstream of MEF2.

**Discussion**

In this study we sought to characterize the regulation of localization of HDAC5 under self-administration cocaine experience. We followed this analysis with a rat experimenter-administered cocaine to confirm the response is conserved across species and analyzed total levels of HDAC5 protein in an independent cohort. These data outline what may be an interesting dissociation in the response of HDAC5 subcellular localization in response to cocaine. While bolus acute experimenter-administered cocaine promotes a weak shift in the dorsal and ventral striatum, chronically self-administered cocaine seems to recruit this mechanism preferentially, albeit weakly, in the ventral striatum. Although a clear interpretation of these findings may require a chronic i.p. condition and/or an acute self-administering group they do suggest a selective nuclear import in ventral striatum that occurs either in response to the chronicity of drug exposure or the experience of self-administration of cocaine. One big limitation of our study is that we did not analyze phospho-status of HDAC5. Although phospho-status is important for localization it is possible it may mediate other interactions and may be informative beyond 'predicting' localization. With this data alone we cannot explain the differences observed between this data and that reported in the literature (Renthal, Maze et al. 2007, Host, Dietrich et al. 2011, Dietrich, Takemori et al. 2012). From our initial study, however, conducted in the pertinent species, mouse, we do observe a discrepancy in regulation the phospho status of S259 and

we attribute this as a likely consequence of whole cell analysis rather than HDAC specific analysis. In following studies outlined in this thesis we analyze the phospho-regulation of HDAC4 by cocaine.

We went on to generate viruses to drive the overexpression of HDAC5, nuclear HDAC5 (HDAC5 3SA), and nuclear HDAC5 deficient for MEF2 binding (HDAC5 3SAΔ). We characterized the expression, subcelullar localization, binding affinity and downstream transcriptional profile of generated constructs. As predicted, the three serine-to-alanine conversions promote robust nuclear accumulation of both HDAC5 3SA and HDAC5 3SAΔ. It is interesting to note that nuclear accumulation is independent of direct association to HDAC5's primary nuclear binding partner. Our own characterization on binding affinity yielded intriguing results in that we observe a non-significant reduction of association by HDAC5 3SA to MEF2D relative to HDAC5 WT. This puzzling result may be an artifact of overexpression studies in a heterologous cell system, which may not reflect basal localization patterns of HDAC5 in striatal cultures or in striatum *in vivo*. These findings are not consistent with the functional observations generated with reporter assays showing a significant reduction of MEF2 activity. Alternatively, the methodology used in this study may be inappropriate to conclude that HDAC5 3SA enhances MEF2 binding affinity. For technical reasons, we analyzed only MEF2D binding and not other isoforms. A more thorough characterization of HDAC5 association to MEF2A, MEF2D and MEF2C in other models may provide a better interpretation of the physical association of HDAC5 3SA to MEF2D transcription factors. These data also shows a decrease of binding in the HDAC5 3SAΔ condition that is not statistically different from GFP control conditions. It is worth noting that a weak association is still observed. This may be consistent with the

observation that HDAC5 3SAΔ also appears to associate in punctate nuclear patterns (data not shown) and may suggest that the direct association of HDAC5 to MEF2 is not sufficient and/or required to drive the regulation of target transcription factors. In line with this idea, one way to interpret the luciferase data may be that in the absence of the local epigenetic landscape the loss of the MBD is sufficient to prevent the direct inhibition. Although we are unable to account for this discrepancy within the current data it will be interesting to look at the regulation of endogenous target HDAC5 genes to help clarify and characterize the effect of deleting the MBD of a nuclear accumulated HDAC5 form.

We went on to perform behavioral characterization of the NAc-selective overexpression of HDAC5 WT or HDAC5 3SA on self-administration behaviors. Our findings show that HDAC5 3SA attenuates cue- and prime-mediated reinstatement in extinguished animals and conditioned place preference. These observations are exciting in that they suggest that nuclear HDAC5 function may be an effective therapeutic avenue to curb relapse-like behaviors. One interesting experimental design would be to test the acute role of nuclear HDAC5 by overexpressing it only during reinstatement but not prior. If nuclear HDAC5 during reinstatement curbs seeking behavior then small molecule activators of HDAC5 function may be a promising next step in the development and translation of this preclinical data.

We next attempted to characterize the role of HDAC5 3SAΔ on self-administration in a four-way comparison but found ex vivo that AAV HDAC5 WT and HDAC5 3SA were no longer effective at overexpressing HDAC5 *in vivo*. Nonetheless, a comparison of control GFP and HDAC5 3SAΔ overexpressing groups reveals no significant differences within group in any measure assayed. These findings are confounded by numerous changes to the

experimental timeline. However, interpreted relative to control conditions they do suggest that overexpression of HDAC5 3SAΔ may not recapitulate the effects observed with HDAC5 3SA. In opposition to these results, we observed a significant reduction to CPP score in both HDAC5 3SA and HDAC5 3SAΔ overexpressing groups. These findings highlight the complex nature of cocaine-related behaviors. It would be interesting to investigate the role of HDAC5 3SAΔ using the same experimental timeline and the appropriate viral groups to confirm the dissociation in our findings. One interpretation of our findings may suggest that HDAC5's ability to bind MEF2 is essential for reinstatement specific effects whereas this interaction may be dispensable for cocaine reward under CPP conditions. This idea may be confirmed by subsequent studies analyzing specifically the role of MEF2 in self-administration behaviors. Our lab has looked at expression of MEF2-VP16, a constitutive active mutant form of MEF2, in NAc on reward behavior and observed enhanced cocaine CPP under these conditions. A characterization of the role of MEF2 in cocaine self-administration is lacking at this time.

**Materials and Methods**

Animals: Charles River Laboratories C57BL/6 mice (8-10 week old) or Sprague Dawley rats weighing 250-300g were housed in standard cages group housed (for mice) and individually (for rats) under controlled temperature (21C) and light conditions (12 h light-dark cycle). All mice and rats were maintained with *ad libitum* food and water. The only exception in rats occurred during sucrose pellet training during which animals were mildly food restricted to <95% but not >85% of the animals initial free-feeding weight. Following sucrose pellet training animals were returned to *ad libitum* food and allowed to gain and

stabilize weight prior to any further manipulations. All animals were habituated to the housing conditions for at least a week prior to any experiments.

Self-Administration Procedures

Intracranial Infusions: Rats were anesthesized with ketamine/xylazine (100/10 mg/kg i.p.) and given acute intracranial infusions of AAV viruses using a 5 µl Hamilton syringe and a 30-gauge needle placed in the medial nucleus accumbens (+1.8 mm anterior to bregma, +/- 1.0 lateral, -7.7 ventral from skull). Bilateral infusions of 1.0 µl/side were delivered at a rate of 0.05 µl/30 seconds with a post-infusion wait time of 7 minutes. Analgesia was achieved with 5mg/kg s.c. ketoprofen delivered at the beginning of the surgical procedure.

Sucrose Pellet training: To facilitate acquisition of cocaine self-administration rats in viral studies were first trained to lever press for sucrose pellets (45mg, BioServe) under restricted feeding conditions until they self-administered 100 pellets within a session for three consecutive sessions. Following inclusion criteria, rats were returned to *ad libitum* feeding for at least three days prior to catheter implantation.

Intravenous catheterization: Animals were anesthesized with ketamine/xylazine (100/10mg/kg i.p.) and implanted with a chronic in-dwelling silastic catheter in the right jugular vein, as described previously (Edwards, Graham et al. 2007, Edwards, Whisler et al. 2007). Analgesia was achieved with 5mg/kg s.c. ketoprofen delivered at the beginning of the surgical procedure and as needed for up to 3 days post-operatively. Catheter patency was achieved with 2x daily catheter flushes of 0.2 ml heparinized saline (30 IU/ml) with

gentamycin (5mg/kg) for 3 days followed by 2x daily 0.2 ml heparinized saline (30 IU/ml) flushes for the remaining self-administration experiments.

Cocaine self-administration studies: Sessions consisted of 3 hours and were performed during the light cycle at the same time of day. Animals were connected to the drug line controlled by an external delivery pump. All chambers contained an active and an inactive lever. Pressing of only the active lever resulted in a 50 µl, 3 second infusion of 0.5 mg/kg cocaine for fixed ratio 5 studies. For the characterization of localization and of HDAC5 3SAΔ we trained animals on a fixed ratio 1 (FR1) schedule using either a 1.0 mg/kg/infusion dose or the following ascending doses: 0.06, 0.125, 0.25, 0.5 and 1.0 mg/kg cocaine/infusion. For all timelines and during the infusion a cue light above the lever was illuminated and followed by a 20 second time-out period signaled by the house light going off; the house light signaled drug availability. Animals on the FR5 studies were allowed to self-administer on a fixed ratio (FR) schedule beginning at FR1 followed by an FR3 and finally an FR5. To increase the FR requirements, intake was analyzed for stability (<20% variability within the last three sessions). Animals that completed 15-20 days of FR training and were stable on the FR1 or FR5 schedule for at least three days were considered for subsequent studies. Animals used for dose response and progressive ratio schedules were then subjected to a 5 dose randomized series: 0, 0.03, 0.1, 0.3 and 1.0 mg/kg cocaine infusion. Subsequently, animals were run on a progressive ratio schedule of reinforcement. For these studies we analyzed two doses, 0.25 and 0.75 mg/kg cocaine/infusion. During a progressive ratio schedule of reinforcement, the requirements for an infusion are increased on subsequent infusions in an exponential manner (Richardson and Roberts 1996).

Animals are allowed to self-administer until they fail to earn an infusion in a 60 min timeframe. The last completed lever pressing requirement is referred to and reported as their breakpoint.

Extinction and Reinstatement of cocaine seeking after withdrawal: Animals were allowed a week of forced abstinence (withdrawal) undisturbed in their home cages. Then, animals were placed back into the operant chambers and lever pressing (in the absence of any drug administration) was measured on both the active (drug paired) and inactive levers. Extinction training continued for 6 days followed by reinstatement sessions. Each reinstatement session consisted of a 3 hr extinction session followed by the administration of a priming stimulus. Priming stimuli included: 1) drug paired non-contingent cue (light), 2) experimenter administered cocaine (0, 5 and 15 mg/kg i.p., counterbalanced), and 3) intermittent foot shock stress (random interval, 30 second average, 1.0mA/0.5 second). Lever pressing was measured for an hour following the priming stimuli.

Conditioned Place Preference: Mice were first allowed to explore the three-chambered apparatus and time on each chamber was recorded. Animals that spent greater than 1/3 of total time in one chamber over another were excluded. All included animals were then balanced across virus group based on magnitude of preference, an average near zero pre-test preference score within group, and side paired with cocaine. The next day animals were given an acute viral infusion by stereotaxic delivery (see methods below) and allowed to recover for three days. On day 3 and 4 post-surgery animals received two daily pairings: i.p. saline and confined on one side of the chamber in the am and i.p. cocaine (5mg/kg) and

confined to the other side of the chamber in the pm. 24 hours later animals were brought back to the chambers and allowed to freely explore all sides. Time in each chamber was recorded and a post-test conditioned place preference score generated for each animals and on average for virus group. Only animals that bilaterally express HSV-mediated gene transfer, assessed by GFP endogenous signal, were considered for analysis.

Viruses and plasmids: Adeno-associated viruses serotype 2 (AAV2) vectors were constructed to deliver either wildtype HDAC5 or 3SA HDAC5 and sent for packaging and purification (NC virus core). Full-length wildtype or mutated human HDAC5 (WT or 3SA) were subcloned into a modified pAAV backbone downstream of a CMWie promoter using MluI and BstEII sites. An SV40 polyA sequence was added downstream using nested sites XbaI and AflII immediately downstream of the HDAC5 open reading frame. Serine to alanine mutations at amino acid sites 259, 279 and 498 were generated by quick-change site-directed mutagenesis in an EGFP-N1 plasmid prior to subcloning into AAV plasmids.
HDAC5 3SAΔMBD was generated by internal cuts using AflII sites from HDAC5 WT ΔMBD construct all in bluescript backbone. Then, generated constructs were subcloned into the modified pAAV backbone (CMVie, SV40 polyA, discussed above).
p1005+ GFP HDAC5 3SA and 3SAΔ were generated by NheI and XbaI digestion (full-length digestion) from the pAAV HDAC5 3SA and pAAV HDAC5 3SAΔ (right?) plasmid and ligation into compatible ends on the p1005+ GFP backbone. Diagnostic digests and sequencing was performed to confirm correct orientation before sending plasmids for packaging (MIT, Rachael Neve)

Additional plasmids: 3X MRE-luciferase, CRE luciferase and TK-Renilla luciferase plasmids have all been previously described (Flavell, Cowan et al. 2006). p1005+ GFP was a generous gift from Rachael Neve. p1005+ GFP HDAC5 WT was previously described (Taniguchi, Carreira et al. 2012).

Immunohistochemistry: To confirm placement and viral mediated gene transfer from AAVs, animals were transcardially perfused with ice-cold 1x PBS followed by 4% paraformaldehyde. Brains were then extracted, post-fixed overnight in 4% paraformaldehyde and cryprotected in 30% sucrose. 30 μM sections were generated, stores in 1xPBS + 0.02% $NaN_3$ and processed for immunohistochemistry. Free-floating sections were washed in 1x PBS, blocked in 3% bovine serum albumin in 1x PBS + 0.02% $NaN_3$ and incubated in anti-HDAC5 antibody (1:200) followed by anti-mouse-Cy3 (1:500) in 3% bovine serum albumin in 1x PBS + 0.02% $NaN_3$. Sections were then counterstained with the nuclear dye Hoechst 33342 and visualized using fluorescent microscopy.

Confirmation of AAV overexpression by plasmid transfections and western blot: HEK-293NT cells were split at 1 million cells/well in 6 well plates. Three hours later cells were transfected via calcium phosphate with 1.8 ug of either pAAV-GFP, pAAV-HDAC5 WT, pAAV-HDAC5 3SA and 0.2ug mCherry. Forty-eight hours laters cells were washed 1 time in ice-cold 1x phosphate buffered saline (PBS) and harvested in 100ul/well of 1x sample buffer (SAB). Lysates were then sonicated at 30% for 20 seconds on ice and boiled for 10 minutes at 98C. Lysates were then processed and visualized by standard western blotting.

Dissociated Primary Neuronal Cultures: For *in vitro* experiments, primary embryonic (E18) striatal cultures were generated from Long Evans rats (Charles River Labs) as previously described (Pulipparacharuvil et al., 2008). Crude striatal cells were plated at $8.0x10^6$ /10 cm PDL (Sigma)-coated plate, $1.3x10^6$/well on PDL coated 6 well plates, or $2.4x10^6$ on PDL and Laminin coated glass coverslips in a 24-well plate in DMEM (Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), penicillin (50μg/ml)-streptomycin (50 units/ml; Sigma) and L-glutamine (4mM; Sigma) and incubated at 37C/5% CO2. 24 hours later, media was changed to supplemented Neurobasal (2% B27 (v/v); Invitrogen, penicillin (50μg/ml)-streptomycin (50 units/ml; Sigma) and L-glutamine (4mM; Sigma)).

Confirmation of HSV overexpression by viral infection *in culture* and western blot: Striatal primary neuronal cultures were infected with 1 μl of HSV virus (titer). Forty-eight hours later cells were washed 1 time in ice-cold 1X PBS and harvested in 300ul/well of 1x sample buffer (SAB). Lysates were then sonicated at 30% for 20 seconds on ice and boiled for 10 minutes at 98C. Lysates were then processed and visualized by standard western blotting.

Co-immunoprecipitation assays: PC-12 cells were plated on 6 well plates and transfected using lipofectamine3000 with 5 ug of either pAAV-GFP, pAAV-HDAC5 WT, pAAV-HDAC5 3SA or pAAV-HDAC5 3SAΔ and 5 ug of either plasmid? PC-DNA3-T7 or T7-MEF2D . Forty-eight hours laters cells were washed 1 time in ice-cold 1x phosphate buffered saline (PBS) and harvested in 100ul/well of IP buffer (1X PBS + 0.5% Triton-X100, 1mM EDTA, 100mM NaF, 10nM okadaic acid and protease inhibitor tablets (Roche)). Lysates were then sonicated at 30% for 15 seconds on ice, IP'd with T7 preconjugated agarose beads (washed

and resuspended in IP buffer), washed 3x with IP buffer, and boiled in 20 µ. of 2X SAB for 10 minutes at 98C. Lysates were then processed and visualized by standard western blotting.

MEF2 (MRE) and CREB (CRE) Reporter Assays: Primary neuronal cultures were transfected using calcium phosphate on day *in vitro* 8. Forty-eight hours later cells were stimulated as indicated, washed with 1x ice-cold PBS and harvested in 1x Passive Lysis Buffer for dual luciferase analysis per manufacturer's instructions (Promega).

Immunocytochemistry: Primary neuronal cultures were transfected using calcium phosphate on day *in vitro* 8. Forty-eight hours later cells were stimulated as indicated, washed with 1x ice-cold PBS and fixed in 4% paraformaldehyde/2% sucrose at room temperature for 20 mins. Fixed cells were permeabilized in 0.4% (v/v) Triton X-100 and blocked with 10% non-fat dry milk, 1% normal donkey serum in 1X TBS containing 0.05% (v/v) Tween-20. Coverslips were incubated in anti-HDAC5 antibody (1:200) followed by anti-mouse-Cy3 (1:500) diluted in 1X TBS containing 0.05% Tween-20 and 3% bovine serum albumin. Cells were then counterstained with the nuclear dye Hoechst 33342. HDAC5 localization was categorized as cytoplasmic, nuclear, or both (evenly distributed across nucleus and cytoplasm) for each GFP-expressing (transfected) cell under experimenter-blind conditions using fluorescent microscopy.

Tissue Harvesting: Animals were quickly decapitated and brains collected on ice at indicated timepoints. Acute dissection, on ice-cold 1xPBS, of a 2-mm coronal section

resulted in ventral and dorsal striatal tissue. Subcellular fractionation of cytoplasmic and nuclear extracts was prepared using the NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology) according to manufacturer's instructions. Samples were collected for protein quantification using detergent compatible Lowry colorimetric assay per manufacturer's instructions (Bio-Rad). 80 and 50 μg nuclear and cytoplasmic amounts, respectively, were used for western blot analysis.

Western Blotting: Protein extracts were subjected to SDS-PAGE, transferred to PVDF membrane, blocked (10% (w/v) non-fat dry milk) and incubated with antibodies. Primary antibodies were diluted in 1X TBS with 0.05% Tween-20 containing 3% (w/v) bovine serum albumin as follows: anti-HDAC5 (1:500), anti-β-tubulin (1:10,000), anti-Lamin A/C (1:500). Secondary antibodies were diluted in 1X TBS with 0.05% (v/v) Tween-20 containing 10% (w/v) non-fat dry milk, as follows: anti-mouse IgG conjugated with HRP (1:10,000), anti-rabbit IgG conjugated with HRP (1:10,000). Blots were developed by enhanced chemiluminescence (ECL-Prime, Amersham Pharmacia Biotech). Densitometry was performed with NIH ImageJ software.

Data Analysis: One-way, two-way or repeated measures ANOVAs with Bonferonni's or Tukey's multiple comparison post hoc tests were used. When appropriate, Student's t-tests were performed. Statistics were processed and graphs were designed using GraphPad Prism software.

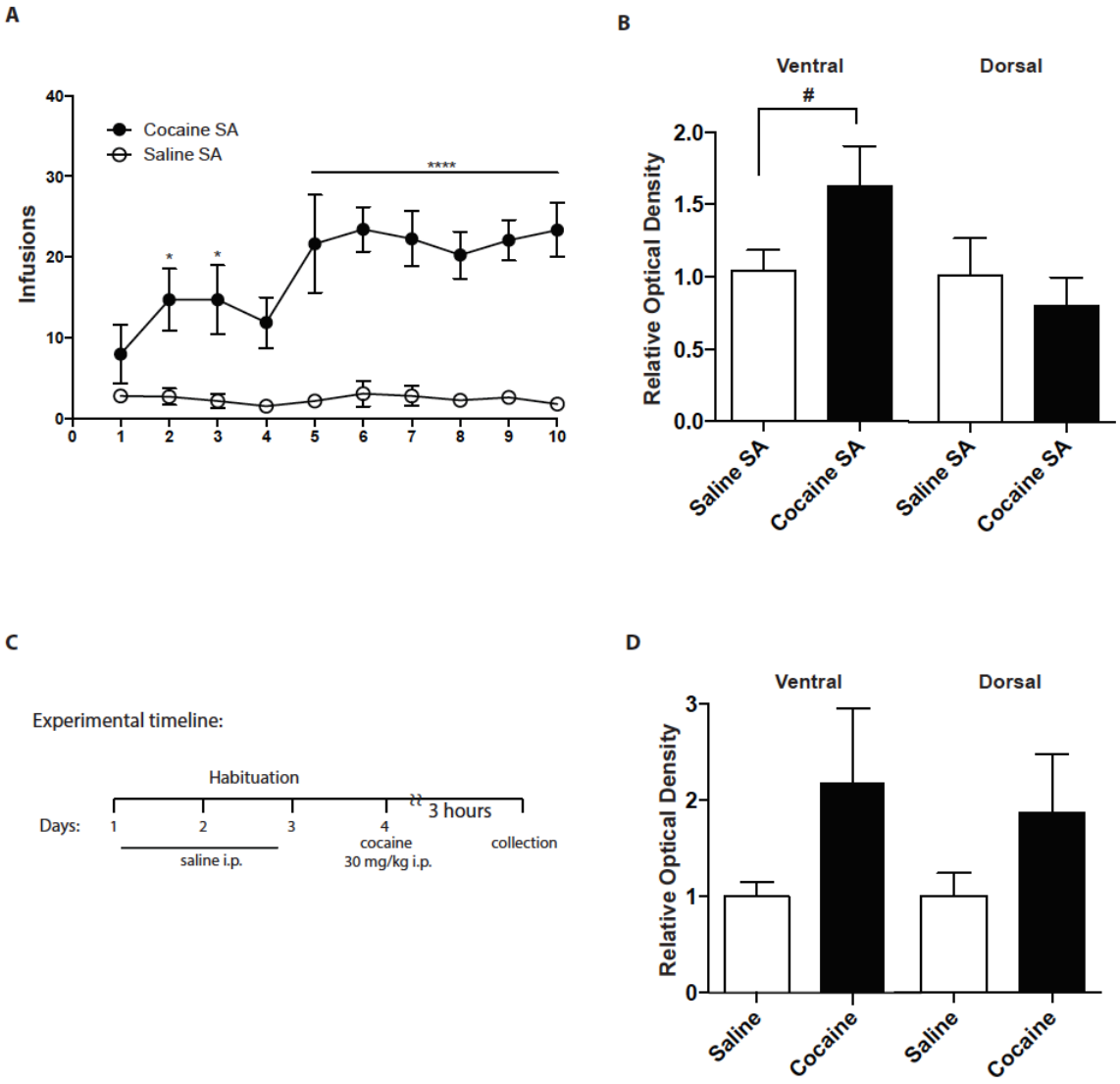


Figure 3-1. Cocaine Self-Administration Weakly Induces Nuclear Accumulation of HDAC5 in the Ventral, but not Dorsal, Striatum.

A. Cocaine but not saline promotes the development of stable intake across sessions. Adult Sprague Dawley rats were allowed to self-administer in daily 3 hr sessions with a 1.0 mg/kg cocaine i.v. dose for 10 days (mean +/- SEM, ANOVA, $F_{1,200}$ = 170.5,*p < 0.05, ****p < 0.0001, Bonferroni's post hoc analysis, n = 11/group).

B. Self-administered cocaine weakly promotes the subcellular redistribution of HDAC5 into the nuclear compartment in the ventral Striatum but not in the dorsal striatum. Striatal tissue was collected immediately following the last self-administration session and fractionated into cytoplasmic and nuclear fractions. Nuclear/cytoplasmic ratios of HDAC5, relative to saline, were calculated by comparing arbitrary units of nuclear and cytoplasmic HDAC5 (normalized to Lamin A/C in nuclear fraction and to β-tubulin in cytoplasmic fraction), (mean +/- SEM, # indicates p = 0.07, Student's t-test, n = 11/condition).

C. Experimental timeline depicting the design for regulation of HDAC5 following acute i.p. administration of cocaine. Adult Sprague Dawley rats were injected with saline for 3 days (1ml/kg i.p.) followed by either a 4$^{th}$ saline injection or a single cocaine injection (30 mg/kg i.p.). Striatal tissue was dissected 3 hours later.

D. Striatal tissue was fractionated into cytoplasmic and nuclear fractions. Acute cocaine does not change the subcellular localization of HDAC5 in the Ventral Striatum but promotes a modest accumulation of HDAC5 in dorsal striatum. Nuclear/cytoplasmic ratios of HDAC5, relative to saline, were calculated by comparing arbitrary units of nuclear and cytoplasmic HDAC5 (normalized to Lamin A/C in nuclear fraction and to β-tubulin in cytoplasmic fraction), (mean +/- SEM, # indicates 1.0 > p > 0.05, Student's t-test, n = 4-6/condition).

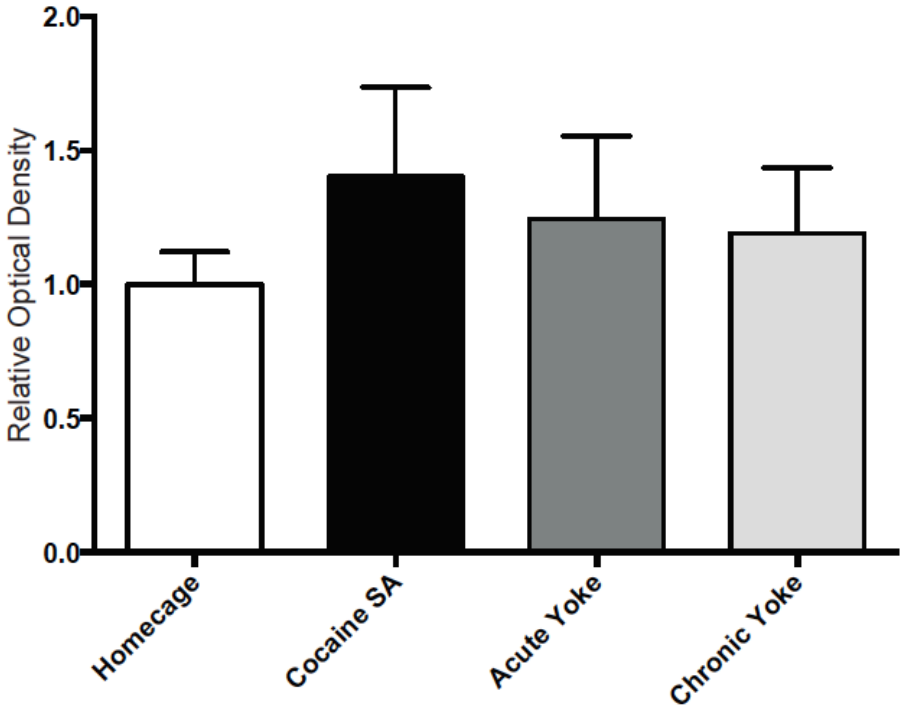


Figure 3-2. Active or Passive Cocaine Self-Administration does not regulate total levels of HDAC5 in the NAc. Adult Sprague Dawley rats were allowed to self-administer saline, cocaine, yoked or acute yoked in daily 3 hr sessions with a 0.5 mg/kg cocaine i.v. dose for 21 days. Cocaine experience does not regulate total HDAC5 levels in NAc. NAc core-enriched punches were processed for standard immunoblotting for HDAC5 and β-tubulin. Normalized optical densities reveal no significant differences across groups (mean +/- SEM, ANOVA $F_{3,34} = 0.5368$, n = 13, 8, 7, and 10 respectively).

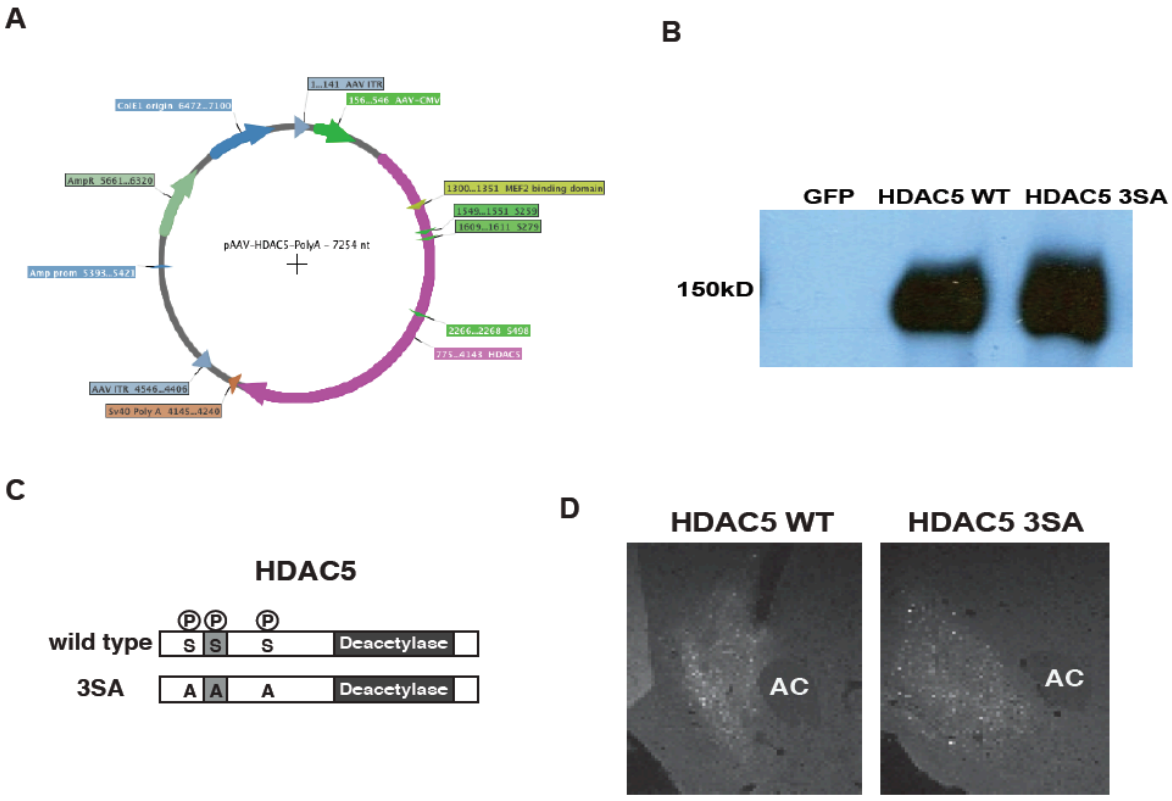


Figure 3-3. Generation of a dephosphomimetic mutant of HDAC5, HDAC5 3SA and a dephosphomimetic mutant deficient for MEF2 binding.
A. Graphic map depicting cloned plasmid overexpressing full-length human HDAC5.
B. Western blotting showing similar expression levels of HDAC5 WT or 3SA mutant in plasmid-transfected HEK cells.
C. Cartoon of full-length human HDAC5 depicting site-directed mutated phosphorylation sites, S259, S279 and S498 and human HDAC5 3SA with the deletion of the MEF2 binding domain (HDAC5 3SAΔ).
D. Representative image of *ex vivo* AAV-mediated gene expression in the NAc. Immunohistochemical expression of HDAC5 was used to confirm virus injection placement. ADD Δ expression?

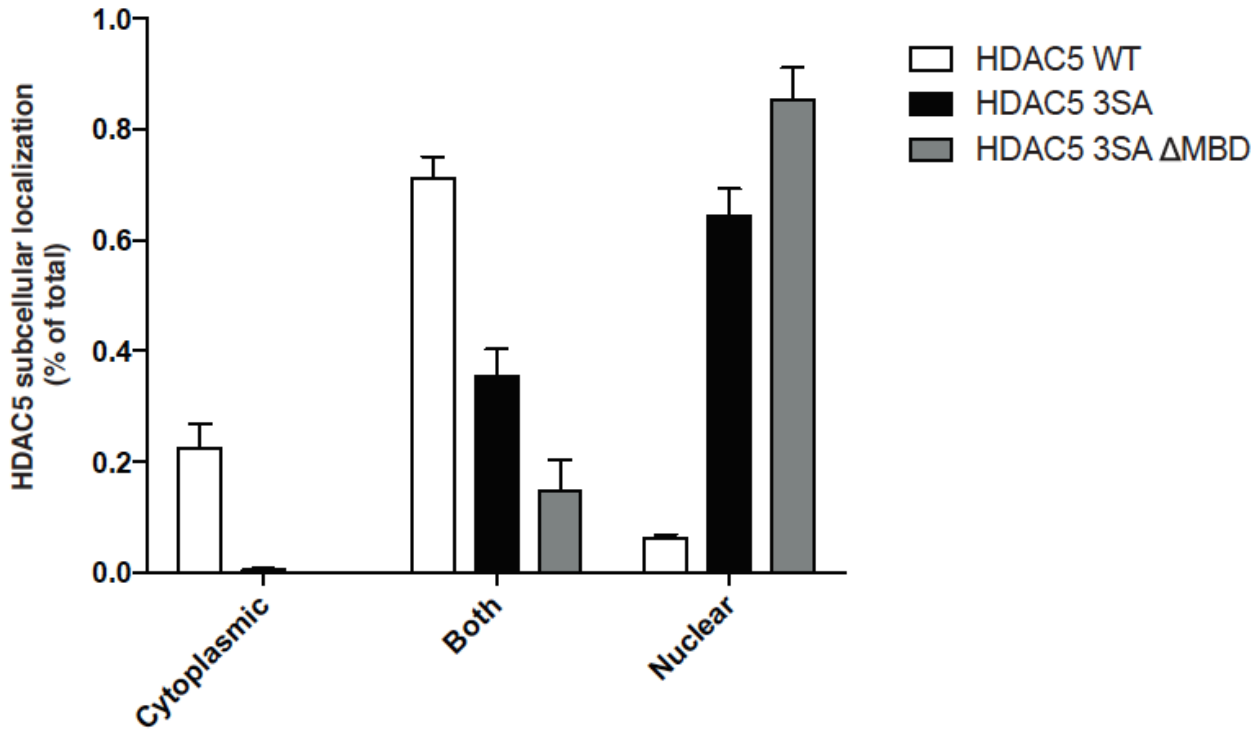


Figure 3-4. HDAC5 3SA or 3SAΔ significantly redistribute HDAC5 to the nuclear compartment.
Over-expression of HDAC5 3SA or 3SAΔ significantly shifts subcellular localization to the nuclear compartment. Neurons transfected with either HDAC5 WT, HDAC5 3SA or HDAC5 3SAΔ p1005+ (GFP expressing vector) were fixed and stained for HDAC5, GFP and DAPI immunocytochemistry (mean normalized percent +/- SEM, n = 3 in each condition, ~90 neurons counted in each well).

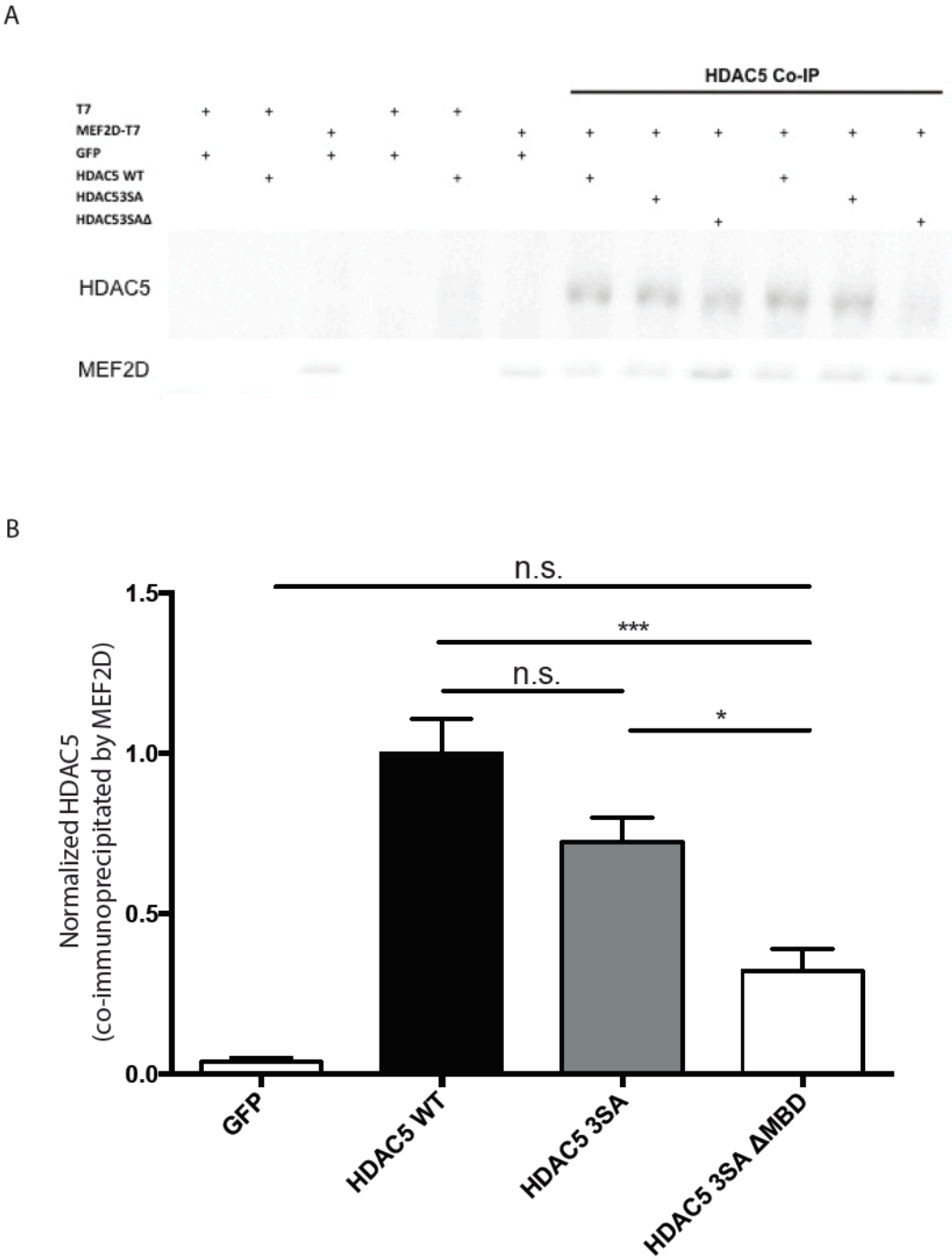


Figure 3-5. HDAC5 3SAΔ significantly decreases the association of HDAC5 and MEF2D by co-immunoprecipitation.

A. Representative image of experimental conditions used to assess the co-immunoprecipitation of MEF2D-T7 and HDAC5. Pull down of T7 tagged MEF2D results in specific binding of HDAC5. PC12 cells were co-transfected with T7 vector control or MEF2-T7 and either GFP vector control, HDAC5 WT, HDAC5 3SA or HDAC5 3SAΔ. 48 hours later cells were harvested and immunoprecipitated with T7-agarose beads. Lysates were processed for western blotting.

B. HDAC5 3SAΔ decreases MEF2D binding. Relative HDAC5 signal normalized to immunoprecipitated MEF2D. HDAC5 3SAΔ, but not HDAC5 3SA, significantly decreases the association of HDAC5 to MEF2 (mean +/- SEM, $F(2,9) = 16.08$, $p = 0.011$, * indicates $p < 0.05$ and *** indicates $p < 0.001$. ANOVA followed by post hoc Bonferoni analysis, n = 4 in each condition).

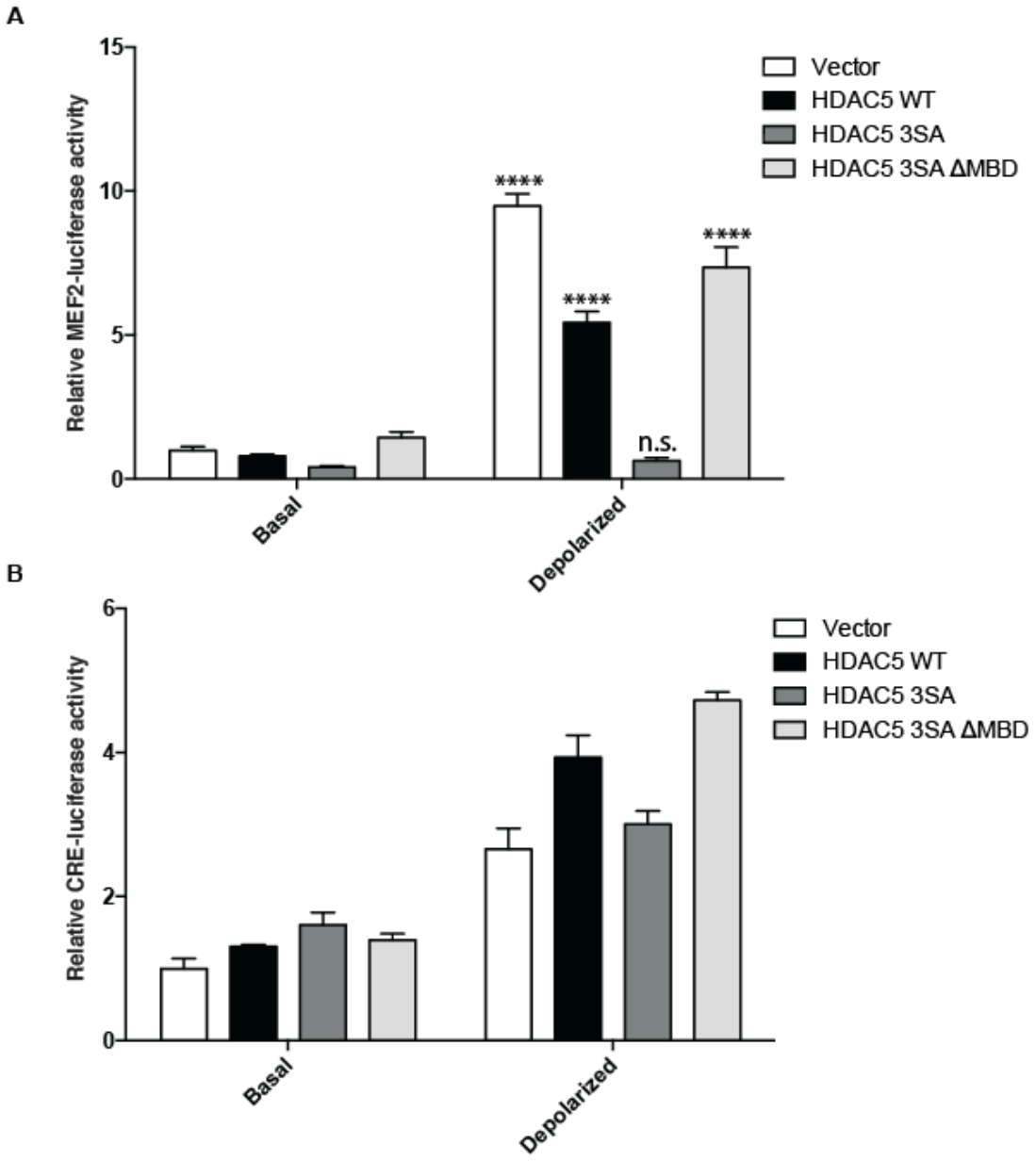


Figure 3-6. HDAC5 3SA drives repression of target transcription factors

Cultured striatal neurons transfected with an (A) MRE-luciferase or (B) CRE-luciferase reporter plasmid and co-transfected with either vector plasmid or plasmid expressing HDAC5 WT, HDAC5 3SA or HDAC5 3SAΔ. Basal or depolarization-induced (60mM KCl) reporter activity was assessed by firefly luciferase and normalized to TK-renilla luciferase.

A) HDAC5 WT reduces inducible MRE-activity in striatal cultures, while HDAC5 3SA reduces basal activity levels and prevents any depolarization-dependent induction. HDAC5 3SAΔ suppresses MRE reporter activity to levels comparable to WT overexpression (mean +/- SEM, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$. ANOVA was performed for basal overexpression effects. Two way ANOVA was performed with all groups with a post hoc analysis within condition to test for induction by depolarization; these results are indicated inside the bars, n = 6 in each condition).

B) The expression of HDAC5 WT, 3SA or 3SAΔ does not regulate CRE-mediated reporter activity under basal conditions in striatal cultures. Overexpression of HDAC5 WT or 3SAΔ significantly enhances depolarization-inducible CRE reporter activity (mean +/- SEM, ** indicates $p < 0.01$, Two way ANOVA with post hoc Bonferonni's analysis was performed, n = 3 in each condition).

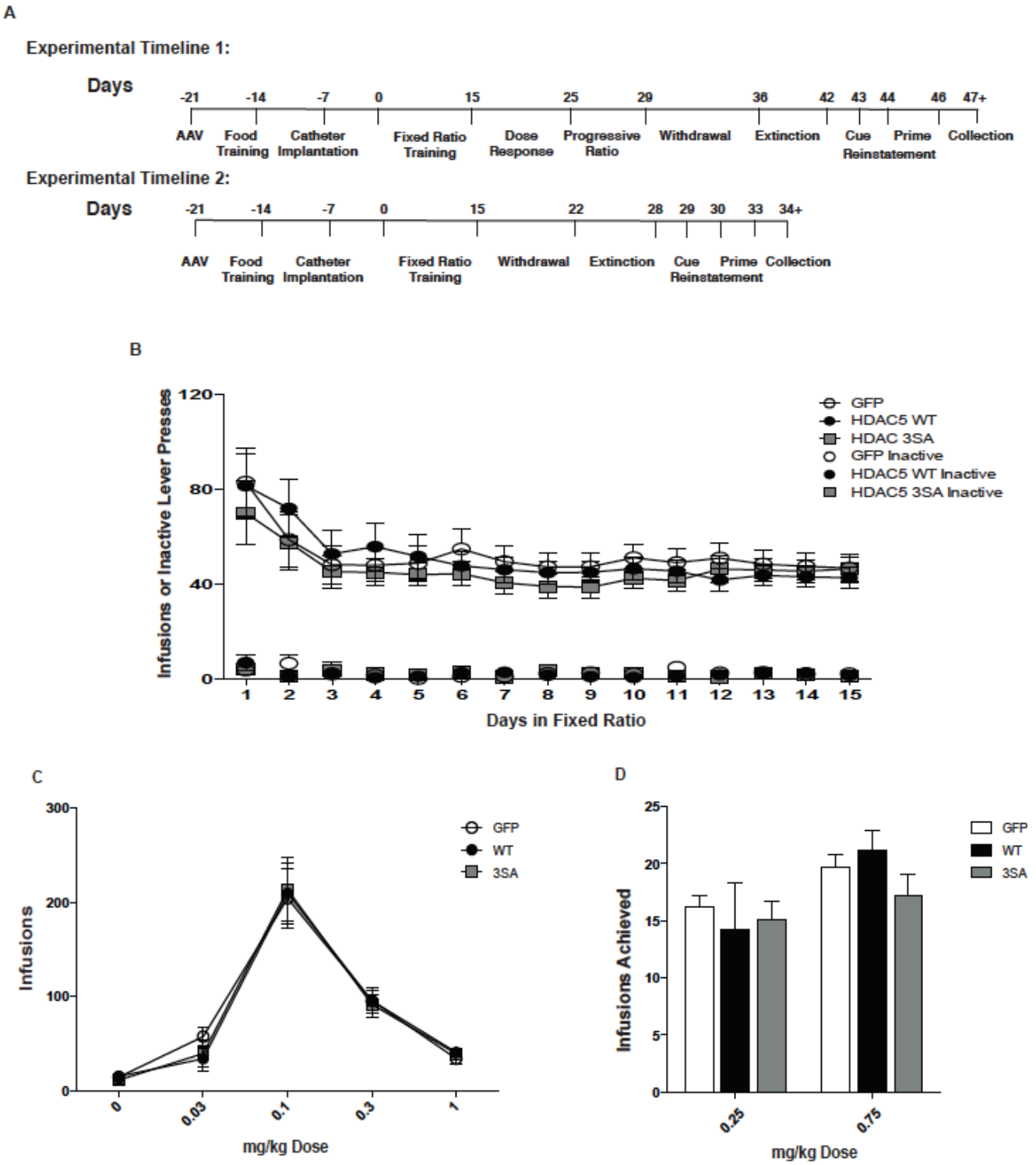


Figure 3-7. HDAC5 3SA does not modulate cocaine-taking behaviors in the intravenous self-administration assay. Figure legend on following page.

Figure 3-7. HDAC5 3SA does not modulate cocaine-taking behaviors in the intravenous self-administration assay. Continued.
A. Time course of IVSA paradigm. (top) First experimental timeline used to assess the effect of HDAC5 WT or HDAC5 3SA overexpression in NAc within animal across fixed ratio 5 (FR5) training, followed by dose response (DR) and progressive ratio (PR). Animals were then allowed to withdraw for a week and assessed for seeking behavior (data shown in next figure). (bottom) Second experimental design used to assess the effect of HDAC5 WT or HDAC5 3SA overexpression in NAc within animal across fixed ratio 5 (FR5) training. Animals were then allowed to withdraw for a week and assessed for seeking behavior (data shown in next figure).
B. Cocaine IVSA promotes stable intake in HDAC5 WT and HDAC5 3SA NAc-overexpressing animals. Fixed ratio training to cocaine (0.5 mg/kg/0.05ml infusion) was monitored in daily 3 hour sessions. Infusions and Inactive Lever Presses were recorded. HDAC5 WT nor HDAC5 3SA modulate stable intake relative to GFP controls (mean +/- SEM, Two way ANOVA of Infusion data only demonstrates no effect of group $F(2, 56) = 0.2954$, n = 19-21).
C. Drug sensitivity is unaffected by NAc overexpression of HDAC5 WT or HDAC5 3SA. Dose response curves were generated in dependent, stable animals using an FR5 schedule. Infusion values are the average within animals of two subsequent daily sessions. All groups displayed similar dose-response curves (mean +/- SEM, Two way ANOVA demonstrates main effect of dose, $F(4, 68) = 75.87$, $p < 0.0001$, but no effect of group, $F(2,17) = 0.01672$, n= 6-7).
D. Motivation to self-administer is unchanged by NAc overexpression of HDAC5 WT or HDAC5 3SA. Progressive ratio was generated using two cocaine doses (0.25 and 0.75 mg/kg/0.05ml infusion) each for two consecutive days and randomized within group. HDAC5 WT nor HDAC5 3SA modulate the number of infusions acquired across groups prior to reaching breakpoint (mean +/- SEM, Two way ANOVA demonstrates main effect of dose, $F(1,17) = 15.89$, $p = 0.0010$, but not group, $F(2,17) = 0.2953$, n = 6-7).

Figure 3-8. HDAC5 3SA attenuates cue- and prime-, but not context-dependent seeking in the reinstatement model. Data shown on following page.
A. Context- induced seeking and extinction is normal in HDAC5 WT and HDAC 3SA NAc overexpressing animals. Following a week of withdrawal animals were returned to the chamber and lever pressing recorded for three hours daily sessions. All animals seek (lever press) on the active, but not the inactive, lever to comparable levels in response to the context. Over the course of six days animals extinguish seeking and presses on the active lever are comparable to inactive lever presses recorded within session (mean +/- SEM, Two way ANOVA demonstrates main effect of day in extinction $F(5, 445) = 28.56$, $p < 0.0001$ and main effect of group, $F(5, 89) = 5.121$, $p = 0.0004$, post hoc Bonferonni's analysis reveals significant differences between active and inactive lever responses for day 1 in all groups, and day 2 in HDAC5 WT and HDAC5 3SA groups but no significant differences across groups on days 3-6, n = 18-20).
B. Cue-mediated reinstatement is attenuated by HDAC5 3SA, but not by HDAC5 WT, overexpression in NAc. Using a within session model, we tested the ability of discrete non-contingent light cues to drive reinstatement of seeking (lever pressing) in extinguished animals (mean +/- SEM, Two way ANOVA demonstrates main effect of cue, $F(1,55) = 29.75$, $p < 0.0001$, post hoc Bonferonni's reveals significant reinstatement in GFP (**) and HDAC5 WT (***) but not HDAC5 3SA group, n = 19-20).
C. Prime-dependent reinstatement is attenuated by HDAC5 3SA, but not by HDAC5 WT, overexpression in NAc. Using a within session model, we tested the ability of experimenter administered cocaine i.p. injections to drive reinstatement of seeking (lever pressing) in extinguished animals (mean +/- SEM, Two way ANOVA reveals main effect of dose, $F(2, 110) = 35.06$, $p < 0.0001$, and group, $F(2, 55) = 3.405$, $p = 0.0403$, post hoc Bonferonni's analysis reveals significant differences between GFP and HDAC5 3SA (**) and HDAC5 WT and HDAC5 3SA (*) in the 15 mg/kg i.p. condition. Student's t-test between GFP and 3SA reveal significant differences in the 5 and 15 mg/kg i.p. cocaine conditions ($p = 0.0348208$ and $0.0417873$, respectively) but not in the 0 mg/kg i.p. saline condition ($p = 0.629698$), n = 19-20).

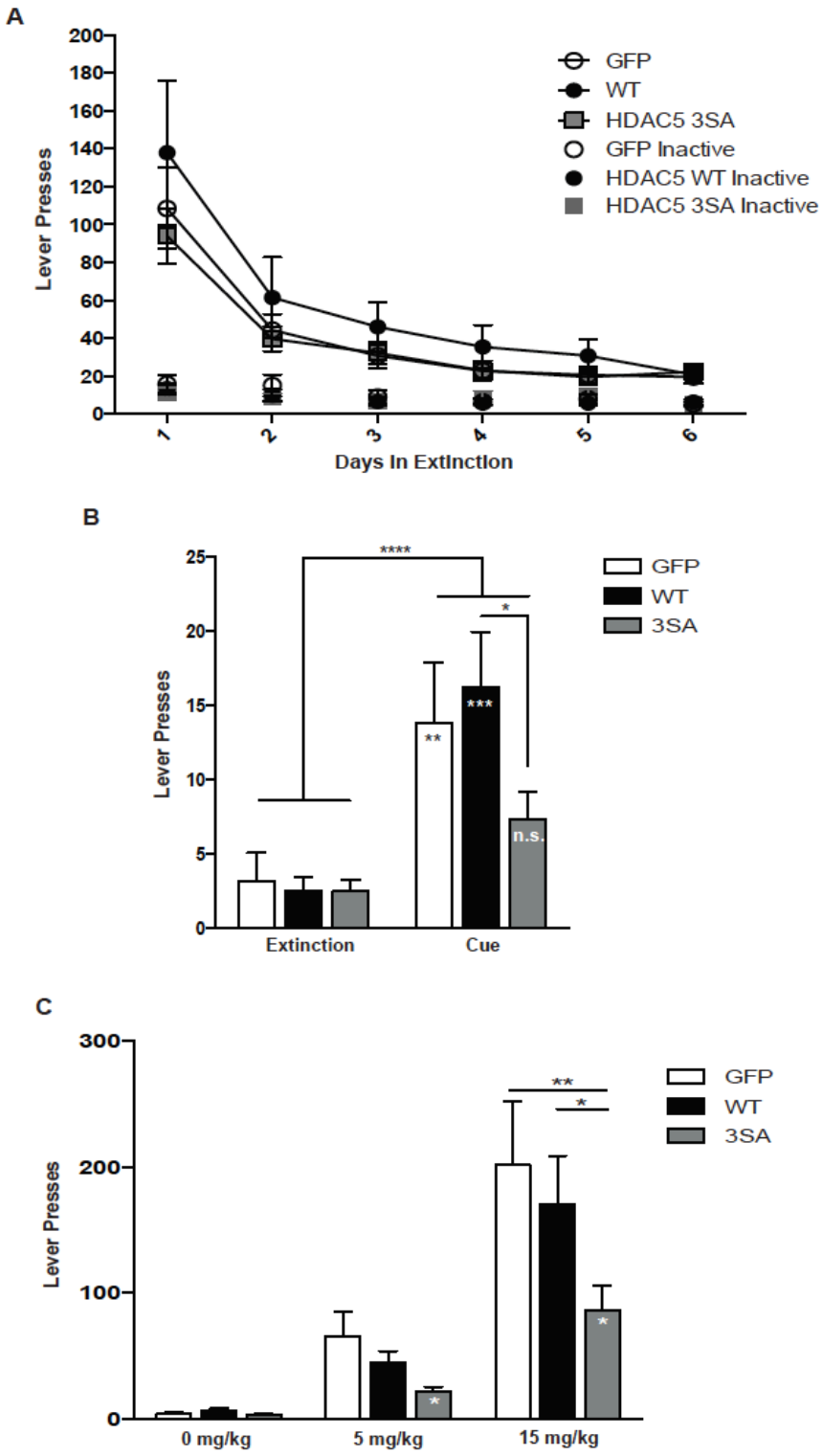


Figure 3-8. HDAC5 3SA attenuates cue- and prime-, but not context-dependent seeking in the reinstatement model. Complete figure legend on previous page.

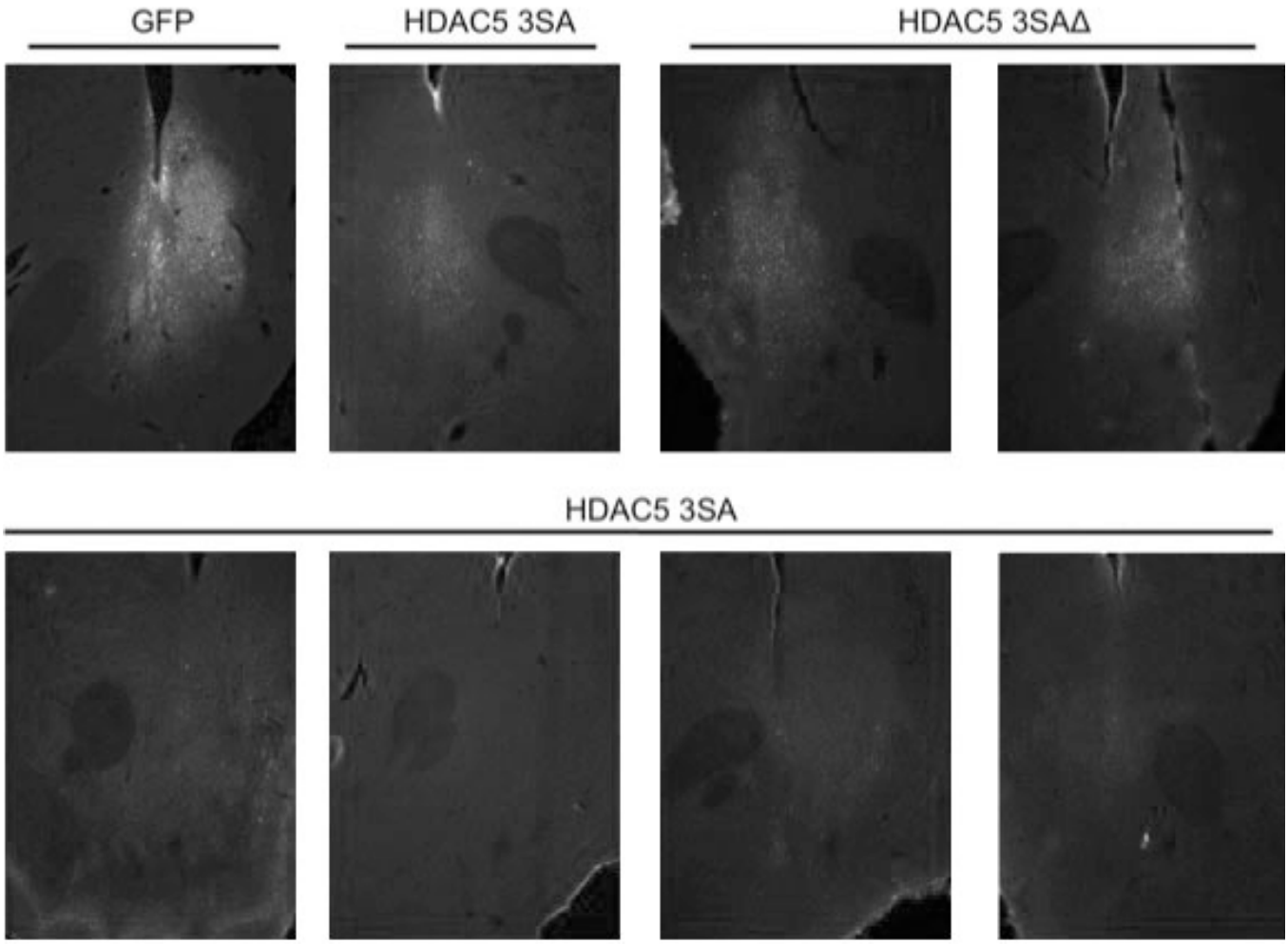


Figure 3-9. Loss of overexpression in WT and 3SA viruses confounds interpretation of studies of HDAC5 3SAΔ. Representative image of *ex vivo* AAV-mediated gene expression in the NAc. Immunohistochemical expression of HDAC5 was used to confirm virus injection placement. Top panel demonstrates expression of either GFP or HDAC5 3SA and HDAC5 3SAΔ by immunohistochemistry for GFP or HDAC5 in the nucleus accumbens of rats following intravenous self-administration behavioral characterization. Botton panel demonstrates ex vivo images of nucleus accumbens that failed to show enhanced immunoreactivity following immunohistochemistry and detection for HDAC5 signal. All images acquired using fluorescent microscopy.

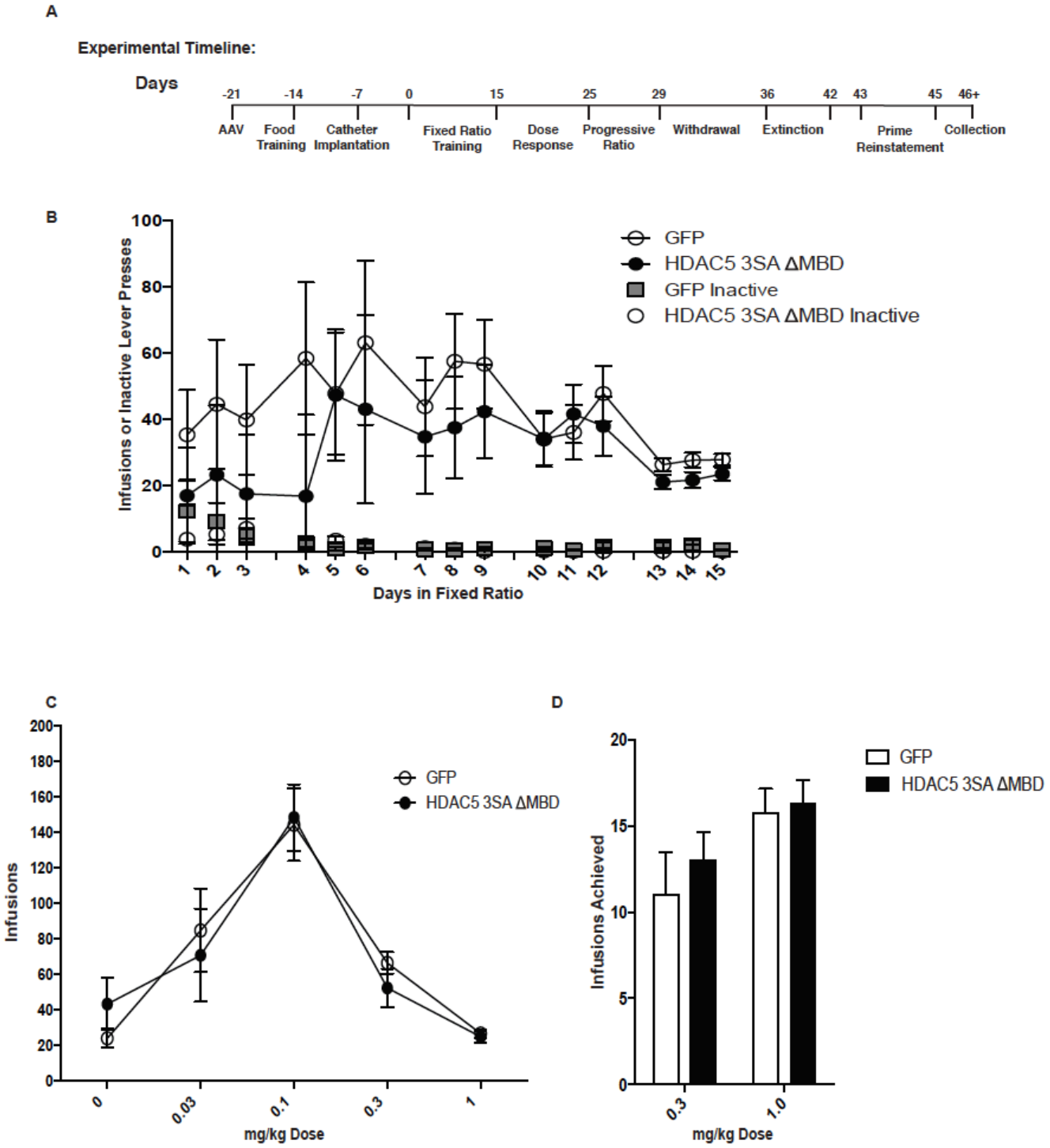


Figure 3-10. HDAC5 3SAΔ does not modulate cocaine taking in the intravenous self-administration assay. Figure legend on following page.

Figure 3-10. HDAC5 3SAΔ does not modulate cocaine taking in the intravenous self-administration assay. Continued.
A. Time course of IVSA paradigm. Experimental timeline used to assess the effect of HDAC5 WT or HDAC5 3SAΔ overexpression in NAc within animal across fixed ratio 1 (FR1) training, followed by dose response (DR) and progressive ratio (PR). Animals were then allowed to withdraw for a week and assessed for seeking behavior (data shown in next figure).
B. Cocaine IVSA promotes stable intake in HDAC5 3SAΔ NAc-overexpressing animals. Fixed ratio training was monitored in daily 3 hour sessions. Increasing cocaine doses were presented each for three consecutive days. Cocaine doses increased as follows: 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/kg/0.05ml infusion. Infusions and inactive lever presses were recorded. HDAC5 3SAΔ does not modulate stable intake relative to GFP controls (mean +/- SEM, $F(1,13) = 1.196$, n = 8 & 7, respectively).
C. Drug sensitivity is unaffected by NAc overexpression of HDAC5 3SAΔ. Dose response curves were generated in dependent, stable animals using an FR1 schedule. Infusion values are the average within animals of two subsequent daily sessions. All groups displayed similar dose-response curves (mean +/- SEM, Two way ANOVA demonstrates main effect of dose, $F(4, 52) = 26.91$, $p < 0.0001$, but no effect of group, $F(1,13) = 0.008464$, n= 7 in each group).
D. Motivation to self-administer is unchanged by NAc overexpression of HDAC5 3SAΔ. Progressive ratio was generated using two cocaine doses (0.25 and 0.75 mg/kg/0.05ml infusion) each for two consecutive days and randomized within group. HDAC5 3SAΔ does not modulate the number of infusions acquired prior to reaching breakpoint (mean +/- SEM, Two way ANOVA demonstrates main effect of dose, $F(1,13) = 14.19$, $p = 0.0024$, but not group, $F(1,13) = 0.2919$, n = 8 & 7, respectively).

Figure 3-11. HDAC5 3SAΔ does not modulate cocaine seeking in the intravenous self-administration assay. Data shown on following page.
A. Context- induced seeking and extinction are normal in HDAC5 3SAΔ NAc-overexpressing animals. Following a week of withdrawal animals were returned to the chamber and lever pressing recorded for three hours daily sessions. All animals seek (lever press) on the active, but not the inactive, lever to comparable levels in response to the context. Over the course of six days animals extinguish seeking and presses on the active lever are comparable to inactive lever presses recorded within session (mean +/- SEM, Two way ANOVA demonstrates main effect of day in extinction $F(5, 130) = 28.66$, $p < 0.0001$ and main effect of group, $F(3, 26) = 6.669$, $p = 0.0017$, post hoc Bonferonni's analysis reveals significant differences between active and inactive lever responses for day 1 in both groups but no significant differences across groups on days 2-6, n = 8 & 7, respectively).
B. Prime-dependent reinstatement is not attenuated by HDAC5 3SAΔ overexpression in NAc. Using a within session model, we tested the ability of experimenter administered cocaine i.p. injections to drive reinstatement of seeking (lever pressing) in extinguished animals (mean +/- SEM, Two way ANOVA reveals main effect of dose, $F(2,39) = 14.86$, $p < 0.0001$, but not group, $F(1, 39) = 0.4024$, n = 8 & 7, respectively).

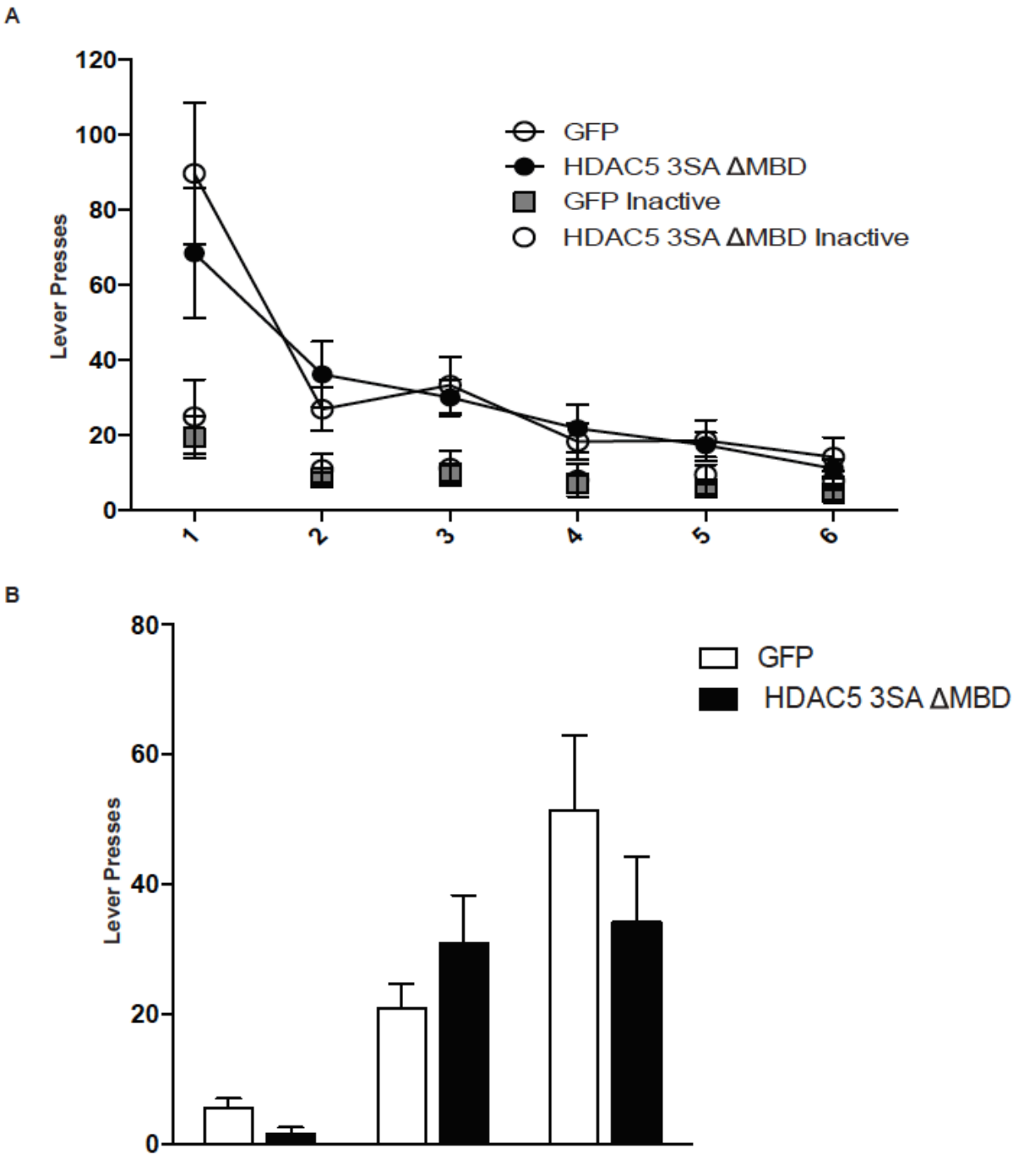


Figure 3-11. HDAC5 3SAΔ does not modulate cocaine seeking in the intravenous self-administration assay. Figure legend in previous page.

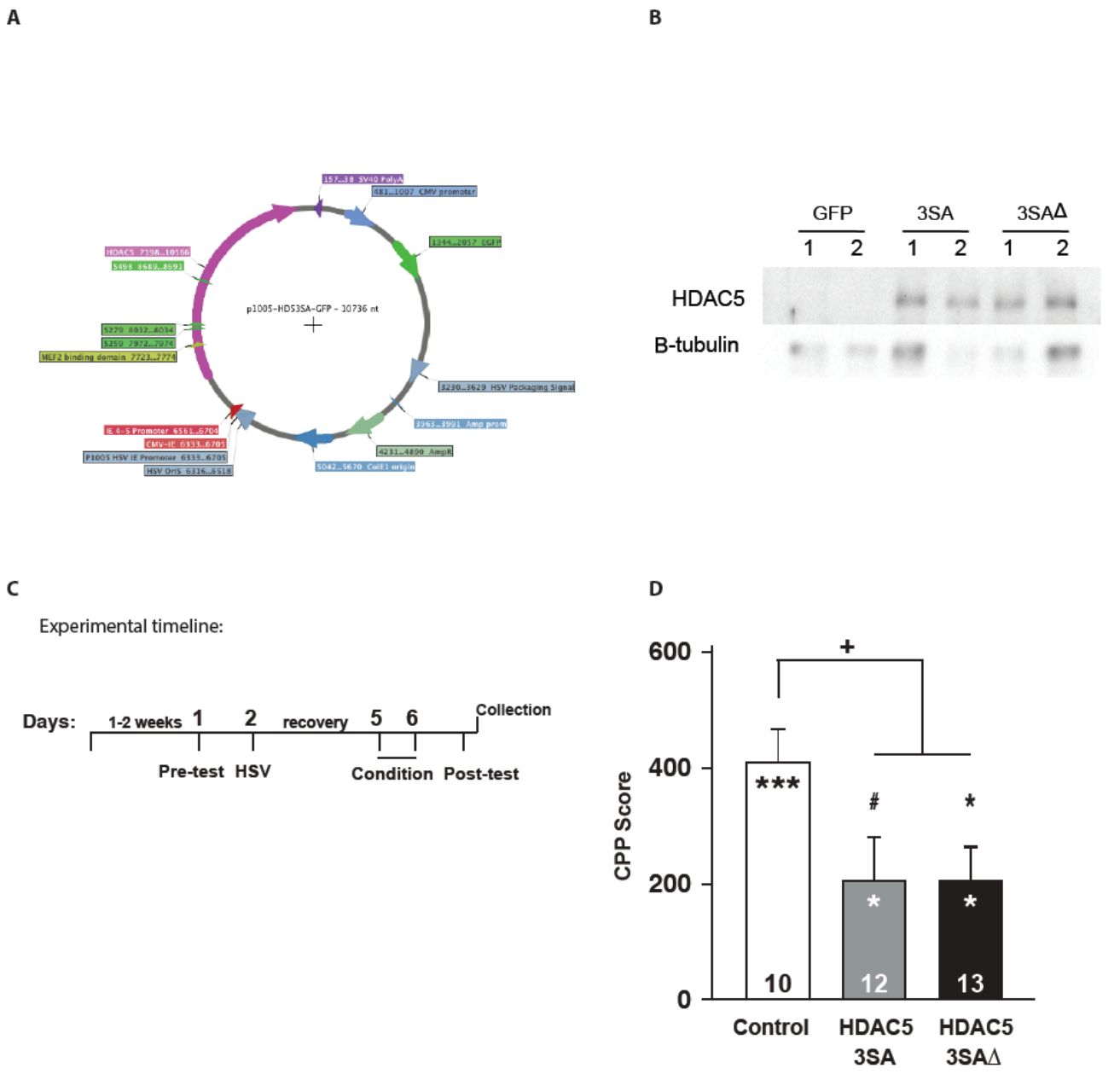


Figure 3-12.HDAC5 3SA and HDAC5 3SAΔ limit the development of conditioned place preference.
A. Graphic map depicting cloned p1005+ plasmid overexpressing full-length human HDAC5. Plasmid was packaged into HSV and GFP expression was used to confirm virus injection placement ex vivo.
B. Representative image of HSV-mediated gene expression in striatal cultures. Western blotting showing similar expression levels of HDAC5 3SA and 3SAΔ in virus-infected cultured dissociated striatal neurons.
C. Time course of CPP paradigm.
D. Conditioned place preference (CPP) to cocaine (5 mg/kg) is attenuated by overexpression of HDAC5 3SA and 3SAΔ (mean +/- SEM, ADD ANOVA, $F(2,32) = 2.804$, $p = 0.0755$, post hoc Student's t-test between GFP and HDAC5 3SA, # indicates $p = 0.0623$, GFP and HDAC5 3SAΔ, * indicates $p = 0.0276$, GFP and combined HDAC5 overexpressing, + indicates $p = 0.0220$). CPP Score was defined as the time (sec) spent in the cocaine side minus the saline side for post-conditioning trials.

# Chapter 4: The class IIa histone deacetylase, HDAC4, does not modulate cocaine-related behaviors *in vivo* in the nucleus accumbens

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RP, MBC, MT, and CWC designed experiments. RP, MBC and MT conducted experiments. Specifically, MBC generated or contributed to figures 4-1, 4-2, 4-3 and 4-6. RLN prepared Herpes Simplex Viruses used in this study. RP and MBC prepared this manuscript.

**Summary**

In recent years a focus on epigenetic mechanisms as mediators of cocaine-related behavioral, structural and functional plasticity has developed. One family of epigenetic molecules that may underlie cocaine behavioral and functional changes is the histone deacetylases, HDACs, which act to mediate transcriptional repression. The class IIa subgroup of HDACs is poised as an intracellular signal detectors and effectors. This feature is conferred by their ability to shuttle subcellularly in a dynamic and activity-dependent manner primarily driven by phosphorylation status of the protein at multiple residues. The class IIa member HDAC4 has been implicated as a regulator of cocaine reward *in vivo* however its regulation in response to cocaine and the role of localization remains unknown. In this study, we report the regulation of phosphorylation and localization of HDAC4 *in culture* in response to increased cAMP activity and *in vivo* following experimenter administer cocaine regimens. We report a modest, and highly variable phosphorylation and nuclear export in response to cocaine experience. We next analyzed the role of endogenous loss of HDAC4 in the NAc and report a modest dose-dependent effect on acute locomotion but not sensitization or conditioned place preference. Finally, we tested the effect of over-expressing full-length wildtype HDAC4 and promoting its nuclear accumulation by over-expressing a phospho-mutant version, HDAC4 3SA. Under these conditions we do not observe an effect on conditioned place preference. These findings are in contrast to those reported for HDAC5, where nuclear HDAC5 function limits aspects of cocaine addiction-like behavior, including cocaine reward. Interestingly, overexpressing nuclear variants of HDAC5 or HDAC4 both suppress the activity of their

target transcription factor myocyte enhancer factor, MEF2. Taken together, these findings propose that MEF2 inhibition is not sufficient to attenuate cocaine reward and highlight the role of HDAC5 but not HDAC4 to limit cocaine reward.

## Introduction

In recent years, one field of study that has gained traction within the field of addiction is that of epigenetic mechanisms (Hyman, Malenka et al. 2006, Renthal and Nestler 2008, Renthal and Nestler 2009, Robison and Nestler 2011). These are molecular events that change the overall genetic landscape, and not genes *per se*, thereby modulating transcriptional activity. One particularly interesting event involves the addition or removal of acetyl groups on histone tails. Acetylated histone tails disrupt histone-DNA complexes resulting in a relaxed structure that allows accessibility to the chromatin machinery. This process is carried out by histone acetyltransferases (HATs) (Renthal and Nestler 2009). The removal of acetyl groups from histone lysine tails is also a regulated process achieved by the histone deacetylases (HDACs). These act as transcriptional repressors as they promote the condensation of DNA within chromatin structures and transcriptional silencing (Bertos, Wang et al. 2001, Martin and Zhang 2007, Jiang, Langley et al. 2008).

Acetylation of histones has been reported as a consequence of cocaine experience (Kumar, Choi et al. 2005, Malvaez, Sanchis-Segura et al. 2010, Wang, Lv et al. 2010, Malvaez, Mhillaj et al. 2011, Rogge, Singh et al. 2013, Rogge and Wood 2013). Studies aimed at characterizing the role of increased acetylation by global or systemic HDAC inhibitor administration have failed to generate consistent, replicable findings suggesting that these effects may be complex and heterogeneous, class- or molecule-specific in nature (Renthal,

Maze et al. 2007, Host, Anglard et al. 2010, Romieu, Deschatrettes et al. 2011, Raybuck, McCleery et al. 2013) (Kumar 2005, Romieu 2008, Host 2010, Hui 2010, Malvaez 2010, Wang 2010, Romieu 2011, Malvaez 2013, Raybuck 2013). Few studies have characterized the role of HDAC inhibitors specifically in NAc. Wang et al 2010 characterized the effect of infusing TSA or SAHA into the shell or core of the nucleus accumbens and found that only shell administration of either pan-HDAC inhibitor enhanced dose response and motivation in the absence of an effect in intake. Similarly, intra-NAc administration of SAHA enhances cocaine conditioned place preference (Renthal, Maze et al. 2007). These findings support a role for HDACs in the NAc in limiting tolerance and motivation as well as cocaine reward. Taken together with systemic studies, however, these findings have encouraged the study of specific HDAC members or classes in driving cocaine-associated plasticity.

HDACs are subdivided into classes based on size, localization, structural and functional homology. Of these, particular interest has been placed on the class IIa HDACs because of their unique ability to act as molecular sensors of multiple signaling/neurotransmitter cascades and subsequently translocate between the cytoplasmic and nuclear compartments (Bertos, Wang et al. 2001, Martin and Zhang 2007, Parra 2015). The class IIa HDACs, when in the nucleus, act as transcriptional co-repressors by deacetylation of targets lysine residues on histones (Kouzarides 1999, Miska, Karlsson et al. 1999) and recruitment of complex co-repressor machinery (Bertos, Wang et al. 2001, Dressel, Bailey et al. 2001, Fischle, Dequiedt et al. 2001, Fischle, Dequiedt et al. 2002). The subcellular localization of the class IIa HDACs has been described as $Ca^{2+}$-responsive as well as cAMP-responsive and these intracellular signaling cascades oppose in their regulation of localization (McKinsey, Zhang et al. 2000, Chawla, Vanhoutte et al. 2003,

Belfield, Whittaker et al. 2006). This effect is primarily driven by the phosphorylation status of a number of serine residues on the class IIa HDACs that include the CaMK sites S259 and S498 of HDAC5 corresponding to sites S246 and S467 and additional residue S632 of HDAC4 (Lu, McKinsey et al. 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2000, Backs, Backs et al. 2008) and the PKA or CdK5 site S279 of HDAC5 corresponding to S266 of HDAC4 as of yet uncharacterized (Ha, Kim et al. 2010, Taniguchi, Carreira et al. 2012). In response to increases in intracellular $Ca^{2+}$, CaMK family phosphorylates the class IIas (Youn, Grozinger et al. 2000, Chawla, Vanhoutte et al. 2003, Linseman, Bartley et al. 2003, Backs, Backs et al. 2008, Schlumm, Mauceri et al. 2013) and promotes cytoplasmic accumulation and 14-3-3 binding (Wang, Bertos et al. 1999, Grozinger and Schreiber 2000, McKinsey, Zhang et al. 2000, McKinsey, Zhang et al. 2001, Nishino, Miyazaki et al. 2008). Conversely, increases in cAMP promote nuclear accumulation (Belfield, Whittaker et al. 2006, Ha, Kim et al. 2010, Taniguchi, Carreira et al. 2012).

Behaviorally, the class IIa HDACs, HDAC4 and HDAC5, have been primarily studied in the context of learning and memory and cocaine behaviors. In a forebrain specific manner, HDAC4 KO results in enhanced locomotion, decreased anxiety in the elevated plus maze and the open field test, decreased memory for a contextual fear-conditioning paradigm and decreased spatial learning and memory in the Morris water maze assay. Authors then demonstrate this effect is HDAC4 specific, as HDAC5 global knockouts do not display impairments in anxiety-based measures or learning and memory tasks (Kim, Akhtar et al. 2012). Further support for a crucial role of HDAC4 in learning and memory, but contrary to the previous proposed role for HDAC4 function, comes from the characterization of a transgenic mouse line designed to express a truncated N-terminal

HDAC4 nuclear variant. Using this genetic model, authors report impaired spatial memory, impaired retention and impaired reversal in a Barnes maze assay (Sando, Gounko et al. 2012). These findings, although puzzling, may be explained by a nuclear, repressor gain of function effect of the truncated N-terminal fragment. Likewise, Agis-Balboa and colleagues reported a significant reduction of spatial learning, context and cued freezing following fear conditioning in aged HDAC5 KO mice relative to wildtype littermates (Agis-Balboa, Pavelka et al. 2013); a contrasting report to that of Kim (Kim, Akhtar et al. 2012) that may be explained by a number of experimental and technical differences across studies. Taken together, however, these studies highlight the compartment-specific function of HDAC4 and propose that the role of HDAC4 may not always be functionally redundant with HDAC5.

The class IIa HDAC4 has been described as playing a role specifically in cocaine related behaviors. Wang et al reported that HDAC4 over-expression in the NAc limits dose response and progressive ratio in self-administration(Wang, Lv et al. 2010). Authors report that this effect is only observed when viral-mediated over-expression of HDAC4 was targeted to the nucleus accumbens shell but not the core. These effects are consistent with the CPP findings described in Kumar et al (2005). In this study authors over-expressed HDAC4 in the NAc and found a significant attenuation of conditioned place preference to cocaine (Kumar, Choi et al. 2005). These findings are in line with a functionally redundant role of HDAC4 and HDAC5 as similar CPP studies have reported that HDAC5 over-expression limits cocaine conditioned place preference (Renthal, Maze et al. 2007). Importantly, studies of HDAC4 function in cocaine have yet to characterize the subcelullar localization of HDAC4 in response to cocaine experience and the behavioral consequence of driving nuclear function of HDAC4; a critical characterization as its role has been primarily

ascribed to its presence in the nuclear compartment (Haberland, Montgomery et al. 2009, Parra and Verdin 2010, Parra 2015). Recently, we reported that cAMP and cocaine trigger delayed nuclear import of HDAC5 in striatal neurons *in culture* and *in vivo*, respectively. Promoting nuclear accumulation of HDAC5 limits cocaine conditioned place preference (Taniguchi, Carreira et al. 2012). We hypothesized that HDAC4, like HDAC5 would be dephosphorylated and nuclear accumulation promoted following cocaine experience. In addition, based on their high sequence homology and apparent cocaine-behavior redundant functions, we proposed that driving HDAC4 nuclear function would serve to limit cocaine reward.

In this study, we characterized the phosphorylation of HDAC4 S266 and subcelullar localization in response to cAMP signaling *in culture* and cocaine experience *in vivo*. We then characterized endogenous HDAC4 function specifically in NAc by viral-mediated conditional deletion in the $HDAC4^{fl/fl}$ background. Next we assessed the consequence on cocaine reward of driving HDAC4 overexpression and nuclear function in NAc using a mutated HDAC4 3SA containing serine to alanine substitutions that promote nuclear accumulation. Finally, we tested the ability of HDAC4 3SA to drive transcriptional repression of the transcription factor MEF2. We aimed at testing the hypothesis that cocaine and cAMP signaling induce dephosphorylation and nuclear accumulation of HDAC4, and that driving nuclear accumulation and function of HDAC4 limits cocaine reward *in vivo*.

## Results

### HDAC4 is weakly regulated by forskolin stimulation *in vitro* or cocaine *in vivo*

Previous work has demonstrated that cAMP signaling *in vitro* can enhance HDAC5 dephosphorylation at multiple residues, including S279. This residue was shown to critically regulate sub-cellular localization, with dephosphorylation of this site required for nuclear accumulation following forskolin treatment. Mass spectrometry analysis of *in vitro* phosphorylated HDAC4 identifies a homologous residue in HDAC4 (S266, data not shown). Given the strong association of this site with HDAC5 trafficking, we probed the phosphorylation state and subcellular localization of HDAC4 following forskolin treatment *in vitro*. Cultured striatal neurons were exposed to 10uM forskolin or DMSO vehicle for 3 hrs. Cultures were lysed and subjected to HDAC5 immunodepletion in order to isolate HDAC4-specific phospho-signal. A small but nonsignificant dephosphorylation was observed at this site in HDAC4 (figure 4-1A), in contrast to the robust dephosphorylation previously observed at this site in HDAC5. In order to determine the effect of forskolin treatment on subcellular localization of HDAC4 *in vitro*, striatal cultures were transfected with hHDAC4-GFP and stimulated with forskolin or DMSO for 3hrs. Consistent with the weak dephosphorylation of S266 HDAC4 produced by forskolin treatment, there was only weak nuclear accumulation observed in hHDAC4-GFP (figure 4-1B).

*In vitro* findings indicate that despite high homology, forskolin/cAMP signaling differentially regulates HDAC4 and HDAC5 phosphorylation and subcellular localization. Given the previously described regulation of HDAC5 by cocaine, we examined if HDAC4 phosphorylation and subcellular localization is similarly or differentially regulated *in vivo*. Previous research demonstrated robust dephosphorylation of multiple HDAC5 serine residues four hours after a cocaine injection in both cocaine-naïve and cocaine-experienced animals. Consistent with this dephosphorylation, HDAC5 demonstrated enhanced nuclear

localization under these same conditions in a separate group of animals (Taniguchi, Carreira et al. 2012). In order to determine the regulation of HDAC4 following cocaine exposure, animals were subjected to 7 daily injections of saline (saline), 7 daily injections of 20mg/kg cocaine injections (cocaine-experienced), or a single 20mg/kg cocaine injection after 6 days of saline injections (cocaine-naïve). NAc tissue was collected four hours after the last injection and examined for HDAC4 phosphorylation and subcellular localization. Consistent with weak forskolin regulation *in vitro*, cocaine treatment *in vivo* produced a weak and non-significant HDAC4 phosphorylation at S266 (figure 4-2A). Interestingly, although forskolin induced HDAC4 S266 regulation is reduced but similar to HDAC5, cocaine induced S266 regulation is opposite that of HDAC4. Although not statistically significant, there was a trend for enhanced phosphorylation of S266 most notably in the cocaine-experienced group. This pattern is consistent with the previous observation that despite robust dephosphorylation of this site in HDAC5, immunoblotting of this site in a non-HDAC specific manner (i.e., without HDAC pre-depletion to isolate the contribution of closely related HDACs) showed phosphorylation at this site (Taniguchi, Carreira et al. 2012). This phosphorylation pattern was partnered with a trend for cytoplasmic localization (figure 4-2B). These findings suggest that despite high homology, HDAC4 is weakly, and oppositely regulated under cocaine treatment conditions that strongly regulate HDAC5.

**HDAC4 and HDAC5 are dispensible for cocaine locomotor sensitization and cocaine conditioned place preference**

Given the altered subcellular localization of both HDAC4 and HDAC5 produced by cocaine exposure, we investigated if loss of either molecule would influence locomotor responses to cocaine. We first investigated the effect of HDAC5 total knockout (Chang, McKinsey et al. 2004) on the development of cocaine locomotor sensitization. Animals received 3 daily injections of 0.9% saline and locomotor responses were monitored thereafter for up to 60 minutes. After 3 days of habituation, animals received 5 daily injections of 10mg/kg cocaine and locomotor responses were monitored for 30 minutes. Although HDAC5 knockout animals show a trend for reduced locomotor activity on the first saline injection, by the last day of habituation both groups show similar locomotor response to the saline injection (figure 4-3A). HDAC5 knockouts do not differ from wild-type littermates in locomotor responses to an acute injection of cocaine (coc 1) nor do they differ significantly in their sensitized response (coc 5). These findings indicate that HDAC5 is dispensable for normal locomotor responding to acute and sensitizing regimens of cocaine. We next investigated the role of HDAC4 in cocaine locomotor sensitization by viral mediated knockout in the nucleus accumbens (Potthoff, Wu et al. 2007). HDAC4 floxed animals received bilateral infusions of AAV-Cre-GFP or AAV-GFP into the NAc and were allowed to recover for three weeks during which time AAV expression peaked and recombination has occurred (data not shown, confirmed in (Potthoff, Wu et al. 2007, Kim, Akhtar et al. 2012)). Animals were then subjected to low dose CPP (5mg/kg, see figure 4-3E) and allowed to remain in the colony undisturbed for 2-3 weeks. Animals were then subjected to 4 consecutive days of saline injections followed by 7 consecutive days of 15mg/kg cocaine. Locomotor activity was monitored for 30mins. HDAC4 knockout in the adult NAc did not alter locomotor responding to any injection (figure 4-3B), indicating that

HDAC4 in the NAc is dispensable for normal cocaine locomotor sensitization. Interestingly, though not statistically significant HDAC4 KO animals appeared to have slightly lower cocaine-induced locomotor responding across the injections (C1, C7 and data not shown). Considering that the previous drug exposure experienced during the cocaine CPP experiment may influence subsequent acute cocaine locomotor responses, a second group of drug naïve animals were subjected to 4 days of saline injections followed by exposure to a single dose of cocaine (7.5, 15, or 30 mg/kg). Interestingly, HDAC4 NAc knockout animals showed significantly reduced locomotor activity to an acute dose of cocaine (figure 4-3C). Taken together these findings indicate that although HDAC4 in the NAc contributes to acute locomotor responses to cocaine, it is not required for the development of cocaine locomotor sensitization. Similarly, HDAC5 is not required for acute or sensitized locomotor responses.

Given the reduced locomotor activation by acute cocaine in HDAC4 animals, we investigated if this reduced cocaine-locomotor sensitivity might translate into reduced cocaine-reward sensitivity as measured by conditioned place preference. Animals received bilateral infusion of AAV-Cre-GFP or AAV-GFP to the NAc and were allowed to recover for three weeks. Drug naïve animals were conditioned over two days, with saline sessions in the morning followed by cocaine sessions (5mg/kg or 10mg/kg) in the afternoon. On the post-test, the difference in time spent in the drug-paired side compared to the saline side was determined. We found that viral-mediated knockout of HDAC4 in the NAc did not influence cocaine CPP at the two doses examined (figure 4-3D). These findings indicate that despite contributing to initial drug locomotor sensitivity, NAc HDAC4 is not required for acquisition and expression of cocaine CPP.

**Nuclear localized HDAC4 does not influence cocaine reward behavior but suppresses its target MEF2**

Previous work has implicated NAc HDAC4 in cocaine reward behavior via over-expression and pan-HDAC inhibitor experiments (Kumar, Choi et al. 2005). Although we find that HDAC4 KO in the NAc does not influence cocaine-reward and that cocaine treatment *in vivo* likely enhances HDAC4 cytoplasmic localization, it is possible that conditions that promote HDAC4 nuclear accumulation may serve to reduce cocaine reward in a manner similar to enhanced nuclear retention of HDAC5 ((Taniguchi, Carreira et al. 2012) and figure 3-12). In order to test this hypothesis we first confirmed the relationship between phosphorylation and subcellular localization of HDAC4, using two point mutant constructs. We generated a phosphomimetic of the S266 site (HD4-S266E) and a phosphomutant at the well-described CaMK sites (HD4-3SA, S246, S467, S632, Backs 2006, McKinsey 2001, Zhang 2002, figure 5-1A). Consistent with previous reports, transfected wild-type HDAC4 was predominately cytoplasmic *in cultured* rat striatal neurons (Darcy, Calvin et al. 2010, Sando, Gounko et al. 2012). As expected the 3SA mutant promoted robust nuclear accumulation and consistent with it's described role in regulating HDAC5 localization, HD4-S266E showed increased cytoplasmic localization compared to HD4-WT (figure 4-1B). Taken together this indicates that phosphorylation of S266 can regulate HDAC4 subcellular localization and demonstrate that the serine to alanine substiutions in HDAC4 3SA are sufficient to drive robust nuclear accumulation of HDAC4.

To test the role of nuclear localized HDAC4 in cocaine reward behavior, HDAC4-3SA-Flag and HDAC4-WT-Flag were packaged in an HSV vector. In order to assess relative

expression of the constructs, striatal neurons were infected with HSV-HDAC4-WT, HSV-HDAC4-3SA, or HSV-GFP for 48 hrs. Lysates were collected and probed for relative HDAC4 expression to confirm comparable expression levels (data not shown). In order to determine if nuclear localized HDAC4 is able to suppress cocaine-reward, we expressed the HSV-constructs in the NAc of adult male mice (Fig 4C). After allowing 3 days for recovery and viral expression, animals were exposed to the testing chamber for a pre-test and 24hrs later subjected to two training days with saline injection injections in the morning and cocaine (5mg/kg) in the afternoon. On the post-test day, the difference between the time spent in the drug-paired side compared to the saline-paired side was calculated. Neither HSV-mediated overexpression of HDAC4-WT nor HDAC4-3SA significantly affected cocaine place preference at the dose examined (Fig 4D). These findings indicate that despite robust suppression of Mef2-dependent transcription when nuclear localized, HDAC4 is unable to influence cocaine-reward when expressed during cocaine conditioned place preference conditioning.

When nuclear, HDAC4 binds primarily to the transcription factor MEF2 to promote repression of target transcription (Miska, Karlsson et al. 1999, McKinsey, Zhang et al. 2000, Bolger and Yao 2005, Zhao, Sternsdorf et al. 2005). To confirm that HDAC4's repressive function is enhanced by overexpression of the HDAC4 3SA construct we tested the relative efficacy of the constructs to suppress MEF2-mediated transcription using an MRE-luciferase assay. Striatal cultures were transfected with HDAC4-WT, HDAC4-3SA, or GFP control along with MRE-luciferase and control constructs. As expected, KCl depolarization robustly induced MRE-luciferase activity in the control condition (Fig 4-6). Expression of

HDAC4-WT did not significantly reduce induction but expression of the nuclear localized form of HDAC4 (Fig 4-6) significantly repressed MRE-luciferase activity.

## Discussion

In this study we characterized the consequence of inducing cAMP signaling in striatal cultures on the phosphorylation status of HDAC4 S266 and nuclear localization. We observed only a weak dephosphorylation of S266 under these conditions and no effect on subcelullar localization. These findings contrast with the robust dephosphorylation we observe at the comparable phospho-site of HDAC5, S279, and robust nuclear accumulation induced in striatal cultures under the same conditions (Taniguchi, Carreira et al. 2012). These findings propose that HDAC5 may be preferentially targeted downstream of cAMP signaling for dephosphorylation. This may be a consequence of either phosphatase or kinase activity and affinity for the S266/279 site. An interesting observation we have made in lab is that HDAC4 is robustly and rapidly phosphorylated by PKA *in vitro* relative to HDAC5 (data not shown). This information may propose a model in which phosphorylation of S266 on HDAC4 is regulated by PKA, also a target of cAMP signaling and in the presence of forskolin both phosphatase and kinase cascades are recruited resulting in a net no change to the phosphorylation level and subsequent compartment accumulation. Importantly, however, the basal and activity-inducible kinase and phosphatases recruited and responsible for the phosphorylation status of S266 remain poorly understood.

Similarly, we analyzed the phosphorylation status of HDAC4 S266 and nuclear localization *in vivo* in response to cocaine experience. Surprisingly, under these conditions we observed

that HDAC4 is weakly phosphorylated and exported from the nuclear compartment. These *in vivo* observations are not consistent with the data on HDAC4 regulation by cAMP from striatal cultures or the *in vivo* characterization of HDAC5 in response to cocaine. Under cocaine *in vivo* conditions HDAC4 seems to be weakly and oppositely regulated-phosphorylated and exported to the cytoplasmic compartment. A big limitation of our study comes from the phospho-analysis and localization after cocaine after 4 hours. It is possible that mechanisms that promote phosphorylation and export occur on a different timescale than those reported for dephosphorylation and nuclear accumulation. In the future, it will be interesting to analyze the response of HDAC4 to cocaine *in vivo* over a time course that will allow for a more complete interpretation of these preliminary studies. These findings support differential regulation of highly homologous proteins. It is also interesting to speculate about the apparent enhanced phosphorylation and export observed under chronic cocaine experience relative to acute experience. In our analyses of HDAC5 regulation, acute and chronic cocaine experience drives this mechanism to comparable extents ((Taniguchi, Carreira et al. 2012) and figure 3-1). In the case of HDAC4 the mechanisms that promote phosphorylation may be recruited over repeated cocaine experience; analyzing HDAC4 phosphorylation and localization following longer experimenter- or self-administered time points may test this hypothesis. A progressive exclusion of HDAC4 from the nucleus in response to extended repeated cocaine experiences may represent an important chronic neuro-adaptation.

Despite structural and functional homology the observation that HDAC4 and HDAC5 are differentially regulated under cocaine *in vivo* conditions brings into question the redundant function of HDAC4 and HDAC5 in the context of cocaine experience and related

behaviors. Therefore, we used conditional deletion to test the effect of knocking out HDAC4 selectively in the nucleus accumbens or HDAC5 globally on cocaine-related behaviors. Using these genetic models we assessed lomocotor activity and sensitization. HDAC4 NAc cKOs and HDAC5 KOs develop normal sensitization. Interestingly, HDAC4 NAc cKOs display a dose-responsive reduction of acute locomotor responses to cocaine that normalizes over the sensitizing regimen. This acute effect was not observed in HDAC5 KOs suggesting that the initial locomotive response to cocaine is modulated by HDAC4 activity. It is also interesting that HDAC4 NAc KO does not affect CPP while HDAC5 KO animals are susceptible to chronic cocaine experience driving cocaine reward (Renthal, Maze et al. 2007). One major caveat in our interpretation of the functional roles of HDAC4 and HDAC5 comes from the use of a NAc-restricted knockout of HDAC4 versus a global knockout of HDAC5. This comparison is made in light of the fact that HDAC5 KOs are viable while HDAC4 KOs die prematurely (Chang, McKinsey et al. 2004, Vega, Matsuda et al. 2004, Potthoff, Wu et al. 2007, Majdzadeh, Wang et al. 2008) but it is important to consider that HDAC5 KOs may be viable, and display normal behavioral phenotypes, due to compensation. One straightforward way to test this is to look at the relative abundance of other class IIa subfamily, and other classes, of HDACs. Previous characterization in muscle and brain suggests no compensation by other HDACs in the HDAC5 KO although a quantification is lacking in the study (Chang, McKinsey et al. 2004). Together these findings propose a role for HDAC4 in the initial response to cocaine experience and a role for HDAC5 under chronic experience.

We continued our characterization by looking at the effect of driving nuclear function of HDAC4 *in vivo*. In prior work we have observed an effect of driving either

nuclear retention or accumulation of HDAC5 in cocaine reward ((Taniguchi, Carreira et al. 2012) and figure 3-12). We tested nuclear HDAC4's ability to limit cocaine conditioned place preference under similar conditions and found that nuclear HDAC4 fails to modulate cocaine CPP. These findings were surprising in light of previous characterizations of over-expression of either class IIa in CPP (Kumar, Choi et al. 2005, Renthal, Maze et al. 2007, Taniguchi, Carreira et al. 2012). This CPP study lends support to the overall observation that HDAC4 and HDAC5 are not redundant molecules; an observation we have generated from our own characterization of regulation and function in cocaine-related behaviors. Although we were unable to recapitulate prior data showing a role in limiting reward for HDAC4 we have not tested the role of HDAC4 in intravenous self-administration behaviors. Previous work demonstrated a significant decrease of both the descending limb of the dose response and of progressive ratio (Wang, Lv et al. 2010). Together, these findings suggest that HDAC4 may play a role in decreasing sensitivity (or opposing tolerance) and decreasing motivation. In our own self-administration characterization of HDAC5 WT or nuclear (HDAC5 3SA), described in chapter 3, we failed to observe modulation of the dose response or progressive ratio; a prediction we would have postulated given the sequence and functional homology described for these two class IIa members. An interesting model emerges from these observations that dissociates the functions of these class IIa homologues. HDAC4 may limit aspects of cocaine taking and its primary reinforcement while HDAC5 is poised to limit aspects of cocaine seeking and 'secondary' reinforcement, that which comes from associated cues to the drug self-administration experience. This hypothesis may be tested directly in a side-by-side comparison of the role of these two molecules in IVSA behaviors.

Finally we confirmed that driving nuclear accumulation of HDAC4 also promoted its transcriptional repressor activity. To test this we looked at the ability of HDAC4 to limit target transcriptional activity by looking at MEF2 transcription factor reporter activity. Under these conditions we report a significant attenuation of MEF2 activity by HDAC5 3SA under basal and depolarized conditions. These findings are consistent with numerous reports demonstrating that MEF2 is the primary nuclear binding partner of HDAC4 and HDAC5 (Miska, Karlsson et al. 1999, Lemercier, Verdel et al. 2000, Zhang, Williams et al. 2002). They are also in line with the observation that HDAC5 3SA significantly suppresses MEF2 activity under identical conditions (figure 3-6). Taken together with the CPP characterization of a MEF2-binding deficient nuclear mutant of HDAC5, HDAC5 3SAΔ (figure 3-12), these findings support a MEF2-independent mechanism of gene transcription in regulating cocaine-associated behavioral plasticity.

**Materials and Methods**

Animals: All C57BL/6 mice (Charles River) used in this study were adult males tested between 10 and 12 weeks old. HDAC4$^{loxP/loxP}$ contained flanked exon 5 of Hdac4 and have been previously characterized to generate an out-of-frame mutation in the presence of Cre recombinase (Pothoff et al 2007). HDAC5 global knockout (KO) are previously characterized (Chang et al 2004) and are viable into adulthood. Experimental littermate animals were generated from heterozygous crosses of HDAC5 ($^{+/-}$). All animals were housed on a 12 hr light-dark cycle with access to food and water ad libitum. All procedures were in accordance with the Institutional Animal Care and Use (IACUC) guidelines.

Dissociated Primary Neuronal Cultures: For *in vitro* experiments, primary embryonic (E18) striatal cultures were generated from Long Evans rats (Charles River Labs) as previously described (Pulipparacharuvil et al., 2008). Crude striatal cells were plated at $8.0x10^6$ /10 cm PDL (Sigma)-coated plate, $1.3x10^6$/well on PDL coated 6 well plates, or $2.4x10^6$ on PDL and Laminin coated glass coverslips in a 24-well plate in DMEM (Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), penicillin (50µg/ml)-streptomycin (50 units/ml; Sigma) and L-glutamine (4mM; Sigma) and incubated at 37C/5% CO2. 24 hours later, media was changed to supplemented Neurobasal (2% B27 (v/v); Invitrogen, penicillin (50µg/ml)-streptomycin (50 units/ml; Sigma) and L-glutamine (4mM; Sigma)).

Viruses and plasmids: p1005+ GFP HDAC4 WT was generated by PCR amplification of hHDAC4-Flag from pBJ-hHDAC4-Flag. PCR primers were designed to generate XbaI restriction sites on 5' and 3' end of full-length hHDAC4-Flag. p1005+ GFP backbone and PCR product were digested with XbaI and compatible cohesive ends were ligated to produce p1005+ GFP HDAC4 WT. p1005+ GFP HDAC4 3SA was generated by PPuMI and PflMI digestion of pcDNA3.1 HDAC4 3SA (Addgene), generating a fragment of HDAC4 coding sequence containing the mutations of interest. p1005+ GFP HDAC4 WT was digested with PPuMI and PflMI to remove the wildtype portion of the HDAC4 coding sequence and compatible cohesive end ligations generated p1005+ GFP HDAC4 3SA. Diagnostic digests and sequencing was performed to confirm correct orientation before sending plasmids for packaging (MIT, Rachael Neve).

Additional plasmids: 3X MRE-luciferase, CRE luciferase and TK-Renilla luciferase plasmids have all been previously described (Flavell, Cowan et al. 2006). p1005+ GFP was a generous gift from Rachael Neve. p1005+ GFP HDAC5 WT was previously described (Taniguchi, Carreira et al. 2012).

Confirmation of HSV overexpression by viral infection *in culture* and western blot: Striatal primary neuronal cultures were infected with 1 μl of HSV virus (titer). Forty-eight hours later cells were washed 1 time in ice-cold 1X PBS and harvested in 300ul/well of 1x sample buffer (SAB). Lysates were then sonicated at 30% for 20 seconds on ice and boiled for 10 minutes at 98C. Lysates were then processed and visualized by standard western blotting.

Immunocytochemistry: Primary neuronal cultures were transfected using calcium phosphate on day *in vitro* 8. Forty-eight hours later cells were stimulated as indicated, washed with 1x ice-cold PBS and fixed in 4% paraformaldehyde/2% sucrose at room temperature for 20 mins. Fixed cells were permeabilized in 0.4% (v/v) Triton X-100 and blocked with 10% non-fat dry milk, 1% normal donkey serum in 1X TBS containing 0.05% (v/v) Tween-20. Coverslips were incubated in anti-Flag antibody (1:100-1:250) followed by anti-mouse-Cy3 (1:500) diluted in 1X TBS containing 0.05% Tween-20 and 3% bovine serum albumin. Cells were then counterstained with the nuclear dye Hoechst 33342. Flag-tagged HDAC4 localization was categorized as cytoplasmic, nuclear, or both (evenly distributed across nucleus and cytoplasm) for each GFP-expressing (transfected) cell under experimenter-blind conditions using fluorescent microscopy.

Sample Preparation from Cocaine-Injected Mice: C57BL/6 mice (Charles River) were injected 1x/day (IP) with saline, for 6 days with saline followed by 1 day of 20mg/kg cocaine (cocaine-naïve), or for 7 days with 20mg/kg cocaine (cocaine-experienced) and sacrificed 4 hours after the last injection. Striata were rapidly dissected out from 1 mm coronal sections sliced by using a mouse brain matrix. For the phosphorylation status of HDAC4, dissected striata were dissolved in Tissue Lysis buffer (5 mM HEPES, pH 7.4, 1% SDS, 0.32 M sucrose, 10 mM NaF, 10 nM okadaic acid and protease inhibitor cocktail tablet (Roche)) and diluted with 9-fold volume of RIPA (without SDS) buffer (50 mM Tris pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% (v/v) NP40, 0.5% (w/v) DOC, 10 mM NaF, 10 nM okadaic acid and protease inhibitor cocktail tablet (Roche)) prior to immunoprecipitation of HDAC5 and HDAC4. Cytosolic and nuclear extracts were prepared using an NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology) according to the manufacturer's instructions. Nuclear/cytoplasmic ratios of HDAC5, relative to saline condition, were calculated in each sample; signal intensities of HDAC4 were normalized to -tubulin in the cytoplasmic fraction and to Lamin A/C in the nuclear fraction.

Immunoprecipitation and western blotting: HDAC4 was immunporecipitated following HDAC5 immunoprecipitation. HDAC5 was immunoprecipitated from striatal neuronal extract and striatal tissue extract in RIPA buffer using anti-HDAC5 monoclonal antibody (Abcam, #50001) and anti-HDAC5 polyclonal antibody (Cell signaling, #2082) then HDAC4 antibody ( ). Protein extracts were subjected to SDS-PAGE, transferred to PVDF membrane, blocked (10% (w/v) non-fat dry milk) and incubated with antibodies. Primary antibodies were diluted in 1X TBS with 0.05% Tween-20 containing 3% (w/v) bovine serum albumin

as follows: anti-HDAC4 (1:5XX00), anti-P-S279 (1:50), anti-β-tubulin (1:10,000), anti-Lamin A/C (1:500). Secondary antibodies were diluted in 1X TBS with 0.05% (v/v) Tween-20 containing 10% (w/v) non-fat dry milk, as follows: anti-mouse IgG conjugated with HRP (1:10,000), anti-rabbit IgG conjugated with HRP (1:10,000). Blots were developed by enhanced chemiluminescence (ECL-Prime, Amersham Pharmacia Biotech). Densitometry was performed with NIH ImageJ software.

Viral-Mediated Gene Transfer: Expression plasmids for HDAC5 WT, S279A, and S279E mutants in HSV vector were packaged into high-titer viral particles as described previously (Barrot et al., 2002). Stereotactic surgery was performed on mice under general anesthesia with a ketamine/xylazine cocktail (10 mg/kg:1 mg/kg). Coordinates to target the NAc (shell and core) were +1.6 mm anterior, +1.5 mm lateral, and −4.4 mm ventral from bregma (relative to dura) at a 10° angle. Virus was delivered bilaterally using Hamilton syringes at a rate of 0.1 µl/min for a total of 0.5 µl. Viral placements were confirmed by GFP signal, which was coexpressed in each virus.

Locomotor Sensitization: At the same time each day, mice were placed in standard plastic cages similar to their home cages for one hour to acclimate. Animals were then picked out of the cages, injected with saline or cocaine (i.p.), and placed back in the chamber for an additional hour. These plastic cages were inside the Photobeam Activity System (San Diego Instruments, San Diego, CA or MedAssociates Inc Instrumentation), where five photobeams measured the mouse's locomotor activity in 5-minute bins during the entire session (habituation and post-injection). Mice received saline injections on day 1–3 for HDAC5

studies or days 1-4 for HDAC4 studies. Both timelines were designed to allow animals to habituate to the novel environment and minimize novelty-induced locomotion. On days 4–10 for HDAC5 studies or on day 5 or days 5-9 for HDAC4 studies mice received cocaine injections (15 mg/kg for HDAC5 studies and 20 mg/kg for HDAC4 studies). Locomotor sensitization was assessed in HDAC4 NAc cKOs at least two weeks after a low dose CPP experiment was performed. For naïve HDAC4 studies (acute locomotor activity, figure 4-4c), day 1 of the experiment was 21 days after viral delivery, a time point at which high levels of expression were verified. For each day, the sum of the first 30 minutes of locomotor activity after injection is displayed.

Conditioned Place Preference: Mice were given an acute viral infusion by stereotaxic delivery (see methods below) and allowed to recover for three days. On day 3 post-surgery animals were allowed to explore the three-chambered apparatus and time on each chamber was recorded. Animals that spent greater than 1/3 of total time in one chamber over another were excluded. All included animals were then balanced across virus group based on magnitude of preference, an average near zero pre-test preference score within group, and side paired with cocaine. On days 4 and 5 post-surgery animals received two daily pairings: i.p. saline and confined on one side of the chamber in the am and i.p. cocaine (5 or 7.5 mg/kg) and confined to the other side of the chamber in the pm. 24 hours later animals were brought back to the chambers and allowed to freely explore all sides. Time in each chamber was recorded and a post-test conditioned place preference score generated for each animals and on average for virus group. Only animals that bilaterally express HSV-mediated gene transfer, assessed by GFP endogenous signal, were considered for analysis.

MEF2 (MRE) Reporter Assays: Primary neuronal cultures were transfected using calcium phosphate on day *in vitro* 8. Forty-eight hours later cells were stimulated as indicated, washed with 1x ice-cold PBS and harvested in 1x Passive Lysis Buffer for dual luciferase analysis per manufacturer's instructions (Promega).

Data Analysis: One-way, two-way or repeated measures ANOVAs with Bonferonni's or Tukey's multiple comparison post hoc tests were used. When appropriate, Student's t-tests were performed. Statistics were processed and graphs were designed using GraphPad Prism software.

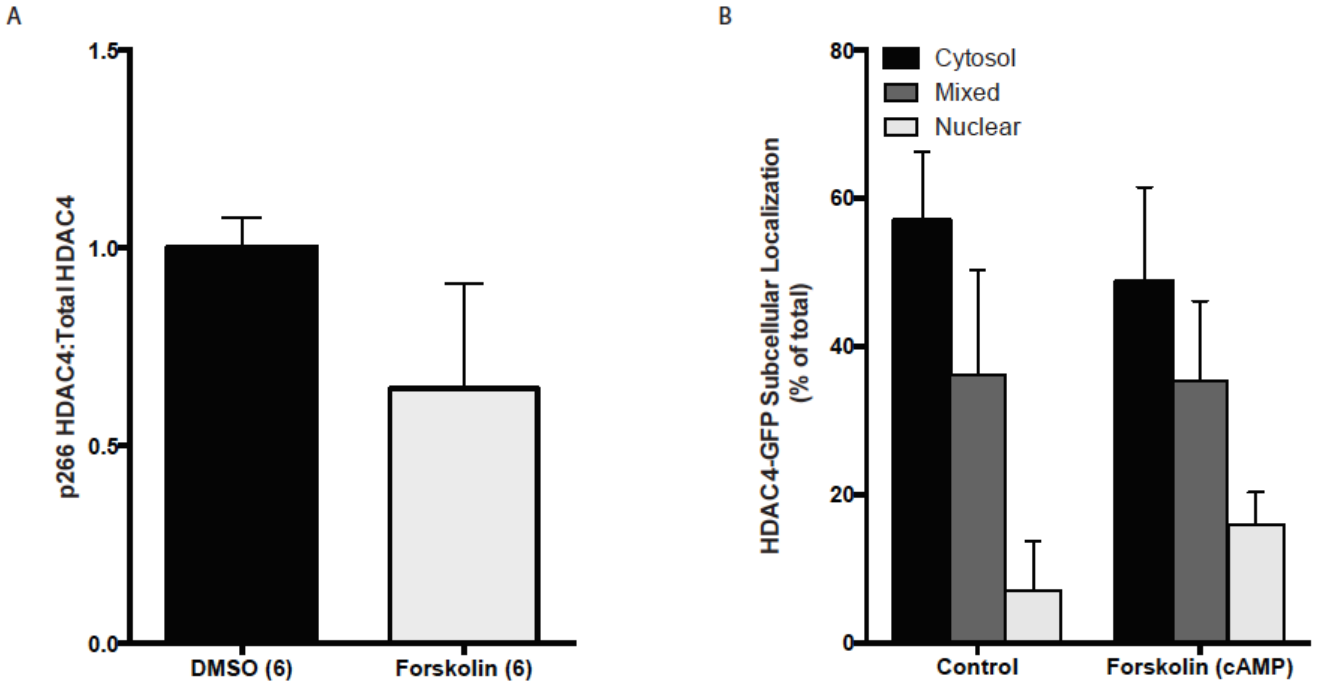


Figure 4-1. HDAC4 phosphorylation and localization are weakly influenced by forskolin signaling in vitro.
A. Cultured striatal neurons were treated with forskolin (10 μM) and lysed 3 hours later. HDAC5 immunodepleted lysates were processed for immunoprecipitation of HDAC4 and analyzed by immunoblotting with anti-P-S266 and anti-HDAC4 antibodies (mean +/− SEM, p = 0.2276, unpaired Student's t-test; n = 6/condition, three independent experiments).
B. The localization of HDAC4-GFP was categorized as cytoplasmic, nuclear, or both for individual cells by an experimenter blind to treatment. The percentage for each category was calculated from the total number of transfected neurons counted in each condition, and the average across experiments is shown (mean normalized percent +/- SEM, Two way ANOVA reveals no main effect of stimulation, F (1,12) = 2.877e-015, = > 0.9999, but a main effect of subcelullar compartment, F (2,12) = 8.187, p = 0.0057; n = 3 wells/condition, ~80 transfected cells were counted in each well).

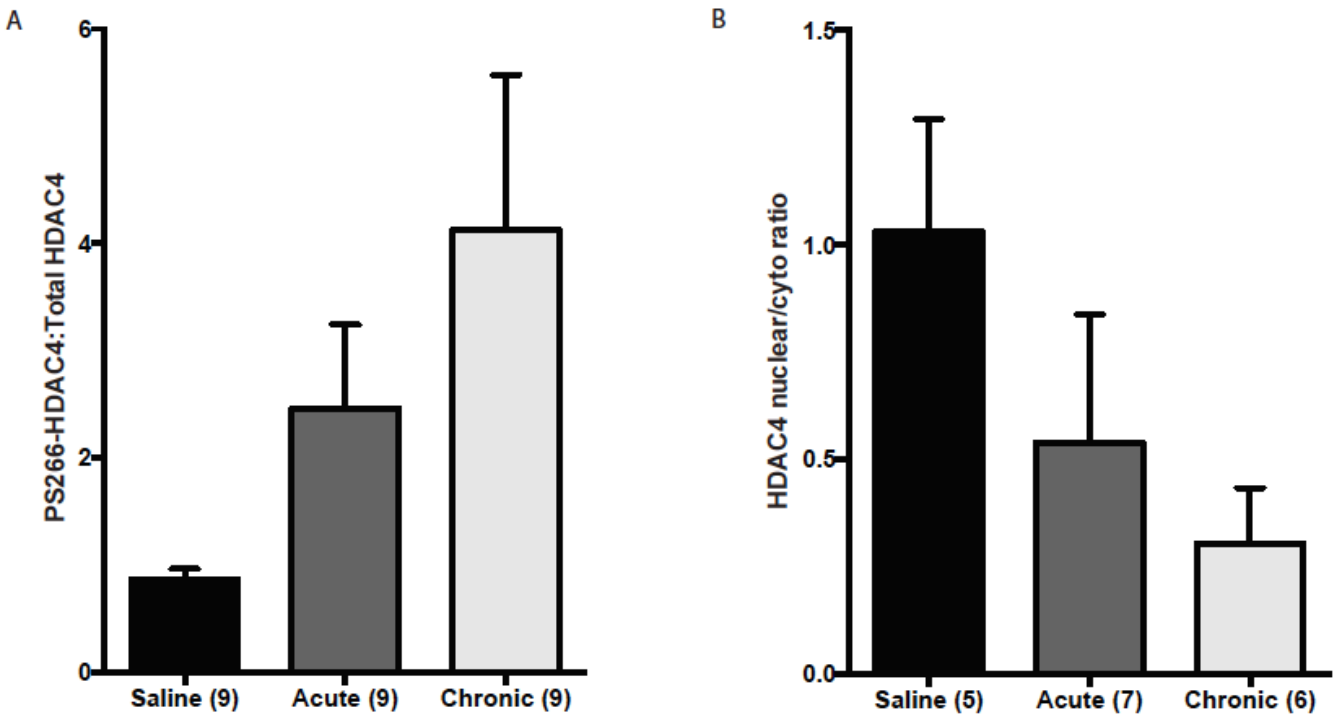


Figure 4-2. HDAC4 phosphorylation and localization are weakly influenced by cocaine exposure in vivo. Adult C57Bl/6 mice were injected with saline for 7 days (1 mL/kg, IP, 1x/day; control), with saline for 6 days followed by 1 day of cocaine (20 mg/kg; IP; cocaine-naïve) or with cocaine for 7 days (20 mg/kg; IP; cocaine-experienced).

A.Cocaine weakly influences phosphorylation of HDAC4. Striatal tissue was dissected out 4 hrs after the last injection and immunoprecipitated HDAC4 was subjected to western blotting using P-S266 and HDAC4 antibodies (mean +/− SEM, Univariate ANOVA reveals a trend of treatment, $F(2,24) = 2.675$, $p = 0.0894$ and no significant differences are detected by Bonferroni's multiple post hoc comparison to the saline control group; n = 9/condition)

B. Subcellular fractionation after cocaine exposure demonstrates that cocaine is unable to modulate localization of HDAC4 in striatum. Nuclear/cytoplasmic ratios of HDAC4, relative to saline, were calculated by comparing arbitrary units of nuclear and cytoplasmic HDAC4 (normalized to Lamin A/C in nuclear fraction and to β-tubulin in cytoplasmic fraction), (mean +/− SEM, Univariate ANOVA, $F(2, 15) = 1.970$, $p = 0.1739$; n = 5, 7 and 6, respectively).

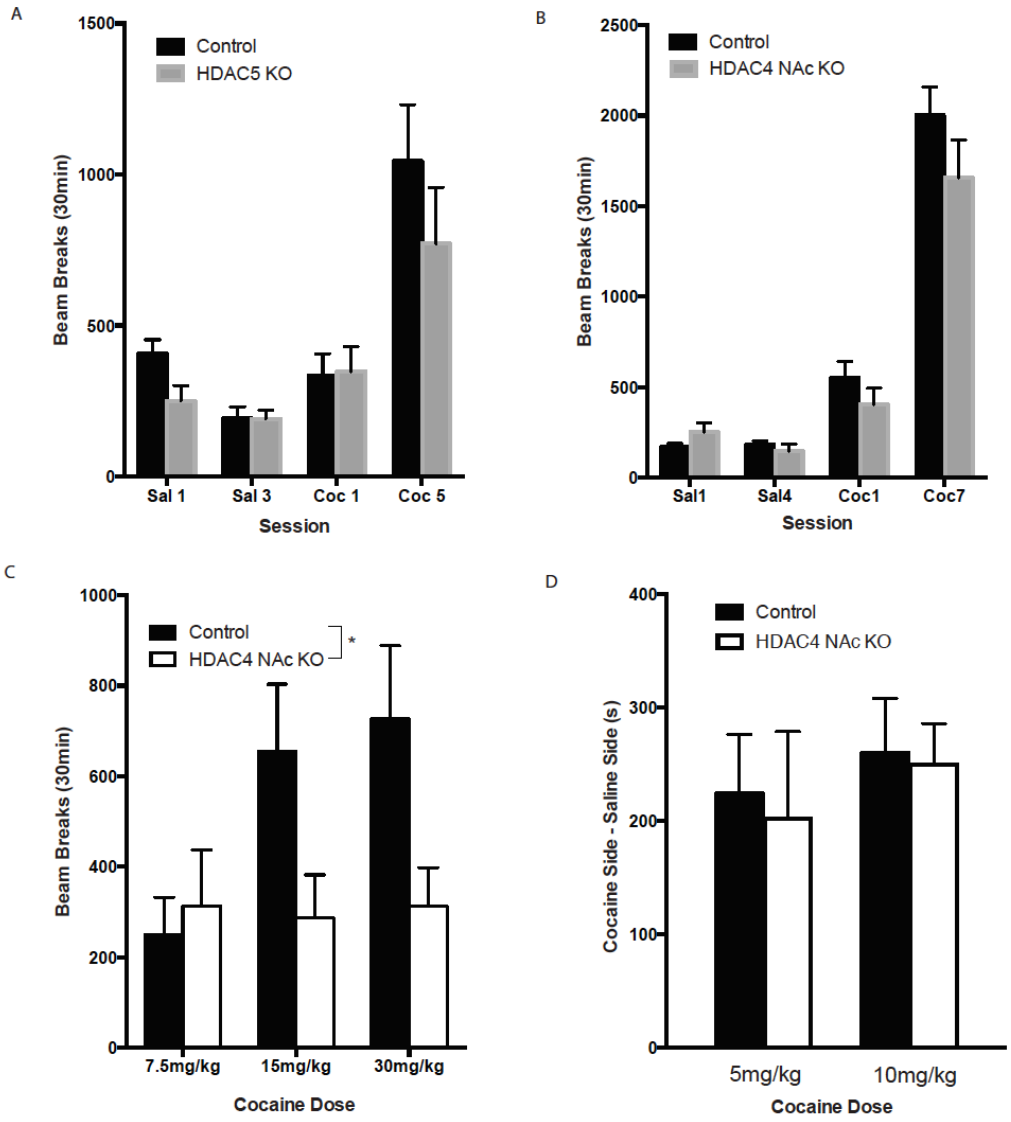


Figure 4-3. Loss of HDAC4 does not influence cocaine-related behavioral plasticity.

A. Loss of HDAC5 does not alter acute or sensitized locomotor responses to cocaine. HDAC5 KO mice have normal locomotor responses to saline and 20mg/kg cocaine injection (mean +/- SEM, Repeated measures Two-way ANOVA reveals main effect of cocaine sensitization, $F(3, 63) = 20.81$, $p < 0.0001$, but not genotype, $F(1,21) = 1.406$, $p = 0.2490$; n = 11 and 12. Post hoc Bonferroni's multiple comparisons reveals no significant differences between genotypes within treatment day).

B. HDAC4 NAc KO does not alter sensitized locomotor responses to cocaine. NAc targeted HDAC4 viral mediated knockout (cKO) animals have normal locomotor responses to saline and 15mg/kg cocaine (mean +/- SEM, Repeated measures Two-way ANOVA main effect of cocaine sensitization, $F(3, 87) = 126.5$, $p < 0.0001$, but not genotype, $F(1, 29) = 1.790$, $p = 0.1913$; n = 19 and 12. Post hoc Bonferroni's multiple comparisons reveals no significant differences between genotypes within treatment day).

C. HDAC4 NAc KO significantly reduces locomotor activation in response to acute cocaine exposure. Control and cKOs were dosed acutely with either 7.5, 15 or 30mg/kg cocaine (mean +/- SEM, Two-way ANOVA reveals main effect of genotype, $F(1, 47) = 5.903$, $p = 0.019$, but not dose, $F(2, 47) = 2.161$, $p = 0.1265$; 7.5mg/kg n = 10 and 8, 15mg/kg n = 9 and 9, and 30mg/kg n = 9 and 8, respectively).

D. HDAC4 NAc KO does not alter cocaine conditioned place preference. CPP to 5 or 10 mg/kg cocaine was not modulated by AAV-mediated Cre recombination of HDAC4 in NAc. (mean +/- SEM, unpaired Student's t-test reveal no significant differences in either 5mg/kg, $p = 0.6197$, or 10mg/kg, $p = 0.431$, CPP).

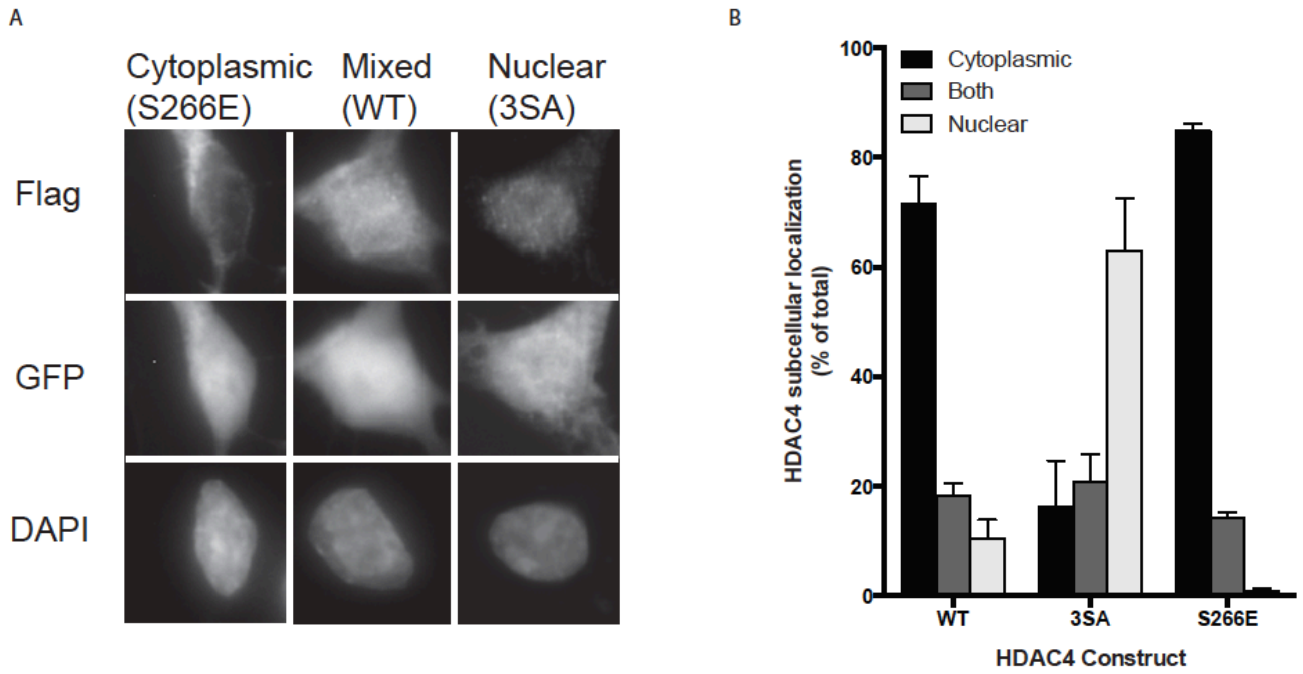


Figure 4H 4. Validation of HDAC5 3SA, a construct that promotes HDAC4 nuclear accumulation

A. Representative image showing subcellular localization of flag-tagged HDAC4 constructs in striatal cultures. Sections were counterstained with Hoechst.

B. Over-expression of HDAC4 3SA, but not HDAC4 S266E, significantly shifts subcellular localization to the nuclear compartment. Neurons transfected with flag-tagged HDAC4 WT, HDAC4 3SA or HDAC4 S266E p1005+ (GFP expressing vector) were fixed and stained for flag, GFP and DAPI immunocytochemistry (mean normalized percent +/- SEM, n = 9 coverslips from three independent experiments in each condition, ~90 neurons counted in each well).

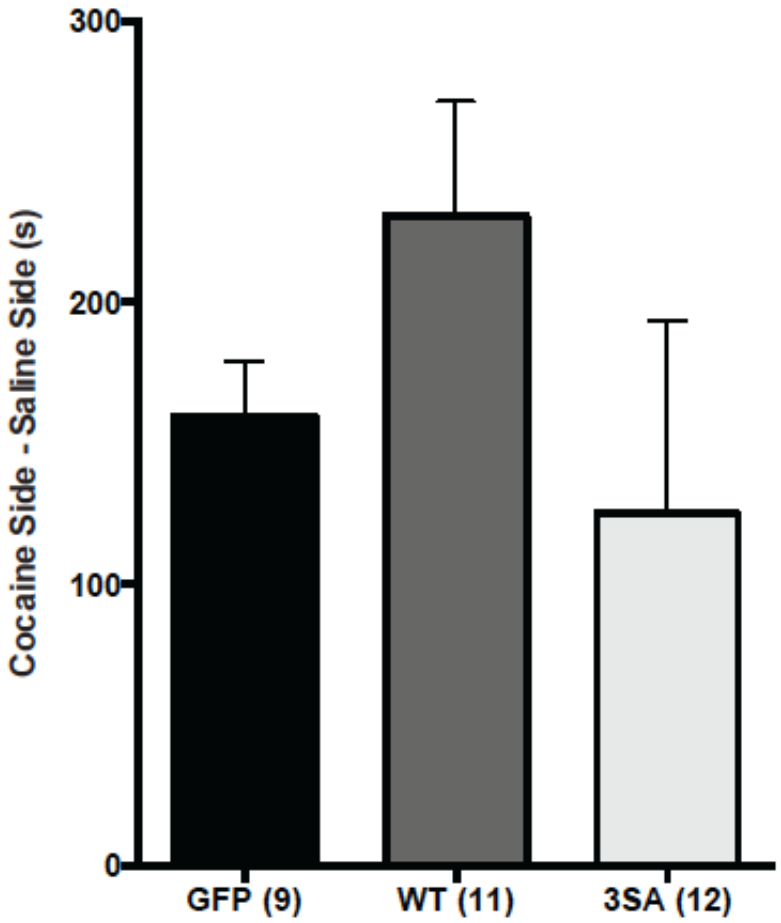


Figure 4-5. Viral Mediated Nuclear HDAC4 accumulation does not influence cocaine reward. HDAC4 OE does not influence cocaine CPP. CPP to 5mg/kg cocaine was not modulated by HSV-mediated overexpression of HDAC4 WT or HDAC4 3SA in NAc. (mean +/− SEM, Univariate ANOVA, $F(2, 30) = 0.3248$, $p = 0.7252$; n = 9, 12 and 12, respectively. CPP Score was defined as the time (sec) spent in the cocaine side minus the saline side post-conditioning.

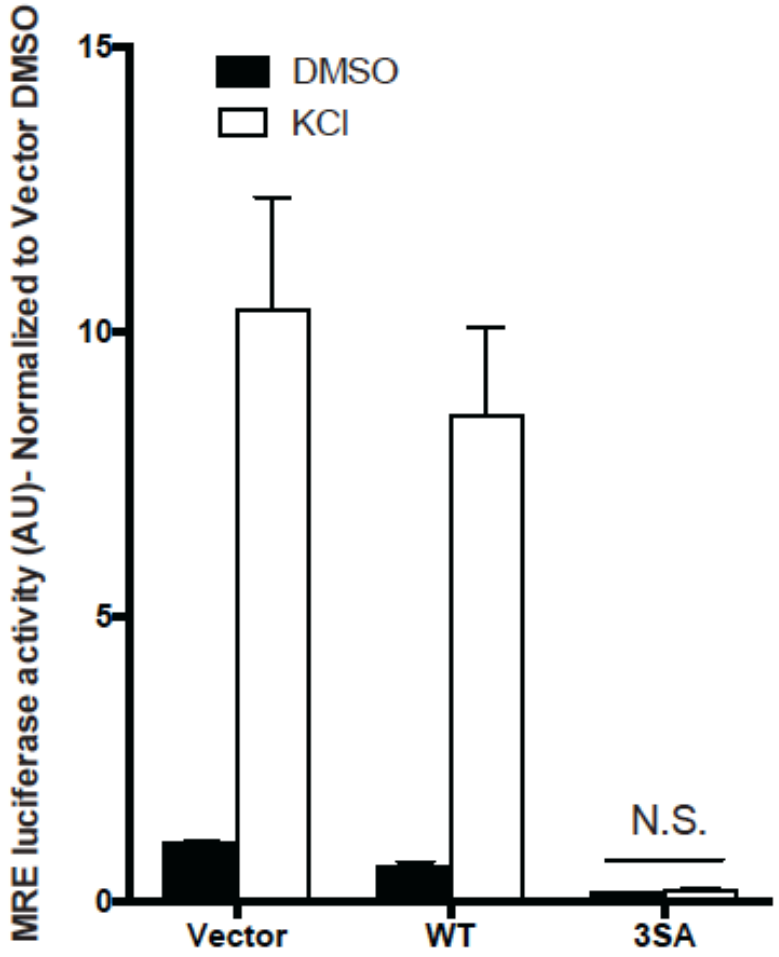


Figure 4-6. HDAC4 3SA suppresses MEF2 activity. Cultured striatal neurons transfected with an MRE-luciferase reporter plasmid and co-transfected with either vector plasmid or plasmid expressing HDAC4 WT or HDAC4 3SA. Basal or depolarization-induced (60mM KCl) reporter activity was assessed by firefly luciferase and normalized to TK-renilla luciferase. HDAC4 WT does not reduce basal or inducible MRE-activity in striatal cultures, while HDAC5 3SA reduces basal activity levels and prevents any depolarization-dependent induction. (mean +/- SEM, Two-way ANOVA was performed with all groups with a post hoc Bonferroni's multiple comparison analysis within condition to test for induction by depolarization; n = 9 in each condition).

## Chapter 5: Discussion and Future directions

This doctoral thesis work focused on two main aims: 1) characterizing the effect of cocaine experience on the regulation of localization of the class IIa HDACs, HDAC4 and HDAC5 and 2) determining the role of the class IIa HDACs, HDAC4 in cocaine-related behaviors. Our findings are summarized in a model below. We identified a preferential nuclear accumulation of HDAC5 and a subtle nuclear exclusion of HDAC4 under similar (cocaine) conditions. Furthermore, we characterized the effect of driving nuclear accumulation of HDAC5 and HDAC4 on cocaine-related behaviors. We observed an attenuation of cocaine reward and cocaine seeking under conditions of nuclear HDAC5 accumulation but not HDAC4. In chapter 2, we reported an extensive characterization of the signaling molecules that regulate HDAC5 downstream of cocaine and/or cAMP signaling *in vivo* and *in vitro*. In this study we characterized the consequence of a single point mutation to one of the regulatory sites for localization of HDAC5 on the conditioned place preference assay (CPP). Under conditions where we promote nuclear retention we observed a decrease in the preference to cocaine; an observation we interpret as a dampening of cocaine reward. In chapter 3, we further characterized the regulation of localization of HDAC5 under self-administered cocaine conditions. Under these conditions animals take comparable levels of cocaine to those analyzed by bolus experimenter delivery via intraperitoneal (i.p.) injections. Unlike experimenter administer cocaine, the delivery of cocaine occurs over a three-hour session. Under these conditions we observed a preferential weak induction of nuclear accumulation of HDAC5 in only the ventral, but not

dorsal, aspect of the striatum- corresponding to a nucleus accumbens enriched sample. We next characterized the functional consequence of driving nuclear accumulation of HDAC5 in the intravenous self-administration paradigm (IVSA) and cocaine conditioned place preference. In both assays, driving nuclear accumulation of HDAC5 (HDAC5 3SA) significantly attenuates cocaine seeking behaviors (IVSA) or cocaine reward (CPP). We pursued the characterization of nuclear HDAC5 in the absence of binding to its primary target transcription factor, MEF2. To this end we generated a mutant deficient for MEF2-binding but containing the serine to alanine substitutions that drive nuclear accumulation (HDAC5 3SAΔMBD). Under these conditions we were able to recapitulate the attenuation of reward (CPP) but not of cocaine seeking in the IVSA paradigm. The straightforward interpretation of these findings is confounded by technical difficulties and differences in the experimental parameters. They, however, propose a downstream dissociation in the MEF2-dependence of nuclear HDAC5's (HDAC5 3SA) effects on cocaine-related behaviors. In chapter 4, we aimed to characterize the HDAC5 homolog, HDAC4 in cocaine-related behaviors. Unlike HDAC5, HDAC4 is either weakly dephosphorylated by cAMP activity *in vitro* or weakly phosphorylated by cocaine *in vivo*. We then characterized the loss of either HDAC5 or HDAC4 for cocaine dependent locomotor sensitization. We observed that loss of HDAC5 or HDAC4 does not affect locomotor sensitization but that loss of HDAC4 in NAc is sufficient to blunt acute cocaine-induced locomotion. We did not observe an effect of deleting HDAC4 in the NAc on CPP. Similarly, driving nuclear accumulation of HDAC4, unlike HDAC5, did not modulate CPP behavior *in vivo*. Consistent with the idea that HDAC5 limits cocaine reward in a MEF2-independent cascade, we observed that nuclear accumulation of HDAC4 significantly blocks MEF2 activity despite not affecting cocaine

reward behaviors. These initial characterizations allow the development of a number of interesting hypothesis and experimental designs to pursue in the future.

Using the IVSA paradigm we identified a unique role for nuclear accumulated HDAC5 in curbing reinstatement to cues and drug primes. These observations were in the absence of an effect on measures of taking, ie. stable intake, sensitivity or motivation. We also did not observe an effect on context-dependent seeking or extinction. A few conclusions can be drawn from these observations: first, animals over-expressing HDAC5 in the nuclear compartment are able to learn and acquire the contingencies associated to a reward. Second, the drug is equally reinforcing to nuclear HDAC5 over-expressing animals. Lastly, driving nuclear HDAC5 function does not change the overall response of the nucleus accumbens to drugs of reward because these animals have normal sensitivity to the drug. It is interesting to consider that nuclear HDAC5 only dampens responses to a discrete external cue (light) and an internal cue (drug experience) but not the re-experience of the drug context. Differences and similarities between contextual, cued, and prime-induced reinstatement are extensive, debated and complex (Crombag and Shaham 2002, Shalev, Grimm et al. 2002, Shaham, Shalev et al. 2003). Importantly, a subregion of the NAc, the core, is heavily implicated in mediating seeking with considerable variability in the relative strength of other drug-associated brain regions in contributing to different reinstating stimuli responses (Kalivas and McFarland 2003). Although an *a priori* hypothesis based on circuitry involved is difficult to make, we may ay least hypothesize that the NAc core is the site of nuclear HDAC5 function in cocaine seeking. Future studies may further refine this hypothesis by analyzing nuclear HDAC5 function in modulating the synaptic strength or efficacy from cortical structures that are required to promote reinstatement behaviors

(Cornish, Duffy et al. 1999, Kalivas and McFarland 2003, Anderson and Pierce 2005, Schmidt, Anderson et al. 2005, Marchant, Rabei et al. 2014). In the study design we allowed animals to withdraw for a week before looking at measure of seeking. This specific withdrawal time may fall short in allowing for the development of drug plasticity-associated mechanisms that are hypothesized to underlie incubation of craving (Loweth, Tseng et al. 2014). One interpretation of our findings, however, is that we have blunted the induction of dynamic changes that occur in response to the drug experience and over withdrawal that may 'sensitize' the system to reinstating stimuli (Hu 2007, Lee and Dong 2011). A limitation is identified in that we did not vary this variable in our studies. An informative experiment may be to test nuclear HDAC5's effect on reinstatement in the absence of withdrawal or following longer withdrawal time-points. Our findings, however, propose that HDAC5's function in the nucleus may act: 1) during reinstatement to prevent some activity-dependent transcriptional program that promotes seeking, 2) during the learning of the association between discrete cues- light stimulus or the interoceptive drug state, and/or 3) during the development of "craving" and associated functional and structural plasticity. This observation proposes the hypothesis that nuclear HDAC5 acts during one or more discrete behavioral time windows to affect reinstating responses. One way to test this idea would involve using HSV-mediated over-expression during discrete periods of the experimental timeline. In separate cohorts, animals could receive HSV-mediated gene transfer only during reinstatement assays, only during fixed ratio training and only during the withdrawal phase. Using this approach, we would identify when nuclear HDAC5 function is sufficient in curbing reinstatement behaviors; an observation that would more easily guide our hypotheses as to its function. This type of study may also

guide future translational work by identifying the critical time window for nuclear HDAC5 function in limiting relapse-like behaviors.

In our IVSA characterization of nuclear HDAC5 function we have uncovered a mechanisms to curb reinstatement, a model of relapse (de Wit and Stewart 1981). Cocaine, and broadly drug, addiction is in nature a chronic disease for which effective treatments are lacking (McLellan, Lewis et al. 2000, Kalivas 2007, Degenhardt and Hall 2012, Quintero 2013). The identification of a molecule and/or treatment that may limit or decrease the likelihood of seeking for drug following abstinent periods may be extremely useful in the clinical setting. With this in mind, one application of or study would involve designing a complex multi-level screen of compounds that may promote HDAC5 nuclear retention or function. Although not an exhaustive design this type of experiment would require at least two screening levels: first, a small molecule screen of compounds that promote/enhance HDAC5 activity *in vitro* that may yield reasonable candidates and secondly, a behavioral assay that may test the efficacy of these molecules *in vivo* (Curtin and Glaser 2003, Duvic and Vu 2007). To this end, a more precise characterization of the necessary and sufficient function of nuclear HDAC5 in IVSA behaviors is informative. It is interesting to note that HDAC5 KO animals are hypersensitive to cocaine CPP following prior cocaine experience. These findings in the context of our own findings outlined above suggest that this transient mechanism of nuclear accumulation serves to limit transcriptional programs that may later promote reward expression (in the CPP paradigm) or seeking (in the IVSA reinstatement paradigm). One hypothesis that may be generated from these combined findings may be that HDAC5 KO mice will develop normal intake under fixed ratio IVSA conditions but show

enhanced seeking behaviors. This characterization may further support the necessary role of HDAC5 function in limiting aspects of cocaine-related behaviors.

One interesting observation to be made from the CPP analyses of HDAC5 and HDAC4 function involves the downstream molecular cascades that may be recruited to limit cocaine reward. Briefly, we found that nuclear HDAC5 limits CPP in a MEF2-independent manner. Meanwhile, nuclear HDAC4 does not limit CPP but is effective at limiting MEF2 transcriptional activity. Taken together, these observations strongly support a function for nuclear HDAC5 that is independent of MEF2 and unique to HDAC5, ie, not induced by HDAC4. Therefore, characterizing the transcription factor/s that mediates the effect of nuclear HDAC5 may be fruitful. From work in our lab we know that driving nuclear HDAC5 accumulation *in vitro* promotes the association of HDAC5 to MEF2 consensus binding motifs as well as AP-1 consensus binding motifs.

AP-1, activator protein-1, is a transcriptional complex that contains both fos and jun family members. These homo or heteromers act to promote transcription in adult brain and are regulated in a complex manner in response to numerous conditions (Herdegen and Waetzig 2001, Ruffle 2014). Regulation and function of members of the fos family of transcription factors is described in the context of cocaine behaviors in an extensive body of literature. Acute cocaine induces c-fos, c-jun, fosB, junB and zif268 in NAc (Hope, Kosofsky et al. 1992, Torres and Rivier 1994, Larson, Akkentli et al. 2010). This effect desensitizes with chronic cocaine experience- the inductions of these genes becomes dampened. However, cocaine increases AP-1 binding activity in the NAc acutely and chronically (Hope, Kosofsky et al. 1992, Hope 1998). The increased AP-1 activity in the context of reduces fos members is in part attributable to the expression of novel delta

FosB-related proteins, or FRAs (chronic Fos-related antigens) (Chen, Nye et al. 1995, Moratalla, Elibol et al. 1996, McClung and Nestler 2003, Perrotti, Weaver et al. 2008). These deltaFosB-like proteins are induced selectively by chronic cocaine (Hope, Nye et al. 1994, Rosen, Chuang et al. 1994, Vialou, Robison et al. 2010). Behaviorally, a number of studies have characterized these molecules. Increases in deltafosB enhance behavioral responses to cocaine: increases in acute locomotion, locomotor sensitization and reward in conditioned place preference or intracranial self-stimulation (ICSS) (Hiroi, Brown et al. 1997, Kelz, Chen et al. 1999, Nestler, Barrot et al. 2001, Muschamp, Nemeth et al. 2012, Robison, Vialou et al. 2013, Ohnishi, Ohnishi et al. 2015). Increases of deltaFosB selectively in dynorphin-containing striatal neurons, primarily D1-type medium spiny neurons, facilitates acquisition of cocaine self-administration and enhances motivation to self-administer cocaine (Colby, Whisler et al. 2003). Interestingly, increases in fosB-like immunoreactivity following chronic cocaine experience correlate positively with enhanced cocaine place preference but negatively with novelty preference suggesting that these changes may drive withdrawal associated reward dysregulation (Harris, Hummel et al. 2007). Similarly, c-fos is induced by response-contingent cue presentations in a reinstatement model of cocaine seeking following abstinence from self-administration (Kufahl, Zavala et al. 2009). Collectively, increases in fos members or in AP-1 activity underlie aspect of cocaine behavioral plasticity. These observations propose that mechamisns that limit either fos induction or fos-dependent AP-1 activity may curb addiction-like behaviors.

Less is known about the regulation and function of members of the jun family of transcription factors in the context of cocaine behaviors. C-jun, jun-B and Jun-D are

robustly induced by acute cocaine and modestly by chronic cocaine experience (Hope, Kosofsky et al. 1992, Couceyro, Pollock et al. 1994). Behaviorally, Delta c-Jun, a dominant negative mutant of c-Jun, in striatum attenuates cocaine CPP but has no effect on acute cocaine locomotion or locomotor sensitization (Peakman, Colby et al. 2003). In the context of our own data on nuclear HDAC5 a testable hypothesis may be made that HDAC5 3SA is dampening the chronic cocaine-dependent FRA response that underlies development of sensitization, reward, increased motivation and correlates with reinstatement. This hypothesis is supported by the effect of HDAC5 3SA in CPP which phenocopies that of ΔC-jun on CPP. Studies designed at identifying the interaction between nuclear HDAC5 and the AP-1 complex members may provide useful insight into novel HDAC5 targets.

Interestingly, other potential binding partners emerge from a review of the literature. One such candidate to explore as a downstream effector and co-repressor for HDAC5 is the transcriptional regulator nucleus accumbens protein 1, NAC1. NAC1 may act as a transcriptional repressor or activator, a function that is determined by its binding partners (Mackler, Korutla et al. 2000, Korutla, Wang et al. 2002, Korutla, Wang et al. 2005, Korutla, Degnan et al. 2007). NAC1 is induced by chronic cocaine (Cha, Pierce et al. 1997) and over-expression of NAC1 prevents behavioral sensitization (Mackler, Korutla et al. 2000) (Mackler 2000). NAC1 is also a target of AP-1 complexes and induced by the co-expression of c-jun and c-fos (Mackler, Homan et al. 2003). In the context of our nuclear HDAC5 model, NAC1 may be a downstream target that may be regulated via AP-1 dependent HDAC5 inhibition. Importantly because HDAC5 is a binding partner of NAC1 it may also act acutely in a heterodimer to limit reward- and reinstatement-promoting genes.

This example highlights alternate binding partners to nuclear HDAC5 that may permit and promote its actions *in vivo*.

It is also informative to make the observation that both HDAC4 and HDAC5 have been shown to require the deacetylase domain to modulate cocaine-related behaviors (Renthal, Maze et al. 2007, Wang, Lv et al. 2010). These studies do not themselves confirm that deacetylase activity *per se* is required but rather that the C-terminal domain is required. This is an especially important distinction to make as some evidence for a weak or inactive catalytic domain has been reported in the literature (Lahm, Paolini et al. 2007, Sando, Gounko et al. 2012). Taken together with our findings, these findings highlight a potential region of interest to identify novel binding partners that may mediate the effect of nuclear HDAC5 *in vivo*.

An alternative strategy to identifying and defining downstream effectors and targets of nuclear HDAC5 may be to further characterize the downstream transcriptional program. Work in our lab has characterized the genomic regions to which nuclear HDAC5 is bound using chromatin immuno-precipitation followed by massive parallel DNA sequencing (ChIP-seq) (Mundade, Ozer et al. 2014). This study, although not presented within the content of this thesis has informed our studies and highlighted potential gene candidates for HDAC5 regulation. Furthermore, this analysis took advantage of our ability to immuno-precipitate complexes that contain endogenous HDAC5. Although it is likely that our HDAC5 3SA mutant behaves similar to WT HDAC5 we cannot discard the possibility that HDAC5 3SA confers some transcriptional repressor gain-of-function or an indirect transcriptional activation gain-of function that may underlie or explain our behavioral effects. It is also noteworthy that roughly ~80% of target genomic regions contained at

least one consensus motif for MEF2 binding but our behavioral effects suggest a MEF2 independent function at least for cocaine reward. Although inconclusive, the effects on IVSA may be in fact mediated by MEF2 transcriptional repression. These observations together allow us to generate a number of conditions to analyze targets of HDAC5. Targets that are MEF2 consensus containing, we may predict, are important for mediating the IVSA behavioral finding while targets that do not contain MEF2 consensus sites may be more relevant as candidates for cocaine reward-related plasticity. Importantly, however, these findings do not provide information on transcriptional regulation of candidate genes. One way to assess the role of HDAC5 3SA therefore would be to look at the transcriptional profile of target genes in the presence or absence of WT HDAC5, HDAC5 3SA and HDAC5 3SAΔ. This may be achieved by isolating RNA from infected cell populations *in vitro* or *in vivo* and performing whole transcriptome shotgun sequencing (WTSS) or RNA-seq (Evans 2015, Jiang, Zhou et al. 2015). This experimental technique, together with the results of ChIP-sequencing may confirm gene targets of HDAC5 as well as elucidate MEF2-dependent and independent HDAC5 targets. This design may also help identify the function of HDAC5 3SA *in vivo* and confirm that this effect is in fact a downstream consequence of accumulating HDAC5 in the nuclear compartment.

Our findings on the role of HDAC5 are exciting in that they provide a molecular mechanism that may be targeted to curb cocaine reinstatement behaviors. There are however, important limitations to our study and our ability to translate the importance of our findings towards a clinical application. Nuclear HDAC5 limits cue- and prime-mediated reinstatement behaviors following cocaine IVSA and attenuates cocaine reward in the CPP assay. Within the scope of these studies, however, we have failed to characterize the role of

HDAC5 in other drugs of abuse. Importantly, circuitry that drives drug consumption and more specifically reinstatement in preclinical models or relapse in addicts is similar across drug kinds (Shalev, Highfield et al. 2000, Kalivas and McFarland 2003, Shaham, Shalev et al. 2003, Bossert, Marchant et al. 2013). Similarly, animals contextually condition (develop CPP) to a multitude of drugs of abuse, but not all (Bardo and Bevins 2000, Napier, Herrold et al. 2013). Future studies should explore the role of HDAC5 in other drugs of abuse. Whether HDAC5 acts similarly in other drug models or not will be informative in identifying the relative value of pursuing this mechanism as a therapeutic avenue for addiction.

In the same light, it is important to acknowledge that our studies were performed on a short access design of self-administration. This experimental design exposes animals to the operant chamber for 1-3 hours daily and yields stable intake over days. This design allows for the interpretation of drug sensitivity and reinforcing properties but fails to model certain aspects of cocaine addiction. Other models and experimental parameters have better face validity for some aspects of cocaine addiction such as the development of tolerance to the drug of abuse (escalation), the incubation of craving over prolonged periods of withdrawal, and seeking as a consequence of stressors or in the face of punishment (Lu, Grimm et al. 2004, Lu, Grimm et al. 2004, Roberts, Morgan et al. 2007, Zernig, Ahmed et al. 2007, Erb 2010, Bossert, Marchant et al. 2013). An interesting direction would be to test the hypothesis that nuclear HDAC5 limits aspects of addiction in these models. These studies may further add value to developing more translation work.

Our studies employ viral mediated gene transfer in the NAc. Under these conditions, we observed over-expression in both the core and shell of the NAc. It is important to

highlight that the NAc is not a homogeneous nucleus but rather has at least two functionally relevant distributions. First, the NAc is subdivided into the core and shell. Although still debated, the shell is primarily described as driving reinforcement-based behaviors while the core has been shown to play a key role in seeking and reward (McFarland, Lapish et al. 2003, Di Chiara and Bassareo 2007, Ikemoto 2007, Graham, Krishnan et al. 2009, Wang, Lv et al. 2010, Larson, Graham et al. 2011). Second, the medium spiny neurons (MSNs) of the NAc are sub-classified as either dynorphin and D1- or enkephalin and D2-receptor type expressing. Important distinctions arise from this categorization, D1R expressing MSNs signal via $G_s$ or $G_\alpha$- coupled receptor proteins and promote cAMP and PKA signaling as well as $Ca^{2+}$ signaling via L-type calcium channels. Meanwhile D2R expressing MSNs signal via $G_i$ or $G_o$–coupled receptor proteins and dampen cAMP signaling cascades and $Ca^{2+}$ but promote $K^+$ channel activity (Lobo and Nestler 2011, Smith, Lobo et al. 2013). Behaviorally, D1 MSNs predominate in driving reinforcing and sensitizing effects of drugs of abuse while D2 MSNs activity antagonize these. Cell-type specific roles in models of relapse are less well characterized but pharmacological and genetic studies strongly suggest a preferential role for D2R-expressing MSNs in driving these behaviors (Self, Barnhart et al. 1996, Bachtell, Whisler et al. 2005, Bachtell, Choi et al. 2008, Larson, Akkentli et al. 2010, Self 2010, Smith, Lobo et al. 2013). Future studies on the role of nuclear HDAC5 in the NAc may benefit from cell-type and sub-NAc regional specificity.

Our study on the regulation and role of HDAC4 in cocaine-related behaviors provided some interesting observations. We speculated that HDAC4 and HDAC5 would have redundant regulation and roles. Surprisingly, we find that the regulation *in vivo* may

be opposing and the function of these homologues dissociable. The conclusions in this study may benefit from a more extensive characterization. The regulation of localization was only analyzed at one time-point, 4 hours after cocaine administration. This time-point was chosen because in the HDAC5 study (chapter 2) it yielded robust observations. It is however reasonable to analyze the localization of HDAC4 at other time-points following cocaine administration as we observe a weak induction of nuclear export 4 hours later. It is possible that in this study we have missed the 'peak of export' as this process may recruit kinases to phosphorylate HDAC4 or phosphorylation-independent mechanisms (Soriano, Chawla et al. 2013) on a completely different time scale (authors show nuclear export of HDAC5 peaks at 2 hours after synaptic activity (McKenzie, Stevenson et al. 2005)) as that characterized for the dephosphorylation and nuclear import of HDAC5 (Taniguchi, Carreira et al. 2012).

Our analysis of HDAC4 and HDAC5 phosphorylation following cocaine experience yields a composite regulation within the class IIa HDACs, ie, phosphorylation of HDAC4 and dephosphorylation of HDAC5. It seems likely that the differences of regulation of phosphorylation in our characterization and those previously reported may be due to the complex regulation of the class IIa family members and distinct experimental approaches. To recapitulate those data, authors report that cocaine enhances phosphorylation at S259 of HDAC5 (or S246 of HDAC4) (Renthal, Maze et al. 2007, Host, Dietrich et al. 2011, Dietrich, Takemori et al. 2012). A limitation in this interpretation comes from the fact that this site is conserved within the class IIa HDACs and the antibodies are likely to recognize the phospho-status of all class IIa family members, therefore, one straightforward explanation of this discrepancy is simply that as a population the net movement of the class

IIa's appears to favor the cytoplasmic compartment. This idea is supported by the fact that HDAC5 becomes enriched in the nucleus while HDAC4 seems to be preferentially sequestered in the cytoplasm (chapters 2 through 4). The effect of cocaine or our manipulations on other class IIa HDACs is as of yet unknown. A thorough analysis of the regulation of the other class IIa members, HDAC9 and HDAC7 may help identify and explain incompatibilities in our results and those published.

Similarly, the analysis of the nuclear function of HDAC4 on reward was only analyzed at one cocaine dose, 5mg/kg. Although we attempted to characterize CPP to 7.5mg/kg these data were not interpretable due to technical limitations. In these behavioral cohorts we observed significant toxicity due to HSV delivery that we are unable to account for at this time. Nonetheless, our analysis of cocaine reward under comparable conditions for nuclear HDAC5 and nuclear HDAC4 suggest that these are not redundant manipulations and only nuclear HDAC5 attenuates cocaine reward. Although we were unable to recapitulate prior data showing a role in limiting reward for HDAC4 we have not tested the role of HDAC4 in intravenous self-administration behaviors. Previous work demonstrated a significant decrease of both the descending limb of the dose response and of progressive ratio (Wang, Lv et al. 2010). Together, these findings suggest that HDAC4 may play a role in decreasing sensitivity (or opposing tolerance) and decreasing motivation. In our own self-administration characterization of HDAC5 WT or nuclear (HDAC5 3SA), described in chapter 3, we failed to observe modulation of the dose response or progressive ratio; a prediction we would have postulated given the sequence and functional homology described for these two class IIa members. These separate observations may suggest a non-overlapping role for HDAC4 and HDAC5 in limiting

different aspects of addiction like behaviors. Interesting models emerges that dissociate the functions of these class IIa homologues. HDAC4 may limit aspects of cocaine taking and its primary reinforcement while HDAC5 is poised to limit aspects of cocaine seeking and 'secondary' reinforcement, that which comes from associated cues to the drug self-administration experience. Alternatively, HDAC5 may play a central role in NAc and cocaine-related behaviors while HDAC4 may be dispensable under these conditions. A similar observation is reported in learning and memory studies showing a preferential central role for HDAC4 but not HDAC5 in these behaviors (Kim, Akhtar et al. 2012). These hypotheses on the functional dissociation of HDAC4 and HDAC5 may be tested directly in a side-by-side comparison of the role in NAc of these two molecules in cocaine and other drugs of abuse using IVSA behavioral assays.

In conclusion, our studies describe a novel, unique role for the nuclear function of HDAC5 in cocaine-related behaviors. These findings provide an initial characterization on the nuclear role of HDAC5 that further support its future studies. These findings also lend support to the pursuit of epigenetic mechanisms as regulators of behavioral and functional plasticity associated to cocaine experience. On a personal note, the development of this project alongside incredibly talented scientists and mentors has not only represented an incredibly satisfying thesis work but has also allowed me to develop my capacities as a researcher. I look forward to continuing my professional growth in molecular neuroscience and in understanding epigenetic regulation of complex biological and behavioral phenomena.

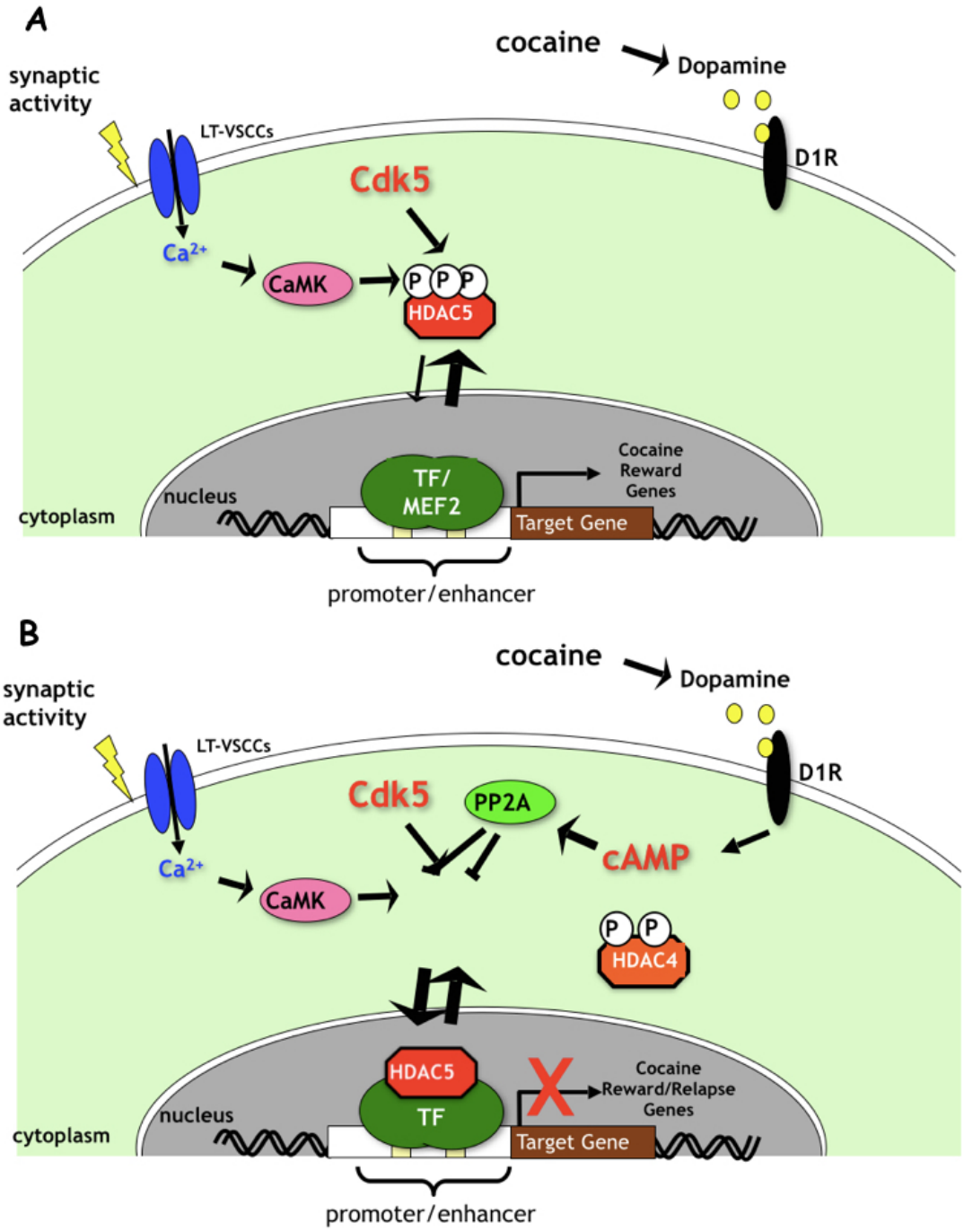


**Figure 5-1. Model for the regulation of HDAC5 and HDAC4 by cocaine**. Under basal conditions (panel A) HDAC5 is phosphorylated by $Ca^{2+}$-calmodulin dependent kinase (CamK) and cyclin dependent kinase 5 (Cdk5) and maintained primarily in the cytoplasmic compartment. In the presence of cocaine and/or dopaminergic signaling via D1 receptor type, cyclic AMP (cAMP) via protein phosphatase 2A (PP2A)-dependent mechanisms dephosphorylates HDAC5, but not HDAC4. This results in nuclear accumulation of HDAC5, a condition that inhibits cocaine reward (CPP) and relapse (IVSA reinstatement) promoting transcription.

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