POSTNATAL ROLE FOR HISTONE DEACETYLASE 1 AND 2 IN BEHAVIORAL AND NEURONAL HOMEOSTASIS

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Dedicated to my parents,
Mohamed and Nadia Mahgoub,
my brother Michael,
my husband Nabil,
my children Ibrahim and Mariam,
and my family and friends for their
unconditional love and continued support.

POSTNATAL ROLE FOR HISTONE DEACETYLASE 1 AND 2 IN BEHAVIORAL AND NEURONAL HOMEOSTASIS

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Epigenetics is a dynamic process that can change gene expression without alterations in the DNA sequence. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) can influence gene activity by inducing either an active or inactive chromatin state, respectively. Accumulating *in vitro* data has demonstrated a crucial function for histone acetylation and deacetylation in regulating the cellular and behavioral mechanisms underlying synaptic plasticity and learning and memory. In trying to delineate the roles of individual HDACs, genetic tools have been used to manipulate HDAC expression in rodents, uncovering distinct contributions of separate HDACs in regulating the processes of memory formation.

Moreover, recent findings have suggested an important role for inhibitors of HDACs in enhancing learning and memory as well as ameliorating symptoms related to neurodegenerative diseases with recent attention focused on HDAC1 and HDAC2.

The overlying goal of my Ph.D. thesis has been to further delineate how the loss of the HDAC1 and HDAC2 genes affects learning and memory and other complex behaviors. We accomplished this in three separate studies. First, we examined whether the individual loss of HDAC1 or HDAC2 postnatally could recapitulate the memory enhancements observed in previous pharmacological studies. We found that a conditional postnatal deletion of HDAC2 improves learning and memory behavior, while no effects were observed in HDAC1 knockout mice. Next, since HDAC1 and HDAC2 share a high degree of sequence homology we examined whether the simultaneous deletion of both genes from the postnatal brain would result in beneficial effects on learning and memory compared to the loss of the individual genes. We found that the loss of both HDAC1 and HDAC2 leads to early lethality in conditional double knockout mice, suggesting redundant functions of these HDACs in postmitotic neurons. Finally, after observing and characterizing an excessive grooming phenotype in conditional HDAC1/2 double knockout mice we mechanistically attributed this phenotype to dysregulation of SAP90/PSD-95-associated protein 3 (SAPAP3), a key protein linked to the development of obsessive-compulsive disorder (OCD). In summary, we have characterized important roles for HDAC1 and HDAC2 in mechanisms underlying learning and memory, and have uncovered a novel role for HDAC1/2 in mediating obsessive-compulsive-like behaviors.

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AAV – adeno-associated virus

AD – antidepressant

BDNF – brain-derived neurotrophic factor

CamKII – calcium/calmodulin-dependent protein kinase

CBL – cerebellum

CBP – CREB binding protein

cDNA – complementary DNA

ChIP – chromatin Immunoprecipitation

CREB – cyclic-AMP response element-binding protein

CTL – control

CTX – cortex

DG – dentate gyrus

DKO – double knockout

DMI – desipramine

Flx – fluoxetine

FST – forced swim test

GFP – Green Fluorescent Protein

H&E - Hematoxylin and Eosin

HAT – histone acetyltransferase

HC – hippocampus

HDAC – histone deacetylase

IP - intraperitoneal

KO – knockout

LTD – long term depression

LTP – long term potentiation

MeCP2 – methyl-CpG binding protein 2

OCD – obsessive-compulsive disorder

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PFA – paraformaldehyde

PPI – prepulse inhibition

Q-PCR – quantitative PCR

qRT – quantitative reverse transcription

RTT – Rett syndrome

SAHA – suberanilohydroxamic acid

SAPAP3 - SAP90/PSD-95-associated protein 3

Slitrk5 - SLIT and NTRK-like protein-5

SSRI - selective serotonin reuptake inhibitor

STR – striatum

TSA – trichostatin A

TUNEL - Terminal deoxynucleotidyltransferase-mediated UTP end labeling

VPA – Valproic acid

WT – wild type

CHAPTER 1

INTRODUCTION

Epigenetics is a complex process that regulates changes in gene expression via mechanisms other than alterations in the DNA sequence. These modifications can promote stable and long-lasting changes to chromatin structure therefore regulating transcriptional activation states in cells. Epigenetic processes can work though molecular modifications of the DNA such as DNA methylation, or modifications of the histone core as is seen in processes including but not limited to acetylation, deacetylation, and phosphorylation (Jaenisch and Bird, 2003) (Goldberg et al., 2007). In eukaryotic cells, DNA is wrapped around histone octamers, which contain one dimer each of the core histones H2A, H2B, H3, and H4, to make up the chromatin material (Felsenfeld and Groudine, 2003) (Borrelli et al., 2008). Histone acetyltransferases (HATs) work to relax the DNA/histone complex through the addition of an acetyl group to histone proteins, allowing more access of transcription factors to DNA, and therefore increasing gene expression. Histone deacetylases (HDACs) work in the opposite fashion by compressing the DNA/histone complex, restricting access of transcription factors to the DNA and consequentially repressing gene expression (Figure 1-1).

There are 11 known HDACs and they are grouped into 4 classes according to sequence homology and subcellular localization and function; Class I HDACs (HDAC1, 2, 3, and 8), Class IIa HDACs (HDAC4, 5, 7, 9), Class IIb HDACs (HDAC6, 10), and Class IV HDACs

(HDAC11) (Haberland et al., 2009). A separate family of HDACs referred to as sirtuins make up the Class III HDACs; this class of HDACs possesses deacetlyase activity, however function in a mechanism distinct from that of the rest of the HDAC family. Extensive reviews have been written on sirtuins (Michan and Sinclair, 2007) (Finkel et al., 2009), therefore they will not be discussed here. Class I HDACs are mostly localized to the nucleus with the exception of HDAC3, which can shuttle out to the cytoplasm, with HDAC1, HDAC2, and HDAC3 widely expressed throughout the brain. Class II HDACs can shuttle out of the nucleus to modify non-histone proteins. Little is known about the Class IV HDAC, HDAC11, as data has yet to emerge about its role in the adult brain. Many of the individual HDACs are expressed throughout the rodent adult CNS with Class I HDACs having the highest prevalence.

Studies have demonstrated that chromatin remodeling plays an important role in regulating synaptic plasticity and learning and memory (Alarcon et al., 2004) (Levenson et al., 2004) (Vecsey et al., 2007). The circuitry of the hippocampus has been previously implicated in regulating memory formation via the CA1-CA3 Schaffer-collateral pathway, a synaptic connection that is involved in long term potentiation (LTP) (Malenka and Bear, 2004). On a systems level, a battery of learning and memory behavioral paradigms including fear conditioning, Morris Water Maze, object recognition tasks, and motor coordination tasks are used to assess the cognitive abilities of rodents and provide preclinical animal models of diseases associated with memory deficits (Crawley, 1999). These electrophysiological and behavioral approaches have been used to examine a role for HDACs, both *in vitro* and *in vivo*, in contributing to the mechanisms that underlie learning and memory processes.

Epigenetic mechanisms have also been suggested to underlie several different psychiatric and neurodevelopmental disorders. Mutations in human CREB binding protein (CBP), a well-established HAT, was shown early on to result in Rubenstein-Taybi syndrome, and patients with this diagnosis suffer from severe learning impairments (Petrij et al., 1995). Moreover, mutations in human histone deacetylases can also lead to intellectual disabilities. It was recently reported that haploinsufficiency of HDAC4 results in Brachydactyl mental retardation syndrome, a debilitating disease encompassing several learning and memory deficits (Williams et al., 2010). Our studies will focus on preclinical data related to histone regulation and its role in learning and memory, however, a more detailed list of data from human studies can be found in a review from Egger and colleagues (Egger et al., 2004).

Histone acetyltransferases (HATs) and learning and memory

In talking about learning and memory processes, it's important to understand the mechanisms occurring at the level of the synapse. Synaptic maturation and strengthening can play important roles in increasing or decreasing synaptic activity. These activity-dependent changes are widely implicated in underlying the cellular basis of learning and memory as seen in the processes of long-term potentiation (LTP) and long-term depression (LTD) (Alkon and Nelson, 1990) (Kandel, 1997) (Malinow and Malenka, 2002). LTP is a well characterized form of plasticity in which strengthening of synapses between neurons can lead to long-term enhancements in synaptic transmission (Malenka and Nicoll, 1999) (Malenka and Bear, 2004). LTP was first discovered in the hippocampus, a brain region essential for learning and memory, and early studies demonstrated that repeated activation of excitatory

synapses results in increased strength and lasting potentiation of synapses that lasts for several hours (Bliss and Lomo, 1973). It is widely believed that these modifications in synaptic strength, particularly at Schaffer collateral synapses within the CA1 region, are a key mechanism in how memories are encoded and stored thus establishing LTP as a critical regulator in learning and memory processes. Conversely, LTD is a selective weakening of synapses that results in a lasting depression of synaptic activity. Similar to LTP, LTD can be experimentally induced by repetitive low frequency stimulation of Schaffer collateral-CA1 synapses (Dudek and Bear, 1992). Accumulating evidence has suggested that LTD functions to regulate synaptic strengthening which results from LTP processes in order to allow for new memories to be encoded (Malenka and Bear, 2004). Collectively, LTP and LTD are crucial mediators of synaptic transmission and offer useful means to study mechanisms underlying memory formation and storage.

Acetylation is carried out via HATs which function by neutralizing the tight interaction between DNA and histones thereby relaxing the chromatin structure and allowing for access of transcription factors to bind DNA and carry out gene expression (Grunstein, 1997; Guan et al., 2009). The most widely studied HAT in the brain is p300/CBP, which was originally identified as two separate coactivators, E1A binding protein (p300) and CREB-binding protein (CBP). Due to similar structure and function, they are now often referred to as p300/CBP (Arany et al., 1994; Shiama, 1997). Several studies report that mice with mutations in p300/CBP show deficits in various learning and memory paradigms such as fear conditioning, novel object recognition, and Morris Water Maze, as well as impairments in synaptic plasticity. A comprehensive list of these data can be found in a review by Barrett

and Wood (Barrett and Wood, 2008). Later studies showed that these learning and memory deficits could be attenuated by blocking deacetylation with pharmacological agents targeted at inhibiting HDAC activity. It was found that impairments in fear conditioning and novel object recognition caused by mutations in CBP could be rescued using Class I HDAC inhibitors giving rise to the idea HDACs play an important role in regulating learning and memory (Alarcon et al., 2004) (Korzus et al., 2004).

In separate work, it was shown that contextual fear conditioning in rats, a paradigm used to assess the early stages of long term memory consolidation, was associated with increases in acetylation of H3 within the CA1 subregion of the hippocampus (Levenson et al., 2004). The authors hypothesized that elevating acetylation levels could also lead to enhancements of long-term memory formation and synaptic plasticity. They showed that blocking HDAC activity in hippocampal slices with two different pharmacological inhibitors, trichostatin A (TSA) and sodium butyrate resulted in an induction of LTP at Schaffer-collateral synapses. Hippocampal LTP is an important phenomenon which measures activity dependent increases in synaptic strength and is considered a valuable tool in assessing synaptic changes associated with long term memory formation (Malenka, 1994) (Bliss and Collingridge, 1993). Other work has expanded on this data and shown that HDAC inhibitors enhance long-form LTP in a transcription-dependent manner. A more recent study found that HDAC inhibitors enhance memory processes by activating two key genes involved in memory formation, Nr4a1 *and* Nr4a2 (Vecsey et al., 2007). Both genes code for the immediate early transcription factors Nurr77 and Nurr1, respectively, are regulated by the CREB:CBP transcriptional complex, and have been previously implicated in learning

enhancements during a fear conditioning paradigm (Colon-Cesario et al., 2006) (von Hertzen and Giese, 2005) demonstrating additional evidence for a role of acetylase activation and specifically p300/CBP in memory formation. Collectively, these data support the premise that chromatin modification via histone acetylation and deacetylation plays a crucial role in regulating learning and memory processes (Table 1-1).

Histone deacetylases and synaptic mechanisms

Accumulating data has suggested an important role for Class I HDACs, most notably HDAC1 and HDAC2, in CNS development as well as synaptic transmission (Table 1-2). It was first reported that deletion of both HDAC1 and HDAC2 in mice during embryogenesis results in major abnormalities in hippocampal, cortical, and cerebellar development, as well as lethality at postnatal day 7 due to triggered apoptosis (Montgomery et al., 2009). Interestingly, no abnormalities occurred following the deletion of either HDAC1 or HDAC2 alone, suggesting redundant roles during neuronal development. In a different study looking at roles of HDAC1/2 *in vitro*, it was demonstrated that HDAC1 and HDAC2 function as a developmental switch that regulates synapse maturation and formation depending on the stage of synapse development (Akhtar et al., 2009). In immature hippocampal neurons, knockdown of HDAC1 and HDAC2 using the pharmacological inhibitor, TSA, results in increased synapse formation and function. However, in mature hippocampal neurons, knockdown of HDAC2 led to a decrease in excitatory synaptic activity with no effects seen following the deletion of HDAC1. This result is in agreement with the differential expression patterns of HDAC1 and HDAC2 during development. Both HDAC1 and HDAC2 are

expressed in neural progenitor cells, however as neuronal maturation occurs HDAC1 expression is mostly limited to glial cells while HDAC2 becomes highly expressed in mature neurons with little presence in glia (MacDonald and Roskams, 2008).

The aforementioned studies implicate an important role for Class I HDACs in regulating memory formation. However, most of this work was carried out with pharmacological agents and used "pan" HDAC inhibitors, offering no insight to the roles of individual HDACs. To overcome this caveat of selectivity, several laboratories have employed genetic tools and generated mutant or conditional knockout mice to examine individual HDAC contributions. Using these genetic mouse models, several studies have suggested a role for HDAC1 and HDAC2 in learning and memory behaviors. A notable study demonstrated that an embryonic deletion of HDAC2 leads to improvements in different types of learning and memory (Guan et al., 2009). Mice lacking HDAC2 perform better in a fear-conditioning paradigm and freeze less, as well as show enhancements in LTP. However, a deletion of HDAC1 had no overt phenotypes suggesting HDAC2 modulates these changes in memory formation. Moreover, overexpression of HDAC2 leads to impairments in fear memory as well as spatial memory as assessed in the Morris Water Maze providing first evidence that HDAC2 plays a crucial role in multiple cognitive tasks. More recent work has looked at a postnatal role for HDAC1 and HDAC2 and yielded similar results. A forebrain-specific conditional knockout of HDAC2 accelerated extinction learning in the fear-conditioning paradigm and resulted in learning enhancements specifically in associative learning tasks (Morris et al., 2013) providing further support for HDAC2 as a potential target in alleviating learning and memory deficits. Another Class I HDAC, HDAC3 has also been

demonstrated as a negative regulator of learning and memory. Mice with deletion of HDAC3 in the CA1 sub-region of the hippocampus displayed improvements in long-term memory when tested in an object recognition task (McQuown et al., 2011).

Less is known about the roles of Class II HDACs. Mice with a forebrain-specific deletion of HDAC4 display learning impairments, perform poorly in the Morris Water Maze, and have deficits in LTP (Kim et al., 2012), opposite to what has been shown with Class I HDAC inhibition. No overt phenotypes were found when deleting HDAC5, suggesting varying roles for the individual HDACs in regulating learning processes and synaptic plasticity. A recent study examining a role for HDACs in the regulation of fear memories in animal models of traumatic memories found that an intraperitoneal (IP) injection of the HDAC2-specific inhibitor, CI-994, during reconsolidation of the fear conditioning paradigm, could attenuate remote fear responses (Graff et al., 2014). They also concluded that the mechanism was likely though increased neuroplasticity within the hippocampus during memory extinction as animals treated with the CI-994 compound had enhanced LTP. Other individual HDACs such as HDAC6 and HDAC7 may function indirectly in regulating memory formation, but currently there is not enough data to establish specific roles for these HDACs in the adult brain or their contribution to learning and memory processes (Fischer et al., 2010).

HDAC inhibitors as therapies for neurodegenerative diseases

The data above provides strong support for HDAC inhibitors as potential therapeutic tools in the treatment of neurodegenerative disorders associated with learning and memory

deficits such as Alzheimer's, Huntington's and Parkinson's disease. Indeed an accumulating number of studies have provided evidence in agreement with this hypothesis using preclinical mouse models of these disorders. One of the first drugs discovered to inhibit histone deacetylase activity was Valproic acid (VPA), originally used in clinical settings as an anticonvulsant and mood stabilizer (Tunnicliff, 1999) (Johannessen, 2000) (Phiel et al., 2001). Later work revealed that VPA can enhance long-term memory and, more specifically, strengthen acquisition, extinction, and reconsolidation of conditioned fear memories (Bredy and Barad, 2008). Moreover, VPA has been demonstrated to enhance learning in various mouse models of Alzheimer's disease: APPswe/PS1dE9 double-transgenic mice, which have impairments in contextual memory beginning at six months of age, benefit from chronic intraperitoneal (IP) injections of VPA (Kilgore et al., 2010), while newly consolidated memories are stable for a period of two weeks following VPA injections as assessed by the fear-conditioning paradigm. A separate study linked VPA to having neuroprotective properties in a different Alzheimer's model, APP23 transgenic mice. IP injections of VPA led to a robust decrease in amyloid beta plaque number and ameliorated the learning and memory impairments characteristic to these mice (Qing et al., 2008). It is important to note, however, that VPA acts as a nonselective HDAC inhibitor, has other mechanisms of action such as targeting the GABAergic system, and can have adverse side effects in clinical patients, making it less than ideal as a therapeutic tool. However, the findings from these *in vivo* studies have further highlighted a role for HDAC inhibitors as potential targets in the treatment of neurodegenerative disease.

Two other well-known HDAC inhibitors, trichostatin A (TSA) and suberanilohydroxamic acid (SAHA), have more selective HDAC action against Class I and Class II HDACs, with SAHA recently shown to have higher potency in inhibiting Class I over Class II HDACs (Kilgore et al., 2010). R6/2 mice, a model of Huntington's disease, mimic symptoms of the disease including deficits in learning and memory as well as motor coordination (Hockly et al., 2002). Intraperitoneal injections of SAHA resulted in increased acetylation of histones H2B and H4 in the brain of R6/2 mice, and SAHA dramatically improved motor coordination when administered orally into drinking water (Hockly et al., 2003). Additional work has described similar findings in Parkinson's mouse models in which MPP+-induced dopaminergic cell death can be prevented with treatment with SAHA or TSA, suggesting a neuroprotective role for HDAC inhibitors (Wu et al., 2008). These data provide further support for the potential of HDAC inhibitors as a beneficial therapeutics to treat patients suffering from neurodegenerative disease (Table 1-3), yet highlight the need for HDAC-selective compounds as there could be unwanted off-target effects.

Concluding remarks

A growing body of work suggests that epigenetic processes, namely histone acetylation and histone deacetylation, play a critical role in the regulation of learning and memory. The studies outlined above provide a strong link between inhibition of HDAC function and enhancements in various forms of memory and synaptic plasticity. While pharmacological studies have started to provide valuable information in our understanding of the differing roles of distinct classes of HDACs in these processes, less insight has been gained in regards

to individual HDAC contributions. Conversely, studies from mutant and conditional knockout mice have uncovered unique roles for individual HDACs, and future studies looking at single deletions of other Class I HDACs in mutant mice could be helpful as those genetic models become available. Another interesting avenue to pursue would be more brain region specific deletions of individual HDACs. For example, the amygdala is known to be involved in the circuitry underlying fear memory, however it is unknown whether a selective loss of individual HDACs in the amygdala would recapitulate the learning and memory enhancements seen in conditional knockouts.

Data from both *in vitro* and in *vivo studies* implicate HDAC2 as the most promising target in regulating learning and memory behavior. Because of this, much attention has been focused on synthesizing HDAC2 selective compounds as therapeutic tools in treating learning and memory impairments related to neurodegenerative disease. It is important to note however, that HDAC1 and HDAC2 share 85% sequence identity (Grozinger and Schreiber, 2002) (Montgomery et al., 2007), making it difficult to develop HDAC2-specific inhibitors (Graff and Tsai, 2013a) without impacting HDAC1 function. Nevertheless, there is much interest and enthusiasm in the development of HDAC inhibitors for the treatment of psychiatric and neurological disorders, with increasing focus on trying to achieve selective targeting of individual HDACS to avoid issues of selectivity and potential adverse side effects.

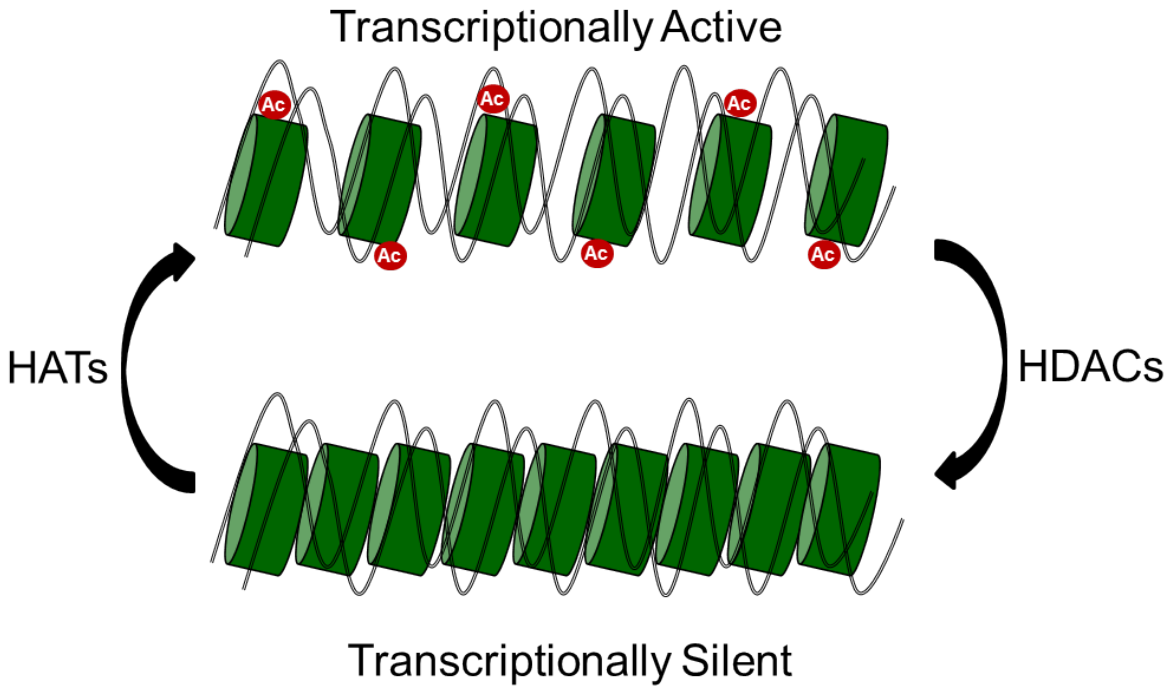

Figure 1-1. Histone acetylation and deacetylation. Histone acetyltransferases (HATs) activate gene transcription by adding acetyl groups to lysine residues of histone tails and relaxing the chromatin structure. Removal of acetyl groups via histone deacetylases (HDACs) compresses the DNA/histone complex and represses gene transcription.

Table 1-1. Histone acetyltransferases (HATs) and learning and memory

Modification	Learning and memory change	Reference
Mutations in p300/CBP	Impaired fear conditioning, novel object recognition, Morris water maze	Barrett & Wood, 2008
Rescue of p300/CBP mutations	Rescues impairments in learning and memory	Alarcon et al., 2004 Korzus et al., 2004
↑ acetylation of H3 in CA1	Enhanced contextual fear conditioning	Levenson et al., 2004
↑ H3 and H4 in HC	Enhanced LTP	Levenson et al., 2004

Table 1-2. Histone deacetylases (HDACs) and learning and memory

Modification	Effect	Reference
in vitro deletion of HDAC1 and HDAC2	Immature neurons – ↑ synapse formation Mature neurons – no change	Akhtar et al., 2009
Embryonic deletion of HDAC2 *in vivo*	Enhanced LTP and fear conditioning	Guan et al., 2009
Overexpression of HDAC2	Impairments in fear conditioning and Morris Water Maze	Guan et al., 2009
Postnatal deletion of HDAC2 *in vivo*	Accelerated extinction in fear conditioning, enhanced associative learning, enhanced LTP	Morris et al., 2013
in vivo deletion of HDAC3 in CA1	Enhanced long-term memory in object recognition task	McQuown et al., 2011

Table 1-3. HDAC inhibitors as therapies for neurodegenerative diseases

Drug	Effect	Reference
SAHA	Improves motor coordination in R6/2 Huntington's mouse model	Hockly et al., 2003
	Ameliorates MPP+ toxicity in Parkinson's mouse model	Wu et al., 2008
Valproic acid	Enhanced long-term memory and reconsolidation of fear memory	Bredy & Barad 2008
	Enhanced fear conditioning in mouse model of Alzheimer's disease (APPsew/PS1dE9 mice)	Kilgore et al., 2010
	Neuroprotective effects and enhanced learning in APP23 Alzheimer's mouse model	Qing et al., 2008

CHAPTER 2

DISSOCIABLE ROLES FOR HDAC1 AND HDAC2 IN LEARNING AND MEMORY

Introduction

Histone deacetylases (HDACs) compress the DNA/histone complex, restricting access of transcription factors to the DNA and consequentially repressing gene expression (Shahbazian and Grunstein, 2007). The Class I HDAC family consists of HDAC1, 2, 3 and 8, and are ubiquitously expressed, predominately localized to the nucleus and display high enzymatic activity toward histone substrates. HDAC1 and HDAC2 are nearly identical in sequence identity, and do not directly bind target genes but rather often form repressor complexes with sin3, NuRD, CoREST, and MeCP2 to control gene expression (Jones et al., 1998) (Yang and Seto, 2003).

Accumulating evidence indicates that remodeling of chromatin structure can affect mechanisms underlying learning and memory. Broad-acting pharmacological inhibitors of Class I HDACs enhance memory formation in animal models of neurodegenerative disease (Levenson et al., 2004) (Vecsey et al., 2007) (Barrett and Wood, 2008). These findings gave rise to the hypothesis that inhibiting HDAC activity can be a beneficial therapy in treating diseases related to cognitive impairments, however, offer no insight to roles of individual HDACs in regulating these mechanisms. While the individual HDAC genes are widely expressed throughout the body and possess differing deacetylase activity, their specific function in various tissues is only now starting to be examined (Brunmeir et al., 2009) (Montgomery et al., 2009) (Guan et al., 2009) (Kim et al., 2012).

Previous work dissecting out the individual roles of HDAC1 and HDAC2 has shown that HDAC1 null mice die at E10.5 (Lagger et al., 2002) and HDAC2 null mice don't survive past 24 hours after birth (Montgomery et al., 2007). Additionally, it has been demonstrated that conditional HDAC2 knockout mice, which were generated using a Nestin-Cre line, a CNS specific driver of Cre expression, to delete HDAC2 during embryonic development, show enhanced memory formation and induced LTP while overexpression of HDAC2 leads to impaired memory formation (Guan et al., 2009). More recent data has suggested that HDAC1 and HDAC2 are functionally redundant in proliferating neurons in that the deletion of both HDAC1 and HDAC2 using the GFAP-Cre driver line, which drives the expression of Cre in the CNS at embryonic day 13.5, results in major abnormalities of cortical, hippocampal, and cerebellar development in mice at E14.5-E15.5 and subsequent death at P7 (Montgomery et al., 2009). Previous work from our laboratory has also demonstrated *in vitro*, that HDAC1 and HDAC2 are important regulators of synaptic excitation–inhibition balance, and form a developmental switch which controls synapse function in a contrasting manner, depending on the maturational state of the neuronal network (Akhtar et al., 2009). Taken together, these data highlight a crucial role for HDAC1 and HDAC2 in neuronal proliferation and development but it remains unclear how the deletion of these genes in postmitotic neurons impacts neuronal function and ultimately behavior.

The goal of this study is to look beyond the early developmental stages, and elucidate a role for HDAC 1 and HDAC2 in complex behaviors as well as learning and memory tasks. We crossed floxed HDAC1 or floxed HDAC2 mice with calcium-calmodulin-dependent protein kinase II (CaMKII)93-Cre transgenic mice to delete these HDACs selectively in the

forebrain at approximately postnatal day 10–14 (Chen et al., 2001). We find that a conditional deletion of HDAC1 does not lead to any behavioral abnormalities or learning and memory impairments. However, conditional HDAC2 KO mice have impairments in associative learning tasks, providing dissociable roles for HDAC1 and HDAC1 in postnatal development and mechanisms related to learning and memory.

Materials and Methods

HDAC1 and HDAC2 KO mouse lines.

Floxed HDAC1, floxed HDAC2, and the CaMKII-Cre93 lines were on a mixed 129/BALBC background that were each backcrossed to a C57BL/6 line for at least 10 generations. The CaMKII-Cre93 mice express Cre recombinase in excitatory neurons in broad forebrain regions (Chen et al., 2001) (Fan et al., 2001). Homozygous floxed HDAC1 and homozygous HDAC2 lines have been previously reported (Montgomery et al., 2009). Genomic DNA was isolated from tails for genotyping by PCR analysis. The primer sequences used were as follows: Cre, forward (5′-CCC GCA GAA CCT GAAGAT GTT C-3′), reverse (5′-CGG CTA TAC GTA ACA GGGTG-3′); HDAC1, forward (5′-TCT ACC GCC CTC ACA AGG C3′), reverse (5′- ACA GAA CTC AAA CAA GCC ATC-3′); HDAC2, forward (5′-GCG TAC AGT CAA GGA GGC GG-3′), reverse (5′-GCT TCA TGG GAT GAC CCT GGC-3′). For all experiments, control (CTL) mice were wild-type littermates of either HDAC1 or HDAC2 KOs. Adult (8–20 weeks of age) male mice were used in all experiments. Mice were maintained on a 12 h light/dark cycle. HDAC1 and HDAC2 KOs and their CTL littermates were run in cohorts for the various behavioral tests. All experiments were performed and scored by an observer who was blind to mouse genotype. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

Fluorescent immunohistochemistry

Mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS) and brains were removed from the skull. Following post-fixation in 4% PFA overnight, the brains were cryo-protected in 30% sucrose in 0.1M PBS prior to sectioning on a freezing microtome. The brains were coronally sectioned at 30 μm and subjected to immunohistochemistry. Briefly, free floating sections were incubated overnight in primary antibody solution composed of 3% normal goat serum, and 0.3% Triton X-100 in PBS. Dilution for the primary antibody was 1:250 for rabbit anti-HDAC1 (Abcam). The sections were treated in 10 mM citric acid (pH 6) for 15 min at 95˚C for antigen retrieval prior to the primary antibody incubation. Immunoreactivity was visualized by secondary antibodies conjugated with Alexa Fluor 594. The sections were incubated at a 1:200 dilution at room temperature for 2 hours, and then mounted in Vectashield mounting media (Vector Laboratories).

Protein Quantification

To confirm region-specific KO, brain regions were dissected out and homogenized in a lysis buffer composed of 25 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM PMSF, 20 mM NaF, 1 mM DTT, 0.1% NP40, and proteinase inhibitor cocktails (Sigma), and spun down to isolate the lysate. Protein concentrations were determined by Bradford assays, and 20 μg of the protein was loaded and resolved on 10% SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes and then blocked with 5% nonfat milk before incubation with primary antibodies. Dilutions of primary antibodies were 1:2000 HDAC1 and HDAC2

(Abcam), 1:2000 for MeCP2 (Affinity Bioreagents), 1:50,000 for GAPDH (Cell Signaling Technology), and peroxidase-labeled goat anti-rabbit secondary antibodies were used at 1:2000 (Vector Laboratories). The signals were visualized by enhanced chemiluminescense. The immunoreactivity was quantified using NIH image J analysis software.

Behavioral Overview

For all behavior testing, adult male mice aged 4-6 months were used unless otherwise specified. Wild type littermates of the HDAC1 conditional KO mice not carrying the Cre recombinase transgene regardless of loxP alleles were used as control (CTL) mice. All experiments were conducted and scored by an observer blind to group assignments. Mice were habituated to testing facilities one hour prior to behavioral assessment. Behavior was performed sequentially in the same cohort of mice from least to most stressful as follows: locomotor, open field, elevated plus maze, rotorod, social interaction, fear conditioning, foot shock, prepulse inhibition, startle response, and forced swim test. For all experiments, data was presented as mean ± SEM and significance was $P<0.05$.

Locomotor activity

Mice were placed individually in a standard mouse cage (18 cm x 28 cm) with fresh bedding, and activity was monitored over 2 hours by five horizontal photobeams linked to data acquisition software (Photobeam Activity System, San Diego Instruments, San Diego, CA). Ambulatory activity was measured by counting the number of consecutive beam breaks in 5-minute increments. Data were analyzed with the Student's T-test. Data are

presented as mean ± SEM.

Open field

Mice were placed in the periphery of a novel open field environment (44 cm x 44 cm, walls 30 cm high) in a dimly lit room and allowed to explore for 10 minutes. The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision 3.0, Noldus, Leesburg, Virginia) to determine the total time spent in the periphery (5 cm from the walls) and the center (14 cm x 14cm). The open field arenas were cleaned between mice. Data were analyzed with the Student's T-test. Data are presented as mean ± SEM.

Elevated Plus Maze

Mice were placed in the center of a cross-shaped maze (each arm 33 cm × 5 cm) that was elevated 2 ft above the floor in a dimly lit room. The maze had two open arms and two closed arms (25-cm-tall walls on the closed arms) and mice were allowed to freely explore for 5 minutes. The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision 3.0, Noldus, Leesburg, Virginia) to determine the total time spent in the closed and open. Data were analyzed with the Student's T-test. Data are presented as mean ± SEM.

Rotorod

Each mouse was placed on the rotarod (IITC Life Science, Woodland Hills, California), which accelerated from 0 to 45 rpm in over the course of 60 seconds. Each session ended when the mouse fell off the rod and the total time spent on the rotorod before falling was measured. The mouse was returned to its original cage for 1 hour. The test was repeated for a total of 8 trials over the course of 2 days (4 trials per day). Data were analyzed by a two-way analysis of variance.

Social Interaction

To determine social interaction behavior HDAC1 KO or mice were exposed to an unknown social target. Experimental mice were placed in an open-field arena (42 × 42 cm) containing an empty wire mesh enclosure (10 × 6.5 cm) located against one of the walls (no target), and its movements recorded with a video tracking system (Ethovision) for 5 minutes. Immediately thereafter, a naïve, adult (12 weeks old) C57BL/6 male mouse (Target) was placed behind the wire mesh, and the movements of the experimental animal were recorded for an additional 5 minutes. The wire mesh allowed visual and olfactory interactions between the mice but prevented direct physical contact. During the No Target and Target sessions, everything was identical except for the absence or presence of the C57BL/6 mouse. The duration of time spent in the interaction zone (8-cm-wide area surrounding the wire mesh enclosure) was measured during both No Target and Target sessions.

Fear-conditioning

The fear-conditioning paradigm was performed as previously described (Monteggia et al., 2004). Briefly, mice went through a training day and were placed in individual chambers for 2 minutes and a loud tone (90 dB) was played for 30 seconds, immediately followed by a 0.5 mA footshock for 2 seconds. Mice remained in the box for 1 minute, at which time they again received a loud tone (90 dB) for 30 seconds and then an immediate 0.5 mA footshock for 2 seconds. Mice were immediately removed and placed back into their home cages. 24 hours later, mice were tested for context-dependent fear conditioning. Mice were placed back in the same chambers without a tone or shock. The amount of time the animal spent freezing was scored by an observer blind to the genotype. Freezing behavior was defined as no movement except for respiration. Four hours later, mice were tested for cue-dependent fear conditioning. Mice were placed in a chamber with a novel environment with no tone or shock for 3 minutes followed by 3 minutes of the tone. The amount of time the mice spent freezing was measured by an observer blind to the genotype.

Pain sensitivity test.

Pain sensitivity was performed as previously described (Gemelli et al., 2006). Mice were allowed to extinguish from the fear-conditioning test for one week, then responses to foot shock were measured. Footshock started at 0.0 mA and increased by 0.05 mA every 20 seconds to 0.25 mA to assess intensity needed to elicit flinch, jump, and vocalization.

Prepulse Inhibition

Prepulse inhibition was performed as previously described (Blundell et al., 2010) Startle chambers (San Diego Instruments) were used to detect the animal's movement, and acoustic stimuli were delivered by high-frequency speakers mounted 33 cm above the cylinders. Mice were subjected to five trial types in a 22 min session: pulse alone (40 ms, 120 dB, white noise pulse), three different prepulse/pulse trials (20 ms prepulse of 4, 8, or 16 dB above background noise level of 70 dB precedes the 120 dB pulse by 100 ms; onset to onset), and no stimulus. All stimuli were presented randomly with an average of 15 seconds between the 62 trials. Mice were allowed to acclimate to the cylinder for 5 minutes followed by four blocks of test trials. The first and last blocks consisted of six pulse-alone trials. Blocks 2 and 3 contained six pulse alone trials, five of each level of prepulse/pulse trials, and five no-stimulus trials. Data were analyzed as percent prepulse inhibition (percentage of decrease in startle amplitude for prepulse/pulse trials compared to pulse-alone trials).

Startle Testing

Startle testing was performed in SR-Lab System startle chambers (San Diego Instruments, San Diego, California) as previously described (Gemelli et al., 2006). Briefly, individual mice were placed into the Plexiglas cylinder. The background noise (70 dB) was presented alone for 5 minutes, after which the animal was presented with 36 trials of stimuli ranging in intensity (six trial blocks repeated six times in a random order). The trial blocks were 70, 80, 90, 100, 110, or 120 dB, with an average inter-trial time of 9 seconds. The startle values were recorded for all trials.

Forced swim test

The forced swim test (FST) was performed as previously described (Porsolt et al., 1977) (Autry et al., 2011). Mice received subchronic treatment with three doses of vehicle (saline) or desipramine at 24 hours, 4 hours, and 1 hour prior to behavioral testing at 15 mg/kg, 15mg/kg, and 20 mg/kg respectively. Mice were placed in 3L of 22-24°C water in a 4L beaker and swimming behavior was videotaped by a camera on the side of the beakers for 6 minutes. The last 4 minutes of the trial were scored for time spent immobile by an observer blind to genotypes and drug assignments. Data were analyzed using one-way ANOVA.

Results

Molecular characterization of conditional HDAC1 knockout mice

CaMKII-Cre93 mice were crossed with floxed HDAC1 mice to induce a forebrain deletion. The CamKII-Cre93 line drives the expression of Cre at approximately postnatal 10-14 in the forebrain areas including cortex, hippocampus, and striatum, however not the hindbrain (Chen et al., 2001), allowing us to achieve regional specificity in deleting HDAC1.

To confirm the deletion of HDAC1 or HDAC2 in conditional KO mice, we used immunohistochemistry to examine regional expression patterns in KO compared to control (CTL) littermate mice. A significant decrease in immunofluorescence was observed in cortex, hippocampus, and striatum in HDAC1 KO mice compared to controls indicating we could selectively reduce HDAC1 expression in KO mice (Figure 2-1). Importantly, no changes in immunofluorescence were observed in the cerebellum of HDAC1 KO mice, consistent with the premise that the CaMKII-Cre93 driver line results in a forebrain specific conditional knockout (Figure 2-1). To assess changes in protein levels in HDAC1 KO mice, we used Western blot analysis on whole cell lysates from frontal cortex, hippocampus, striatum, and cerebellum. We observed over 50% reduction in forebrain regions of HDAC1 in knockout mice, with no changes in expression in the cerebellum compared to control mice, indicating we are able to achieve an efficient deletion of our gene of interest (Figure 2-2). Moreover, we found no significant changes in HDAC2 expression in HDAC1 KO mice suggesting compensation did not occur following the deletion of HDAC1 (Figure 2-3A). Since HDAC1 and HDAC2 are known to come together in a complex with MeCP2 to regulate transcriptional activation states (Grozinger and Schreiber, 2002), we also examined

MeCP2 protein levels in both HDAC1 KO mice. We found no changes in MeCP2 levels in either HDAC1 or HDAC2 KO mice, suggesting that MeCP2 expression is not impacted by the loss of either gene (Figure 2-3B).

Behavioral characterization of HDAC1 knockout mice

To examine the postnatal loss of HDAC1 in behavioral phenotypes, KO and CTL mice were subjected to a battery of behavioral paradigms. Mouse weights were monitored following weaning at 3 weeks of age up until adulthood at 8 weeks old. HDAC1 KO show normal weight gain, indicating the loss of HDAC1 does not impact weight (Figure 2-4A).

Locomoter activity

To assess locomotor activity in the conditional HDAC1 KO mice, animals were individually placed into a novel home cage environment for 2 hours. Activity was recorded by the number of consecutive beam breaks, an indication of horizontal locomotor activity. Conditional HDAC1 KO mice exhibited no changes in locomotor activity over the two hour testing session compared to littermate control mice (Figure 2-4B). The total amount of ambulation over the two hour period was indistinguishable between the two groups (Figure 2-4B, inset), suggesting HDAC1 KO mice do not exhibit deficits in their baseline activity.

Anxiety-related behavior

To determine the effect of HDAC1 deletion on anxiety-like behavior, we tested the mice in the open field paradigm. Mice with an anxiety-like phenotype will spend significantly

more time in the periphery and less time in the center of the open field arena compared to normal control mice. Conditional HDAC1 KO mice spent a similar amount of time exploring the center of the arena compared to littermate controls. Moreover, both groups spent equal amounts of time in the periphery and non-periphery zones of the arena, suggesting no anxiety-like phenotype in HDAC1 KO mice (Figure 2-5A). To confirm this finding, we used a second test, the elevated plus maze, to examine anxiety-related behavior. Mice were placed in the center of a plus maze with two open and two closed arms; mice with anxiety-like phenotypes will spend less time in the open arm and prefer to explore the closed arm when compared to control mice. We found that HDAC1 KO spend a similar amount of time in open and closed arms compared to littermate control mice, reaffirming that the loss of HDAC1 does not impact anxiety-related behavior (Figure 2-5B).

Motor coordination tasks

Mice were next tested on the rotorod task to examine whether a forebrain specific knockout of HDAC1 results in deficits in motor coordination. Conditional HDAC1 KO mice were placed on an accelerating rotorod for 4 trials per day on two consecutive days for a total of 8 trials. In order to excel at this task, mice must have normal motor coordination and learn to stay on the accelerating rod over the course of the 8 trials. Mice with deficits in motor coordination will not improve at the task over time. We found that HDAC1 KO mice are able to learn and improve their time on the rotorod over the 8 trials at comparable levels as littermate control mice, indicating a conditional deletion of HDAC1 does not affect motor coordination and balance or procedural motor learning (Figure 2-6A).

Social interaction

To examine whether conditional HDAC1 KO mice would have deficits in interacting with other mice, we used a two-trial social interaction test in which an experimental mouse was placed in an open-field target with a wire mesh at one end of the field (No Target) for the first task of the paradigm. The amount of time each experimental mouse spent in the interaction in a zone near the wire-mesh target was measured. The conditional HDAC1 KO mice spent a similar amount of time in the interaction zone as control littermate mice (Figure 2-6B). For the second part of the task, a 10-week-old C57BL/6 mouse was placed behind the wire mesh (Target), and the experimental animal's movement was recorded to determine the amount of time the mouse spends interacting with the target. Both conditional HDAC1 KO and CTL mice spend more time in the interaction zone when another mouse (Target) was present compared with the No Target baseline (Figure 2-6B). Moreover, there were no measurable differences in the amount of time spent in the interaction zone with or without a target mouse present between genotypes. Collectively, these results suggest that a postnatal loss of HDAC does not lead to deficits in social interaction behavior with other mice.

Learning and memory tests

To examine whether the loss of HDAC1 would recapitulate the enhanced learning and memory findings reported following administration of HDAC inhibitors, conditional HDAC1 KO mice were tested in learning and memory related tasks. Mice were tested in both context and cue-dependent fear conditioning tasks which are dependent on hippocampal (context) and amygdala (context and cue) function (LeDoux, 2000) (Maren, 2001). To train the

animals to the learn the fear conditioning paradigm, mice are placed inside the fear conditioning box, receive a loud auditory tone (90 dB, 2.8 kHz, 30 sec) followed by a footshock (0.8 mA, 2 sec), then placed back into their home cage. To test context-dependent fear conditioning, mice are placed back into the same fear conditioning environment twenty-four hours later, and the amount of time the animals spent freezing was recorded. To measure cue-dependent fear conditioning, mice are placed in a completely novel environment four hours later, the previous tone from the context dependent task was played, and the amount of time the animal spent freezing was recorded. During the context-dependent fear conditioning, conditional HDAC1 KO mice showed no significant differences in time spent freezing compared with CTL littermates (Figure 2-7A). Moreover, conditional HDAC1 KO mice spent equal amounts of time freezing as control littermates during cue-dependent fear conditioning (Figure 2-7A). As a control measure, both HDAC1 KO mice and CTLs were tested in a startle response paradigm to ensure the fear conditioning results were not confounded by deficits in pain sensitivity. We tested the response of HDAC1 KO and CTL mice to foot shocks over a range of shock intensities (.05 mA to a maximum of .6 mA). We found no differences between the ability of conditional HDAC1 KO mice and CTL wild-type littermates to respond to the foot shock by flinch, jump, or vocalization (Figure 2-7B). Taken together, these data indicate that postnatal loss of HDAC1 does not impact learning and memory, even though the deletion occurs in brain areas associated with the execution of the fear conditioning paradigm.

Reflex tests

To examine reflexive behaviors of HDAC1 KO mice, we used a prepulse inhibition (PPI) paradigm. Under normal conditions, mice will exhibit a startle reflex when exposed to a loud and unexpected stimulus. However, if the startling stimulus (pulse) is preceded by a weaker, non-startling stimulus (prepulse), the reaction to the startle response is weakened or inhibited. Patients suffering from certain neurological disorders such as schizophrenia or Alzheimer's can have deficits in PPI, and exhibit the startle response even when presented with the weaker prepulse (Braff et al., 2001). When presented with three different prepulses at 4, 8, or 16 decibels (dB) above the 70 dB background noise, conditional HDAC1 KO mice exhibit prepulse inhibition at comparable levels as control littermate mice (Figure 2-8A). To ensure the mice do not suffer from hearing deficits, we measured startle responses elicited by various acoustic intensities. The conditional HDAC1 KO and littermate CTL mice exhibited indistinguishable startle responses at all acoustic stimuli (0–120 dB), confirming that the PPI results were not impacted by inability to hear the weaker prepulse stimuli (Figure 2-8B).

Depressive-like behavior

To examine the role of HDAC1 in depressive-like behavior and antidepressant efficacy, we assessed KO and CTL mice in the forced swim test (FST). In this paradigm, mice are placed in a beaker of water and allowed to freely swim for duration of 6 minutes. The more time an animal spends immobile during the testing session interpreted as a measure of despair and considered to be a depressive-like behavior (Porsolt et al., 1977). The FST has predictive validity for antidepressant (AD) efficacy as measured by decreased immobility

time of mouse injected with an AD prior to the test compared to a control animal that received saline. Following subchronic treatment with saline, conditional HDAC1 KO mice spent a similar amount of immobile as control littermate mice mice (Figure 2-9). Similarly, after administration of the antidepressant desipramine (DMI, intraperitoneal injections at 24 hours, 4 hours, and 1 hour prior to behavioral testing at 15 mg/kg, 15mg/kg, and 20 mg/kg respectively), both conditional HDAC1 KO and control animals displayed a significant reduction in immobility time, which is expected following an antidepressant injection (Figure 2-9). Collectively these data suggest that a postnatal loss of HDAC1 in the forebrain does not alter depressive-like behavior in mice.

Discussion

Here we show a detailed characterization of conditional HDAC1 knockout mice. A selective deletion of HDAC1 in postnatal forebrain neurons of adult mice does not result in any overt phenotypes as assessed by several behavioral paradigms such as locomotor activity, anxiety tests, motor coordination tasks, social interaction behaviors, learning and memory tests, reflexive behavior, and antidepressant response. These data reveal a non-redundant role for HDAC1 in development of the adult brain.

Based on previous findings that HDAC1 null mice die at embryonic day 10.5 (Lagger et al., 2002) we used the cre-loxP system to generate conditional knockout mice with a targeted deletion of HDAC1 in the forebrain. Conditional HDAC1 KO mice were viable and born at normal Mendelian ratios. We were able to confirm the selectivity of our deletion by both immunohistochemistry and Western blot analysis, and found a significant reduction of HDAC1 in forebrain regions of frontal cortex, hippocampus, and striatum, with no impact measured in the hindbrain. Previous studies have shown that HDAC1, HDAC2, and MeCP2 can come together in a complex to regulate gene transcription (Grozinger and Schreiber, 2002) but interestingly, we found no changes in HDAC2 or MeCP2 expression in conditional HDAC1 KO mice, indicating no compensatory effects following the deletion.

Mice with a conditional deletion of HDAC1 have normal weight gain, allowing us to test KO mice in an array of behavioral paradigms with age and weight-matched littermate control mice. Conditional HDAC1 KO mice have normal locomotor activity therefore results of additional behavioral tests were not impacted by a hypoactive or hyperactive phenotype. We did not observe any anxiety-like behaviors as assessed by either the open field task or

elevated plus maze. Furthermore, conditional HDAC1 KO mice display normal motor coordination on the rotorod, social interaction with other mice, prepulse inhibition, and response to an antidepressant in the forced swim test when compared to control littermate mice.

Previous findings using pharmacological agents have implicated a role for Class I HDACs in regulating learning and memory behaviors (Levenson et al., 2004) (Vecsey et al., 2007) (Barrett and Wood, 2008). However these studies used "pan" HDAC inhibitors offering little specificity to individual targets. To understand the role of the individual HDACs in learning and memory mechanisms we used a genetic approach and tested conditional HDAC1 KO mice in the fear conditioning paradigm, an associative learning task. Mice learn to associate a novel context or cue to predict aversive events (LeDoux, 2000) (Maren, 2001). Interestingly, we found that HDAC1 KO mice have no impairments in either context or cue dependent fear conditioning. Both HDAC1 is ubiquitously expressed in all tissue types, however following neuronal maturation HDAC1 is expressed primarily in glial cells (MacDonald and Roskams, 2008), in agreement with our immunohistochemistry data that shows limited HDAC1 labeling in control mice. The limited expression of HDAC1 is a possible explanation as to why no behavioral phenotypes are observed following a postnatal deletion in adult mice. Moreover, additional studies have found no measureable phenotypes following manipulation of HDAC1 expression in neurons. Mice overexpressing HDAC1 have no impairments in fear conditioning or Morris water maze, a hippocampal dependent spatial learning task, in agreement with our findings that HDAC1 does not play a role in regulating learning and memory behaviors (Guan et al., 2009).

Since conditional HDAC1 KO mice did not exhibit any behavioral phenotypes, additional studies from our lab were carried out to test conditional HDAC2 KO mice in the same paradigms. It has been shown previously that an embryonic deletion of HDAC2 results in enhanced learning (Guan et al., 2009) however a postnatal role for HDAC2 had yet to be examined. In separate experiments, we found that conditional HDAC2 KO mice do not have any deficits in locomotor activity, anxiety-like behaviors, or motor coordination, similar to what was seen with conditional HDAC1 KO mice (Morris et al., 2013). Interestingly, the conditional HDAC2 KO mice generated with the CaMKII-Cre93 driver line have improved learning in context and cue dependent fear conditioning as well as enhanced LTP compared to control mice. Moreover, conditional HDAC2 KOs extinguish more rapidly following cue dependent fear conditioning and have accelerated extinction in the conditioned taste aversion task, a form of hippocampal-dependent classical conditioning in which mice learn to associate a novel taste with nausea and thereafter learn to avoid the novel taste. Other forms of non-associative learning such as rotorod performance, novel object recognition, and spatial object recognition were not impacted in HDAC2 KOs, suggesting a novel and unique role for HDAC2 in regulating associative forms of learning and memory and synaptic plasticity.

In summary the presented findings identify HDAC1 as having a non-redundant role in postnatal development of the adult brain. Following a conditional deletion of HDAC1 in forebrain neurons, no overt molecular or behavioral phenotypes were measured, indicating that other Class I HDACs may be driving the learning and memory enhancements previously described from pharmacological studies. Conversely, in separate experiments, a deletion of

HDAC2 resulted in improvements in certain types of learning, providing support for the hypothesis that inhibiting HDAC2 may be an effective approach in treating disorders related to cognitive impairment.

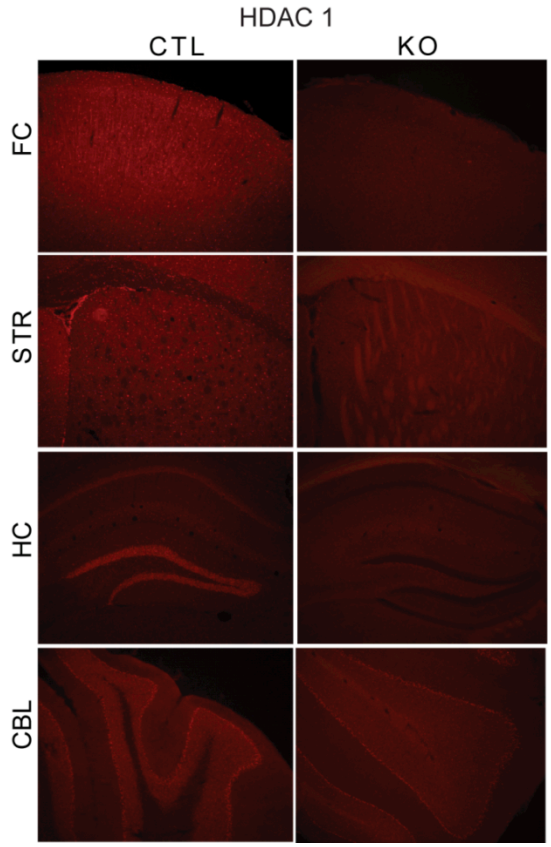

Figure 2-1. Regional deletion of HDAC1 in conditional KO mice. Conditional knockout (KO) mice have a broad, forebrain-specific deletion of HDAC1 compared to littermate control (CTL) mice. Fluorescent immunohistochemistry confirmed a selective loss of HDAC1 in the frontal cortex (FC), striatum (STR), and hippocampus (HC) (CA1, CA3, and dentate gyrus fields) of the conditional HDAC1 KO mice, compared to CTL littermates. Levels of HDAC1 were unaltered in and cerebellum (CBL) of the conditional mice compared to CTL littermates.

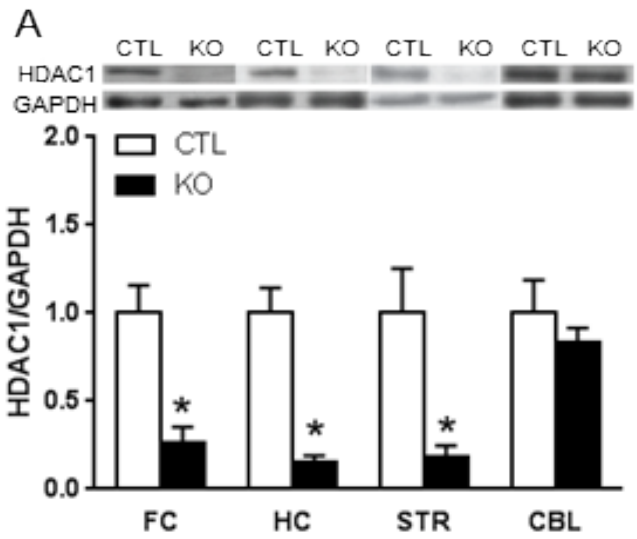

Figure 2-2. Knockdown of HDAC1 in conditional KO mice.
(A) Western blot analysis confirmed knockdown of HDAC1 in frontal cortex (FC), hippocampus (HC), and striatum (STR) to 20–30% of CTL, but not in the cerebellum (CBL). (B) A significant reduction of approximately 70% of HDAC2 protein in FC, HC, and STR was observed in the conditional KO mice compared with CTL, with no change in CBL, confirming a postnatal deletion by our CaMKII-Cre strategy (n=6 per group; *P < 0.05).

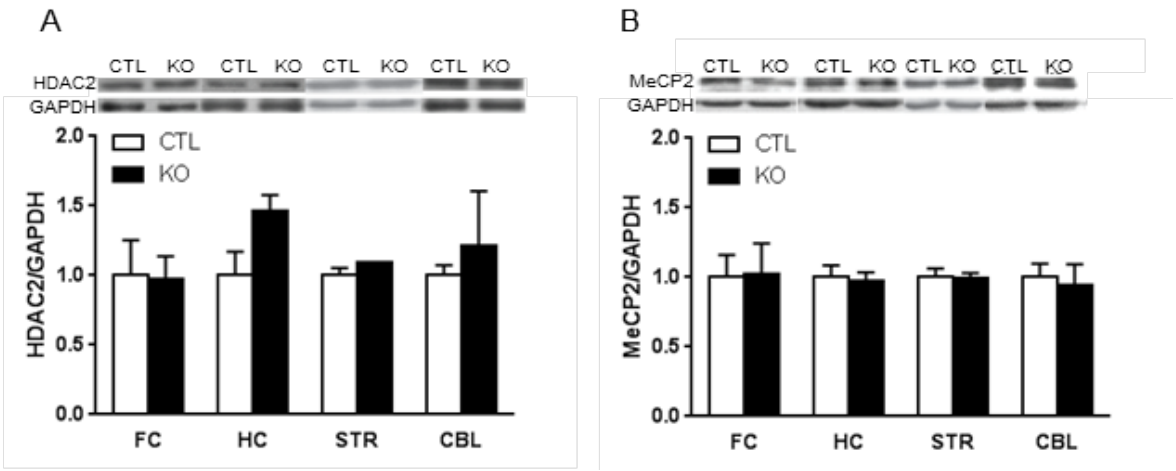

Figure 2-3. Deletion of HDAC1 does not result in compensation by HDAC2 or MeCP2. (A) Western blot analysis shows that conditional HDAC1 KO mice have no significant changes in HDAC2 expression in forebrain or hindbrain regions compared to CTL mice. (B) Forebrain specific loss of HDAC1 does not impact MeCP2 protein levels in regions where the deletion occurs or in the CBL (n=6 per group for all conditions).

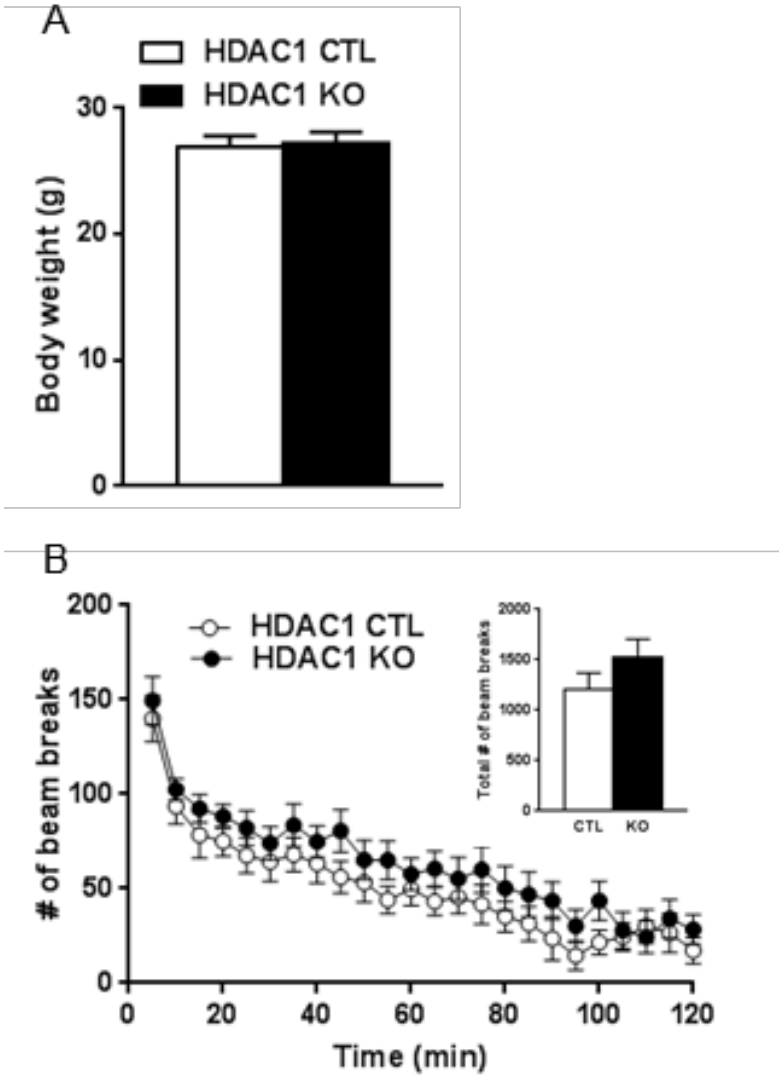

Figure 2-4. Loss of HDAC1 does not impact body weight or locomotor activity. (A) Adult (8 weeks old) conditional HDAC1 KO mice have an average weight that is indistinguishable from CTL mice, therefore loss of HDAC1 does not alter body weight (n=8 per group). (B) Conditional HDAC1 KO mice have normal locomotor activity as assessed by consecutive horizontal beam breaks throughout a 2 hour testing session, and no significant differences in total ambulation were seen between KO and CTL mice (B-inset) (n=12 per group).

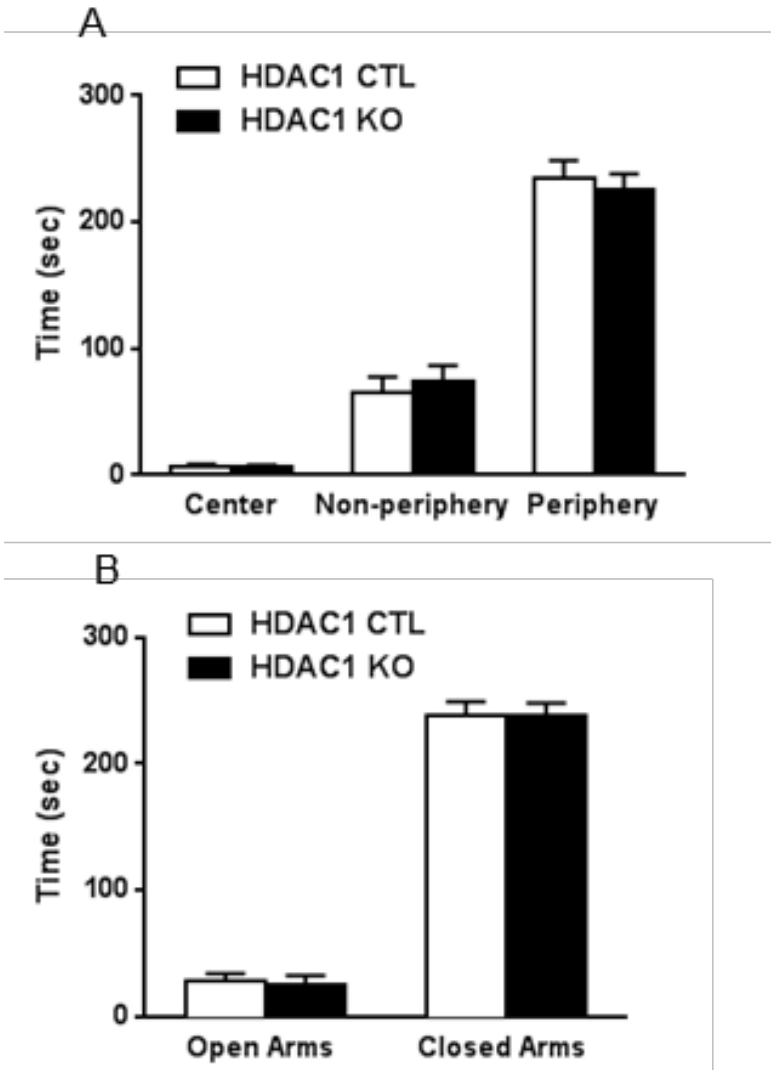

Figure 2-5. Conditional HDAC1 KO mice do not display anxiety-like behavior. (A) Mice with a forebrain deletion of HDAC1 spend equal amounts of time exploring the center, periphery, and non-periphery zones of the open field area compared to littermate control mice (n=12 per group). (B) Time spent in the open and closed arms of the elevated plus maze was not different for HDAC1 KOs relative to their CTL littermates (n = 12 per group).

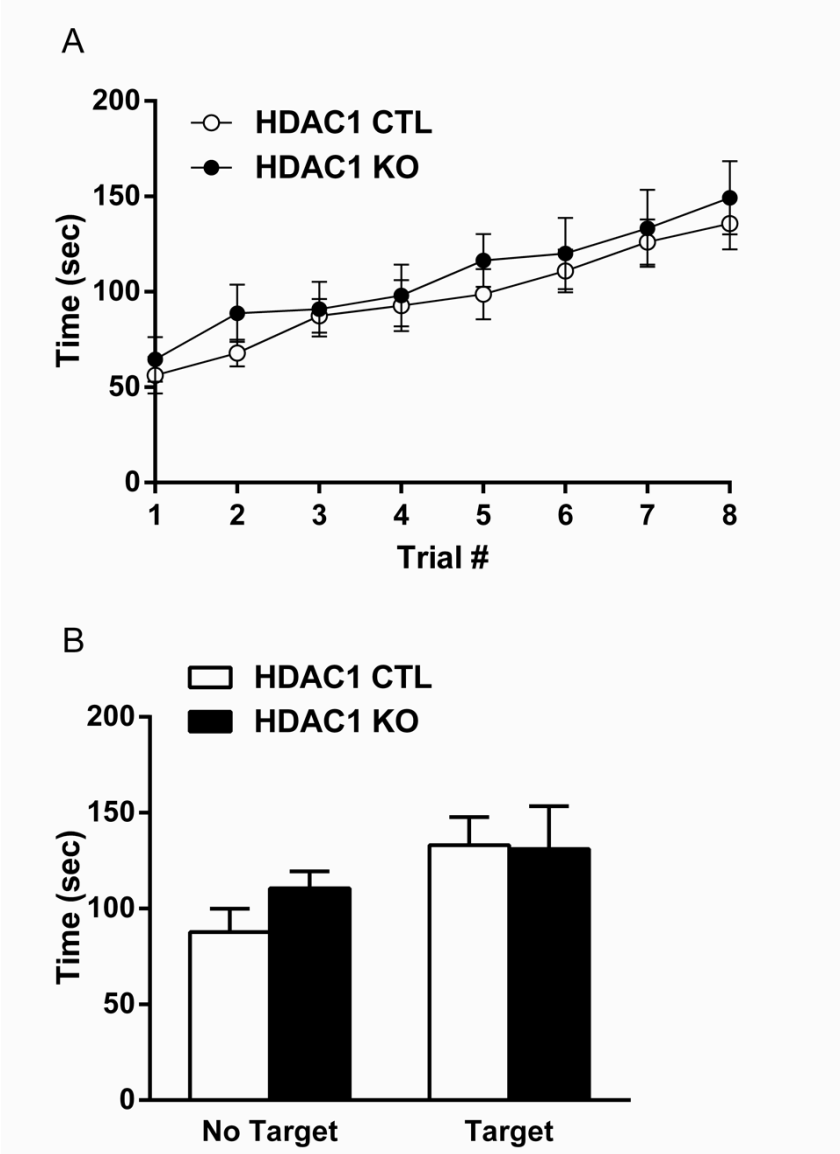

Figure 2-6. Conditional HDAC1 KO mice do not have deficits in motor coordination or social interaction behavior. (A) Motor coordination was assessed using the rotorod task. Conditional HDAC1 KO mice showed improvement over the 8 trials at comparable levels compared to littermate control mice (n=12 per group). (B) In the social interaction paradigm, both HDAC1 KO and CTL mice spend more time in the interaction zone when a target mouse is present compared to no target. No significant differences were measured between HDAC1 and CTL mice when either no target or a target was present (n=12 per group).

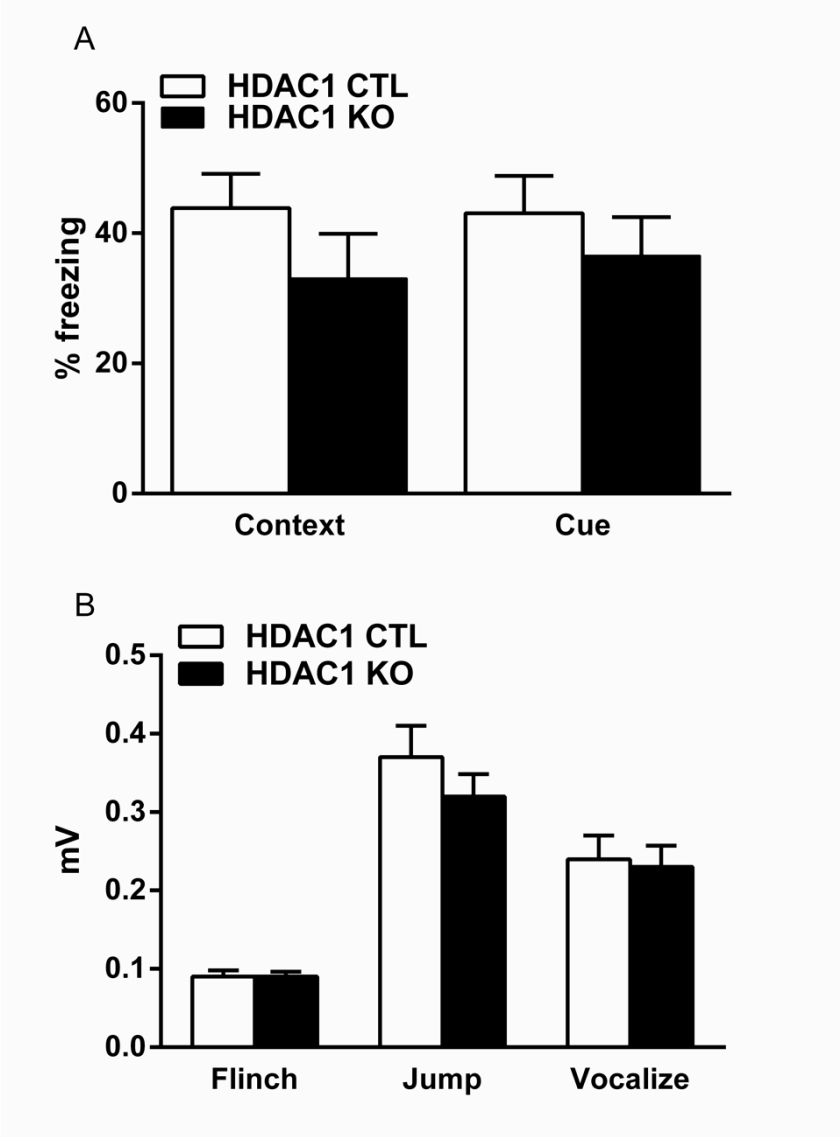

Figure 2-7. Conditional HDAC1 KO mice do not have impairments in fear learning or pain sensitivity. (A) Following one day of training, the conditional HDAC1 KO mice were indistinguishable compared with littermate control (CTL) mice in the average percent time spent freezing for contextual memory. Similarly, conditional HDAC1 KO mice displayed no significant differences in percent freezing time when tested for cue-dependent memory compared to CTL mice (n=12 per group). (B) Conditional HDAC1 KO mice have similar responses to footshock sensitivity measures of flinching, jumping, and vocalization as the littermate control mice (n=12 per group).

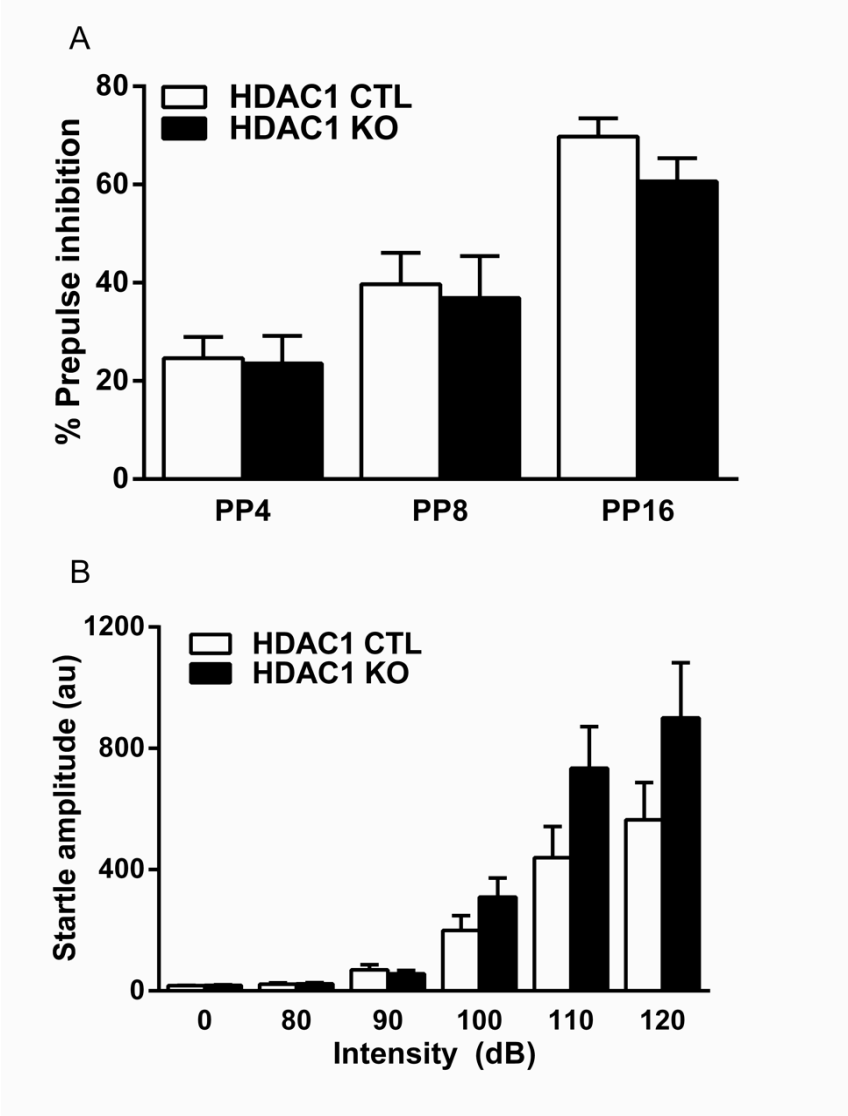

Figure 2-8. Conditional HDAC1 KO mice do not have impairments in reflexive behavior. (A) When presented with a prepulse of either 4, 8, or 16 dB above the 70 dB background noise, conditional HDAC1 KO mice display normal responses and have similar inhibition compared to CTLs (n=12 per group).
(B) Conditional HDAC1 KO mice have comparable startle responses as control mice at multiple stimulus intensities ranging from 0 to 120 dB (n=12 per group).

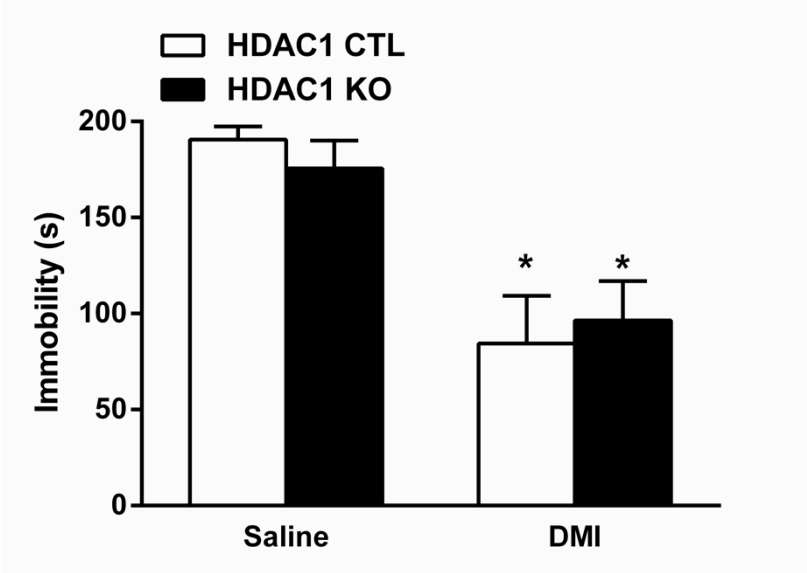

Figure 2-9. Conditional HDAC1 KO mice do not display depressive-like behavior. Following subchronic treatment with three doses of vehicle (saline) or desipramine (DMI) at 24 hr, 4 hr, and 1 hr prior to behavioral testing at 15 mg/kg, 15mg/kg, and 20 mg/kg respectively, both conditional HDAC1 KO and littermate control mice have a significant reduction in immobility time in the forced swim test, indicative of an antidepressant response (CTL Saline n=5; CTL DMI n=6; KO Saline n=5; KO Saline n=5; ANOVA $F_{(3,17)} = 8.205$ for treatment; Tukey's post hoc analysis for CTL Saline versus CTL DMI P = 0.0043, KO Saline versus KO DMI P = 0.0465).

CHAPTER 3

ESSENTIAL ROLE FOR HDAC1/2 IN POSTNATAL DEVELOPMENT

Introduction

Histone deacetylases (HDACs) are a family of enzymes that regulate gene transcription by catalyzing deacetylation of specific lysine residues within histone tails favoring transcriptional repression. HDACs are key mediators of gene expression by allowing for rapid changes in gene transcription in response to environmental stimuli. There are four distinct families of mammalian HDACs that are classified based on their sequence homology, expression patterns, and subcellular localization (Haberland et al., 2009). The class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8) are nearly ubiquitously expressed, localized to the nucleus and have enzymatic activity for histones, with the exception of HDAC3 which can shuttle out to the nucleus and modify non-histone proteins. Compounds that inhibit HDACs, with some specificity to Class I HDACs, are well tolerated in vivo and enhance learning and memory, long-term potentiation (LTP), and improve symptoms in animal models of neurodegenerative diseases suggesting potential therapeutic benefit for CNS disorders (Kim et al., 2008) (Graff et al., 2012). Studies are only now beginning to differentiate between the role of individual HDACs in brain function and processes (Levenson et al., 2004) (Kilgore et al., 2010).

HDAC1 and 2 are often found together in transcriptional repressor complexes such as Sin3, CoREST and NuRD and share approximately 85% sequence homology suggesting they may be functionally redundant (Grozinger and Schreiber, 2002) (Yang and Seto, 2003).

However, recent data suggests that HDAC1 and HDAC2 have dissociable effects in the brain, as improved learning and memory and enhanced LTP was observed following embryonic or postnatal deletion of HDAC2 selectively in the brain, with no observable effects in HDAC1 knockout mice (Guan et al., 2009) (Morris et al., 2013). This data is in agreement with other studies that have also suggested divergent roles for HDAC1 and 2 in mature neurons (Montgomery et al., 2009) (Akhtar et al., 2009). Given the potential beneficial effects observed following deletion of HDAC2 in brain, there has been interest in the development of HDAC2 inhibitors for the treatment of psychiatric and neurological disorders (Kosik et al., 2012). The high degree of sequence homology between HDAC1 and 2 has not been viewed as a potential caveat as embryonic deletion of HDAC1 selectively in brain does not impact measurements of behavior, synaptic plasticity or neurotransmission (Montgomery et al., 2009) (Guan et al., 2009). Thus, it seems reasonable that HDAC2 inhibitors that also target HDAC1 should be well tolerated in the brain. However, a recent study generated conditional HDAC1 and 2 double knockout mice in which the genes were deleted selectively in the brain during embryogenesis and the resulting mice had major abnormalities in cortical, hippocampal, and cerebellar development at E14.5-E15.5 and subsequent death at postnatal day 7, suggesting redundant roles when these genes are embryonically deleted together (Montgomery et al., 2009).

To examine whether HDAC1 and HDAC2 have redundant roles in postnatal brain, we assessed whether the loss of both HDAC1 and HDAC2 selectively in the brain at postnatal days 10-14 impacts behavior compared to the individual conditional deletion of HDAC2. We generated forebrain specific conditional HDAC1/2 double knockout (DKO) mice by

crossing floxed HDAC1/2 mice with the calcium-calmodulin kinase II (CaMKII)-Cre93 transgenic mice to delete the genes of interest selectively in the forebrain at approximately postnatal day 10-14 (Chen et al., 2001) (Akbarian et al., 2002). We also generated separate cohorts of conditional HDAC1 and HDAC2 single KO mice as previously described, to test as a direct comparison to HDAC1/2 DKO mice. The conditional HDAC1/2 DKO mice were viable and appeared normal during early postnatal development, however at approximately 6 weeks of age the HDAC1/2 DKO mice began to lose weight and all died at approximately 9 weeks of age due to apoptosis following deletion of the genes. We observed deficits in locomotor activity and heightened anxiety in HDAC1/2 DKO mice as early as one week after the postnatal deletion of HDAC1 and 2. Our results demonstrate that postnatal deletion of both HDAC1 and HDAC2 impacts neuronal survival and has adverse effects on behavior, highlighting the need for caution in the development of pharmacological inhibitors of HDAC1 and HDAC2.

Materials and Methods

Generation of the conditional KO and DKO mice

The conditional HDAC1 knockout (HDAC1 KO), HDAC2 knockout (HDAC2 KO) and HDAC1/2 double knockout (DKO) mice were generated by breeding transgenic mice expressing Cre recombinase under the control of the Calcium/Calmodulin-dependent kinase II promoter (CaMKII-Cre93 line) with floxed HDAC1, floxed HDAC2, or double floxed HDAC1/2 mice. Previous work has demonstrated that the CaMKII-Cre93 mice express Cre recombinase selectively in forebrain neurons at postnatal day 10-14 (Chen et al., 2001). Floxed HDAC1, HDAC2 and HDAC1/2 lines have been previously reported (Montgomery et al., 2009). Floxed HDAC1, HDAC2, and the CaMKII-Cre93 line were on a mixed 129/BALBC background and backcrossed to a C57BL/6 line for at least 10 generations. Floxed HDAC1/2 lines were on a mixed 129SvEv/C57BL/6 background. The conditional HDAC1 KO mice, HDAC2 KO mice, and HDAC1/2 DKO mice were genotyped using PCR analysis from genomic DNA isolated from tails as previously described (Montgomery et al., 2007). Wild type littermates not carrying the Cre recombinase transgene regardless of loxP alleles for each line were used as control mice in all experiments to minimize the genetic differences. Mice were maintained on a 12 hour light/dark cycle with *ad libitum* access to food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas Southwestern Medical Center.

Protein quantification

Brain regions were dissected out and homogenized in a lysis buffer containing 25 mM

HEPES, pH 7.9, 150 mM NaCl, 1 mM PMSF, 20 mM NaF, 1 mM DTT, 0.1% NP40, and proteinase inhibitor cocktails (Sigma), and spun down to isolate the lysate. Protein concentrations were determined by Bradford assays and 20 ug of the protein was loaded on 10% SDS-PAGE gels, electrophoresed, transferred to nitrocellulose membranes, then blocked with 5% non-fat milk prior to incubation with primary antibodies. Dilutions of primary antibodies were 1:2000 for both HDAC1 and HDAC2 antibodies (Abcam) and 1:50,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology). The signals were visualized by enhanced chemiluminescense. The immunoreactivity was quantitated by the NIH image J analysis software.

Immunohistochemistry

Mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS) and brains were removed from the skull. Following post-fixation in 4% PFA overnight, the brains were cryo-protected in 30% sucrose in 0.1M PBS prior to sectioning on a freezing microtome. The brains were coronally sectioned at 30 μm and subjected to immunohistochemistry. Briefly, free floating sections were incubated overnight in primary antibody solution composed of 3% normal goat serum, and 0.3% Triton X-100 in PBS. Dilutions for the primary antibodies were 1:250 for rabbit anti-HDAC1 (Abcam), and 1:2000 for rabbit anti-HDAC2 (Abcam). For HDAC1 staining, the sections were treated in 10 mM citric acid (pH 6) for 15 min at 95˚C for antigen retrieval prior to the primary antibody incubation. Immunoreactivity was visualized by secondary antibodies conjugated with either Alexa Fluor 594 (HDAC1) or Alexa Flour 488 (HDAC2). The

sections were incubated at a 1:200 dilution at room temperature for 2 hrs, counter-stained with with 4′,6-diamidino-2-phenylindole (DAPI), and then mounted in Vectashield mounting media (Vector Laboratories).

Hematoxylin and Eosin (H&E) Stain

H&E staining was carried out as previously described (Montgomery et al., 2009). Briefly, paraffin sections made from formalin fixed tissue were affixed to microscope slides through sequential room temperature and heated air-drying. Dried sections were deparaffinized and stained with hematoxylin, then destained using 70% ethanol. Sections were then stained with eosin, destained, and dehydrated in ascending ethanol solutions. Sections were rinsed in xylene then cover slipped with synthetic mounting media.

TUNEL

Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) staining for apoptotic cells was done according to the manufacturer's protocol (Promega DeadEnd Fluorometric TUNEL System, Madison, WI). Apoptotic cells were labeled with fluorescein and the sections counterstained with propidium iodide.

Behavioral Overview

For all behavior testing, male mice were age matched littermates and groups were balanced by genotype. All experiments were conducted and scored by an observer blind to group assignments. Mice were habituated to testing facilities one hour prior to behavioral

assessment. For all experiments, data was presented as mean ± SEM and significance was $P<0.05$.

Locomotor Activity

Mice were placed individually in a standard mouse cage (18 cm x 28 cm) with fresh bedding, and activity was monitored over 2 hrs by five horizontal photobeams linked to data acquisition software (Photobeam Activity System, San Diego Instruments, San Diego, CA). Ambulatory activity was measured by counting the number of consecutive beam breaks in 5-minute increments. Data were analyzed with the Student's T-test. Data are presented as mean ± SEM.

Open field

Mice were placed in the periphery of a novel open field environment (44 cm x 44 cm, walls 30 cm high) in a dimly lit room and allowed to explore for 10 minutes. The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision 3.0, Noldus, Leesburg, Virginia) to determine the total time spent in the periphery (5 cm from the walls) and the center (14 cm x 14cm). Open field arenas were cleaned between mice. Data were analyzed with the Student's T-test and presented as mean ± SEM.

Statistical analysis

Data are reported as mean ± SEM. Statistical differences were assessed using the unpaired or paired two-tailed Student's t test, one-way ANOVA, or two-way ANOVA with multiple comparisons when appropriate. Tukey post hoc tests were used following two-way ANOVA when appropriate. Statistical significance was defined as $P < 0.05$.

Results

Characterization of conditional HDAC1/2 double knockout mice

To examine the role of HDAC1 and HDAC2 together in postmitotic neurons, we generated conditional knockout mice lacking both HDAC1 and HDAC2 during postnatal development. Homozygous floxed HDAC1/2 mice were crossed to CaMKII93-Cre transgenic mice, which selectively express Cre recombinase in forebrain regions including hippocampus, striatum, and frontal cortex. The resulting conditional double knockout (DKO) mice have a deletion of both HDAC1 and HDAC2 in broad forebrain regions at postnatal days 10-14 (Chen et al., 2001). The HDAC1/2 double knockout (DKO) mice were born at normal Mendelian ratios and appeared indistinguishable from littermate controls (CTLs) at birth. To confirm the deletion in HDAC1/2 DKO mice we first used immunohistochemistry to examine the regional distribution of HDAC1 and HDAC2 of 8 week old mice. HDAC1/2 DKO mice have a reduction of fluorescently labeled cells in frontal cortex, hippocampus, and striatum compared to CTLs (Figure 3-1A, B). No changes in immunostaining were detected in the cerebellum between DKO and CTL mice indicating the deletion did not occur in the hindbrain. We next used Western blot analysis to assess protein levels of the DKO mice at 8 weeks of age. A significant 50% reduction in both HDAC1 and HDAC2 was observed in DKO mice and was specific to forebrain regions of the brain compared to CTL mice. No significant changes were detected in the cerebellum consistent with the original characterization of the CaMKII-Cre93 line (Chen et al., 2001) (Figure 3-1C, D).

Postnatal loss of HDAC1 and 2 results in gross abnormalities and premature death

The HDAC1/2 DKO mice were indistinguishable in body weight from the CTLs for the first few weeks of life, however, at 6 weeks of age the DKO mice began to lose significant amounts of weight and all DKO mice died at approximately 9 weeks of age (Figure 3-1A). HDAC1 and HDAC2 single KO lines have been previously reported to have no change in weight at the adult stage (8 weeks old) (Morris et al., 2013). To confirm the deletion of HDAC1 or HDAC2 does not impact weight immediately after birth, mice were weighed between 3-8 weeks of age. HDAC1 or HDAC2 conditional knockout mice were indistinguishable from littermate CTLs in weight and had a normal lifespan into adulthood (Figure 3-2B, C). Conditional HDAC1/2 heterozygous mice carrying the Cre transgene and one copy each of HDAC1 and HDAC2 loxP alleles had a normal lifespan and showed no overt phenotypes including no significant change in weight suggesting that HDAC1 and HDAC2 can have redundant functions in postmitotic neurons, which ultimately contributed to the shortened lifespan.

The conditional HDAC1/2 DKO mice underwent pathological analysis to examine the cause of premature death, and necropsy results concluded that the HDAC1/2 DKO mice did not die of liver or kidney failure or other peripheral abnormalities. However, a significant reduction in overall brain size of 8 week old HDAC1/2 DKO mice was observed compared to age matched CTLs that appeared to be due to a decrease in the size of the cortical areas, consistent with the regional deletion of HDAC1 and HDAC2 (Figure 3-3A). Moreover, at 8 weeks of age, conditional HDAC1/2 DKO mice have a significant reduction in brain weight compared to littermate controls (Figure 3-3C). Since the CaMKII-Cre93 line expresses Cre

recombinase starting at postnatal day 10-14 (Chen et al., 2001), we examined the brain mass of HDAC1/2 DKO mice at a time point coinciding with the initial expression of Cre recombinase, postnatal day 16, and found no difference in brain mass in the DKO mice compared to CTLs (Figure 3-3B) suggesting that the changes in brain mass were due to the deletion of HDAC1 and HDAC2. We found no differences in brain weight in 8 week old conditional HDAC1 or HDAC2 single KOs demonstrating that the loss of both HDAC1 and HDAC2 were required for the reduction in brain mass (Figure 3-3D, E).

Conditional deletion of HDAC1 and HDAC2 results in neuronal apoptosis

To more closely examine the brains of the HDAC1/2 DKO mice, we used Hematoxylin and Eosin (H&E) staining to perform histological analysis. H&E staining revealed dramatically abnormal brain morphology in the cortex, with aberrant cortical laminations in HDAC1/2 DKO mice compared to littermate controls (Figure 3-4A, B). Conditional HDAC1/2 DKO mice also showed hippocampal alterations, as the CA1 hippocampal subregion appeared thinner (Figure 3-4F, enlarged) and the dentate gyrus appeared drastically reduced (Figure 3-4F) compared to littermate control animals (Figure 3-4E and enlarged). Rather surprisingly, there were no cell morphology abnormalities observed in the striatum of HDAC1/2 DKO compared to CTL mice (Figure 3-4C, D) although HDAC1 and HDAC2 expression was reduced in this brain region. The CaMKII –Cre93 driver line is known to express Cre recombinase in medium spiny neurons of the striatum (Fan et al., 2001), providing an explanation for the deletion of HDAC1/2 in this brain region. There were

no detectable differences in the cerebellar structure between HDAC1/2 DKO and CTL mice, consistent with the forebrain specific deletion (Figure 3-4G, H).

The results from H&E staining suggested that cell death is occurring in HDAC1/2 DKO mice, therefore we used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to determine whether the cell loss was due to apoptosis. The HDAC1/2 DKO mice showed an increase in TUNEL positive cells in forebrain regions within the cortex and hippocampus, suggesting that deletion of HDAC1 and HDAC2 resulted in neuronal apoptosis (Figure 3-5A-D). We detected no TUNEL positive cells in the striatum in agreement with the H&E analysis that did not detect any cellular morphological changes in this brain region (Figure 3-5E, F). No TUNEL positive neurons were detected in the cerebellum of DKO mice, consistent with a forebrain deletion of the genes of interest (Figure 3-5G, H).

Conditional HDAC1/2 DKO mice are hypoactive

To examine whether the DKO mice displayed any behavioral abnormalities prior to the loss of weight, we tested 6 week old mice for locomotor activity to get a baseline measure of activity. The HDAC1/2 DKO mice were significantly hypoactive over the two hour testing session (Figure 3-6A) and had significantly less total beam breaks compared to CTL mice (Figure 3-6A inset). Since 6 weeks of age is the beginning of the appearance of the overt phenotypes, we tested a separate cohort of mice at an earlier time point of 3 weeks of age, which is approximately one week after the expression of Cre recombinase. Surprisingly, we found that conditional HDAC1/2 mice were significantly hypoactive at 3 weeks old, indicating that the loss of HDAC1/2 impacts locomotor activity (Figure 3-6B and inset). As

a control measure, conditional HDAC1 and HDAC2 single KO mice were tested for locomotor activity at 6 weeks and 3 weeks of age. Conditional HDAC1 and HDAC2 KO mice have no impairments in locomotor activity at 6 weeks (Figure 3-7A,C) or 3 weeks of age (Figure 3-7B, D), suggesting the loss of both HDAC1 and HDAC2 is necessary to impair baseline activity.

Conditional HDAC1/2 DKO mice display anxiety-like behavior

To examine anxiety-like behavior, conditional HDAC1/2 DKO mice were tested in the open field paradigm. During the 10 minute testing session, 6 week old DKO mice spent significantly less time in the center of the arena and more time in the periphery compared to littermate control mice, suggestive of an increase in anxiety-like behavior (Figure 3-8A). As with locomotor activity, 3 week old mice were also tested in the open field paradigm and we found that conditional HDAC1/2 DKO mice have heightened anxiety-related behavior compared to control mice (Figure 3-8C), demonstrating that loss of both HDAC1 and HDAC2 rapidly impacted behavior. To ensure that the heightened anxiety phenotype is not due to hypoactivity, data were also analyzed as duration ratio (time spent in the center over time spent in the periphery). At both 6 and 3 weeks of age, conditional HDAC1/2 DKOs have a duration ratio significantly less than that of controls (Figure 3-8B, D) suggesting the elevated anxiety is not impacted by hypoactive behavior or less time spent moving in the open field arena.

Discussion

We demonstrate that postnatal loss of both HDAC1 and HDAC1 in the brain results in several adverse effects. We show that conditional HDAC1/2 DKO mice have dramatic structural abnormalities in forebrain regions following the gene deletion and die at approximately 9 weeks of age. The conditional HDAC1/2 DKO mice also display hypoactivity and heightened anxiety soon after the loss of the genes. The current study provides key evidence for a crucial role of HDAC1 and HDAC2 in postnatal brain development.

Our results indicate that a conditional loss of HDAC1 and HDAC2 is deleterious, suggesting that these genes are functionally redundant when deleted postnatally. Conditional HDAC1/2 DKO mice are viable and grow normally compared to littermate controls until 6 weeks old, after which mice significantly lose weight and die at 9 weeks old. Hematoxylin and Eosin analysis of coronal brain sections of conditional HDAC1/2 DKO mice reveals aberrant cellular morphology and pattern formation, suggestive of cell death processes. Further analysis was done using TUNEL staining, and we found evidence of neuronal death in conditional HDAC1/2 DKO mice, but not in littermate controls. The timing of death in conditional HDAC1/2 DKO mice remains an interesting point of study, as it does not coincide with the time of birth or timing of Cre-mediated recombination at postnatal day 14. Moreover, apoptosis does not occur in all forebrain areas, rather is selective for the cortex and hippocampus suggesting postnatal loss of HDAC1 and HDAC2 does not have global effects. More detailed time course studies to determine precisely when apoptosis is triggered

could provide additional insight into how deleting HDAC1/2 postnatally results in early lethality.

The severe weight loss, neuronal apoptosis, and early lethality of the conditional HDAC1/2 DKO mice precluded our ability to test these animals in learning and memory paradigms. We therefore tested conditional HDAC1/2 DKO mice in non-stressful paradigms to assess baseline behavior. At 6 weeks old, just before severe weight loss occurs, conditional HDAC1/2 DKO mice are hypoactive when tested for locomotor activity compared to littermate controls. Surprisingly, at 3 weeks old, about one week after Cre-mediated deletion of the genes, we also found DKO mice to display a hyperactive phenotype. Moreover, conditional HDAC1/2 DKO mice have heightened anxiety at 6 and 3 week time points, indicating both HDAC1 and HDAC2 act early on to regulate development and baseline behaviors. As a control measure, we tested conditional HDAC1 or HDAC2 single KO mice and no histological abnormalities or behavioral impairments were measured, confirming that the loss of both genes results in detrimental effects compared to the loss of the individual genes.

Accumulating evidence has shown that inhibition of Class I HDACs, specifically HDAC2, can enhance learning and memory and LTP in various *in vitro* and *in vivo* models (Levenson et al., 2004) (Guan et al., 2009) (Morris et al., 2013). With that, much attention has been focused on the development of pharmacological compounds designed to target HDAC2 with greater selectivity than existing Class I HDAC inhibitors (Graff and Tsai, 2013b). Given the high degree of sequence identity between HDAC1 and HDAC2 an important concern has been compounds designed to inhibit HDAC2 could also impact

HDAC1 and result in potential off-target effects. Indeed recent data has shown that an embryonic deletion of both HDAC1 and HDAC2 in mice results in detrimental effects, and lethality occurs at postnatal day 7 (Montgomery et al., 2009). We were therefore interested to understand whether a deletion of HDAC1 and HDAC2 past the stage of neuronal proliferation would have similar learning and memory benefits as seen with the individual loss of HDAC2, or whether these genes have redundant roles postnatally. Here, we show that deletion of HDAC1 and HDAC2 in postnatal brain induces several adverse effects including premature death, neuronal apoptosis, and alterations in behavior suggesting redundancy when deleted together. Our data on the negative impact of HDAC1/2 loss in adult neurons provides important information on the utility of the approach in targeting both of these HDACs in the postnatal brain and further emphasizes the importance of targeting isoform specific HDACs for neurotherapeutics.

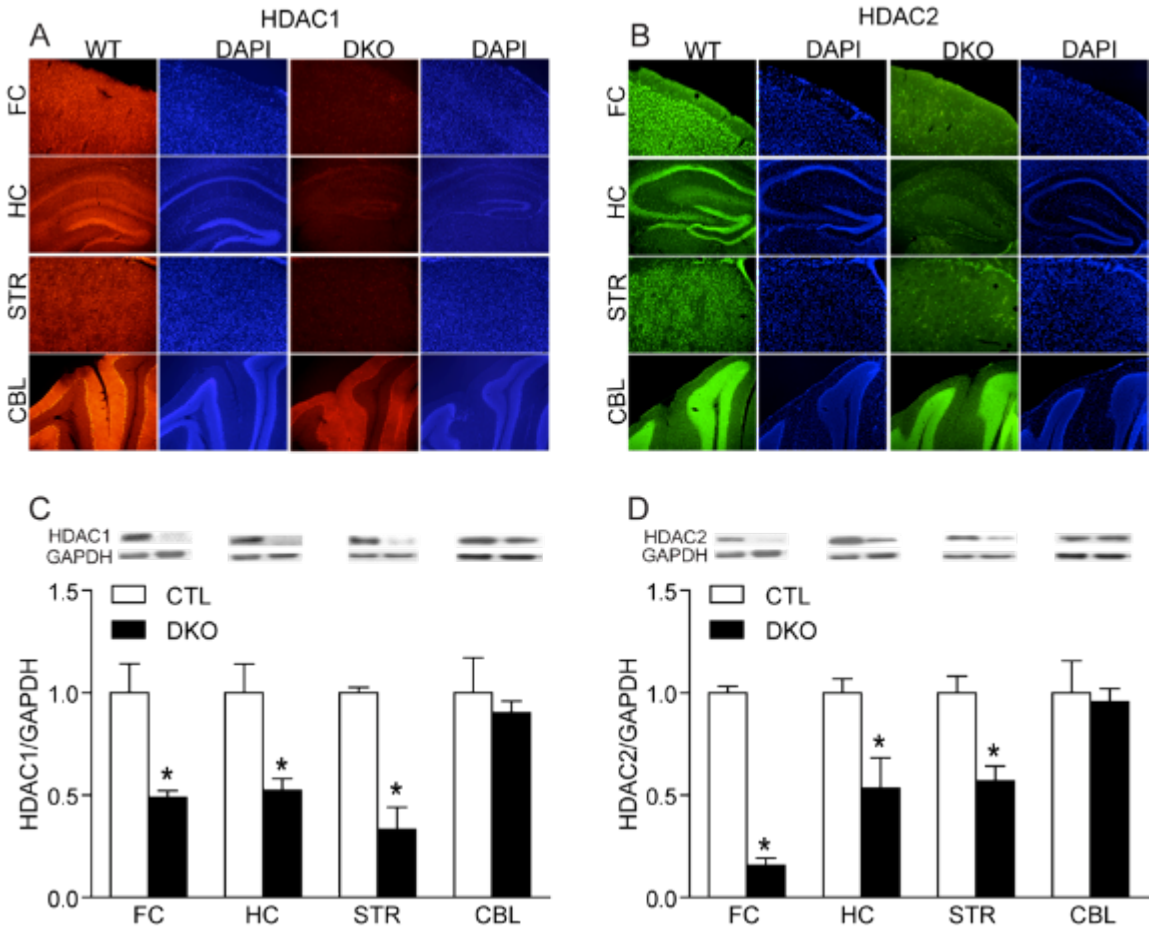

Figure 3-1. Conditional HDAC1/2 double knockout (DKO) mice have a forebrain-specific deletion of both HDAC1 and HDAC2. Fluorescent immunohistochemistry confirmed a selective loss of HDAC1 (A) and HDAC2 (B) in the frontal cortex (FC), hippocampus (HC) including CA1 and dentate gyrus sub-fields, and striatum (STR), but not in the cerebellum (CBL) of the conditional HDAC1/2 DKO compared with CTL mice. (C and D) Protein expression of HDAC1 (C) and HDAC2 (D) were examined by Western blots in frontal cortex (FC), hippocampus (HC), striatum (STR), and cerebellum (CBL) from 8 week old animals. Protein levels were normalized to GAPDH and confirmed over 50% reduction of HDAC1 and HDAC2 in forebrain regions of conditional HDAC1/2 DKO mice, with no alterations in the CBL (n = 5 mice per group; *P < 0.05 by t-test).

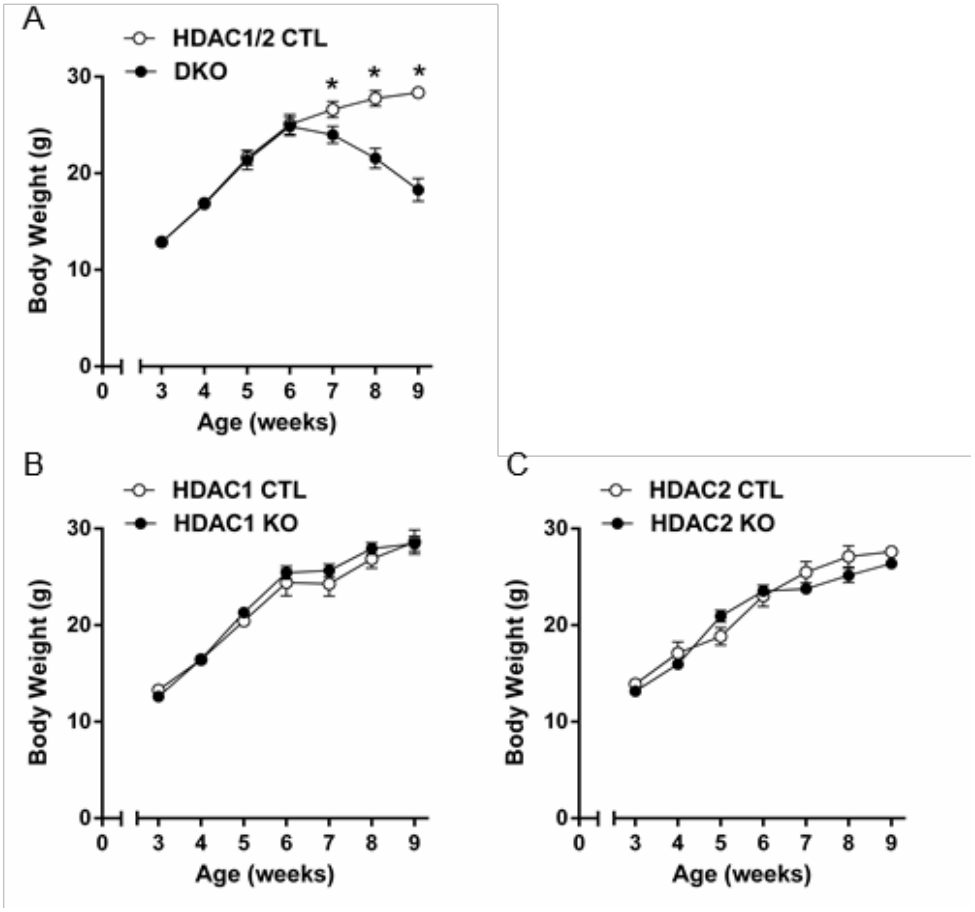

Figure 3-2. Conditional HDAC1/2 DKO mice have a significant reduction in weight. (A) Body weights of HDAC1/2 DKO mice were indistinguishable from littermate CTL mice until 6 weeks of age, after which time significant weight loss was observed in HDAC1/2 DKO mice until their death at approximately 9 weeks of age (n = 6 mice per group; *P < 0.05 by t-test). (B) No differences in body weight were found at any time point between HDAC1 single KO mice and control littermates or (C) HDAC2 single KO mice and control littermate mice (HDAC1 CTL n=5; KO n=7, HDAC2 CTL n=6; KO n=6).

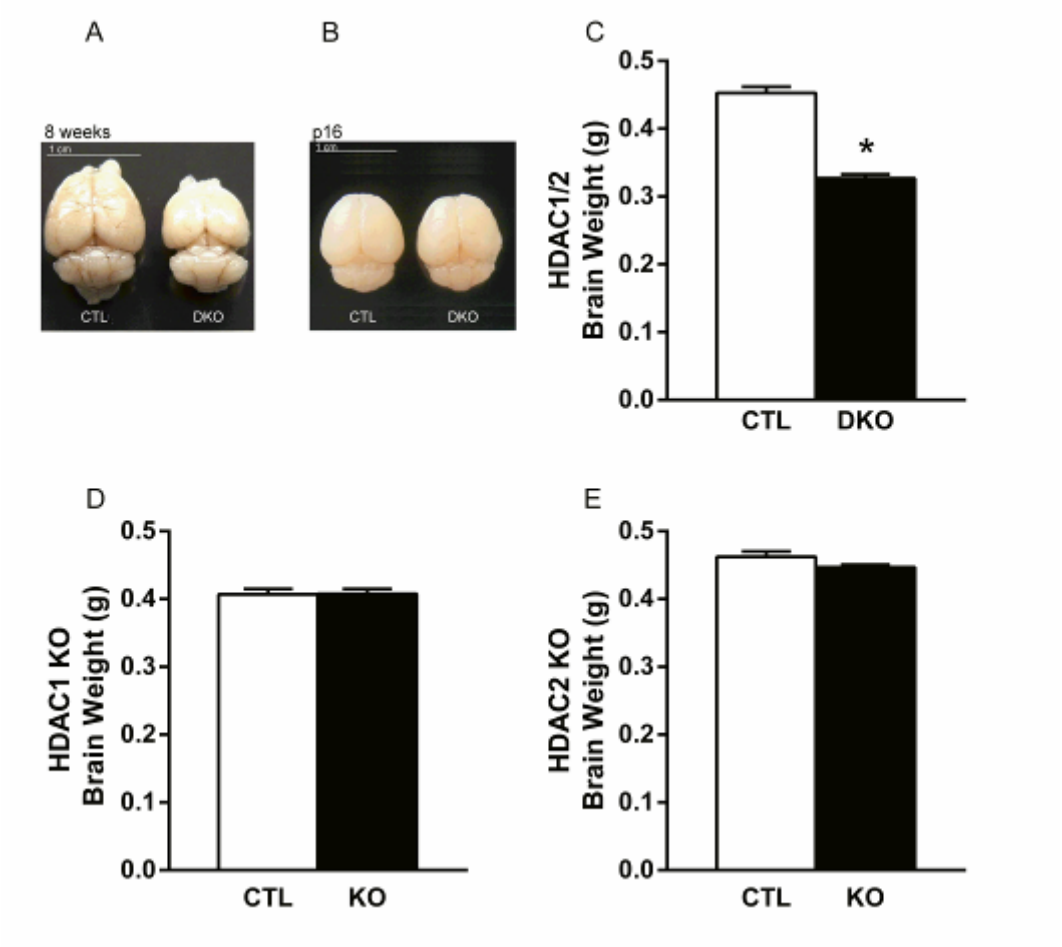

Figure 3-3. Conditional HDAC1/2 DKO mice have a significant reduction in brain size and weight. Shown are representative pictures of the brain at 8 weeks of age (A) and p16 (B) time points from littermate CTL (left) and HDAC1/2 DKO (right) mice. Forebrain volume was much smaller in HDAC1/2 DKO mice than CTL mice in 8 week old mice with no changes observed at p16. (C) Brain weight of conditional HDAC1/2 DKO mice at 8 weeks old was significantly reduced in comparison to littermate CTL mice (n = 6 mice per group; *$P < 0.05$ by t-test). (D) Conditional HDAC1 single KO mice have a similar brain mass to control littermate mice 8 weeks of age with no significant difference in weight (n = 6 mice per group). (E) Conditional HDAC2 single KO mice and control littermates have no measureable differences in brain mass at 8 weeks of age (n = 5 mice per group).

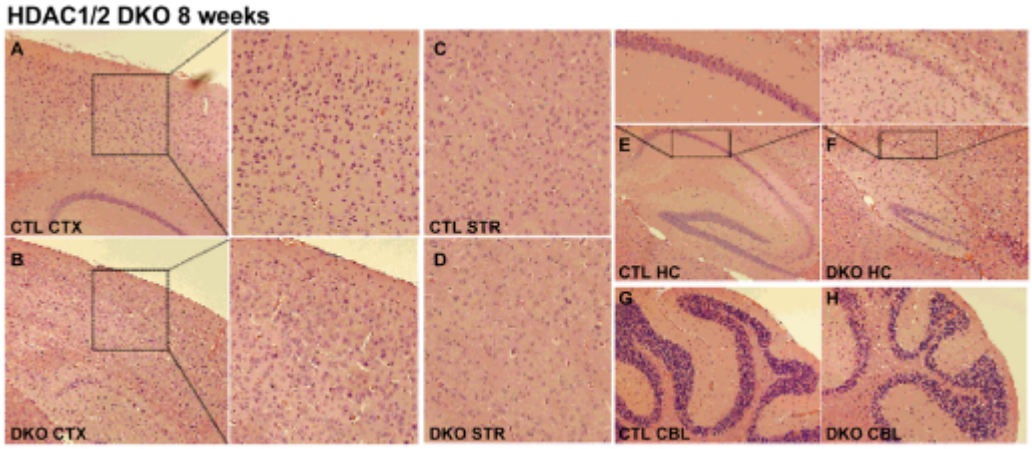

Figure 3-4. Conditional HDAC1/2 DKO mice have abnormal cellular morphology. Presented are H&E staining of 8 week old HDAC1/2 DKO and littermate CTL mice. (A and enlarged) CTL mice displayed normal cortical formation and lamination. (B and enlarged) In contrast, conditional HDAC1/2 DKO mice showed much thinner cortical thickness as well as considerably disrupted cortical laminations. (C and D) Striatal patterning and organization of HDAC1/2 DKO mice (C) appear to be indistinguishable from littermate CTL (D) mice. (E, F and enlarged) The overall size of the hippocampus in HDAC1/2 DKO mice (F) was dramatically smaller than that in littermate CTL mice (E) and granule cell layers of CA1 subregion were drastically thinner in HDAC1/2 DKO mice (F enlarged), compared to littermate CTL mice (E enlarged). (G and H) No histological differences were observed in cerebellum (CBL) between HDAC1/2 DKO (H) and littermate CTL mice (G).

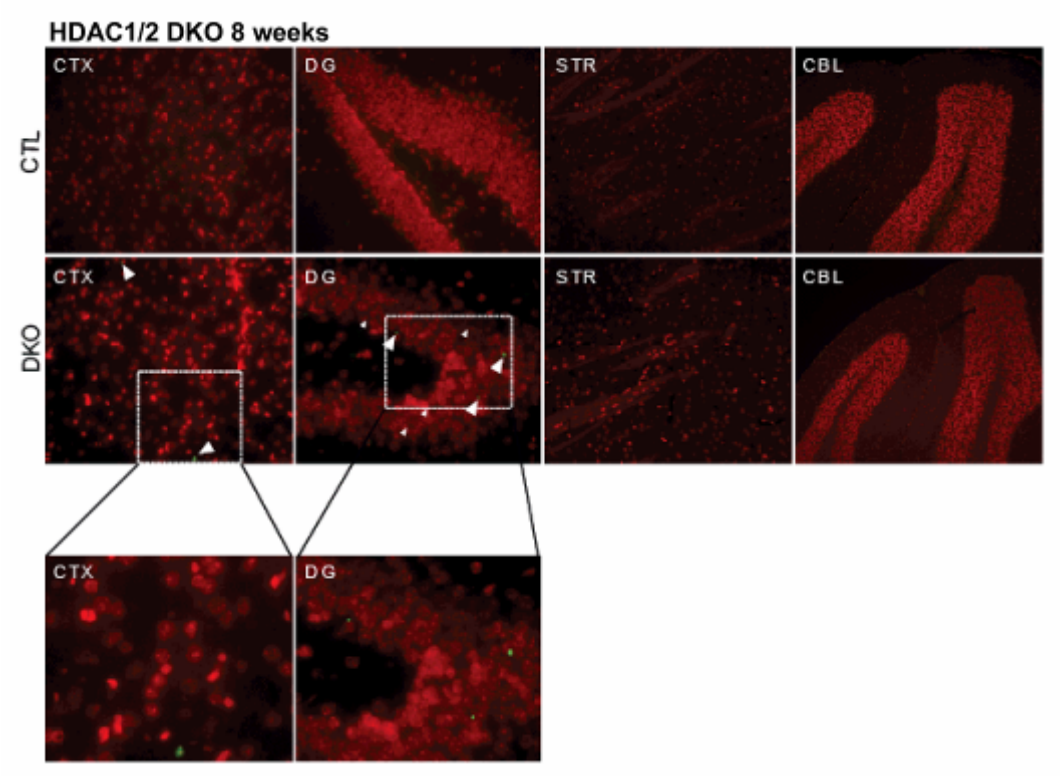

Figure 3-5. Postnatal deletion of HDAC1 and HDAC2 triggers neuronal apoptosis in forebrain neurons. Presented are TUNEL staining on HDAC1/2 CTL and conditional DKO mice at 8 weeks old. No TUNEL positive neurons appear in HDAC1/2 CTL mice indicating an absence of neuronal death. HDAC1/2 DKO mice have several TUNEL positive cells in the cortex and the dentate gyrus (DG) region of the hippocamous suggestive of cell death occuring in the forebrain as a result of apoptosis. No TUNEL positive neurons are detected in the striatum or cerebellum of HDAC1/2 DKO mice.

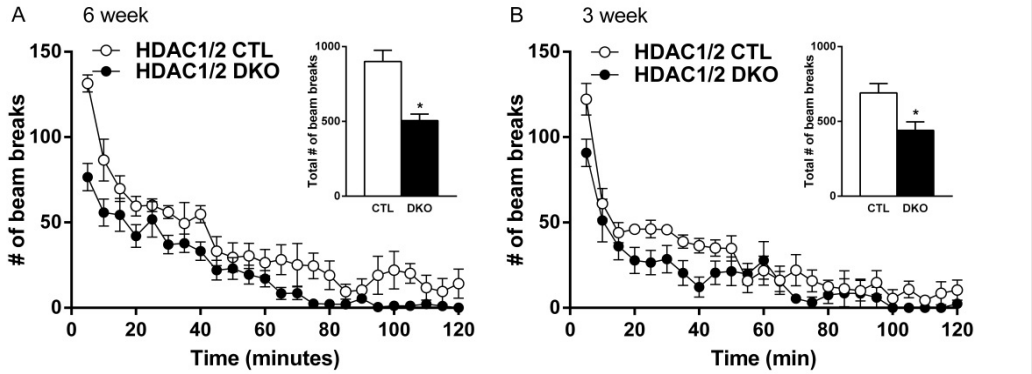

Figure 3-6. Conditional HDAC1/2 DKO mice are hypoactive. Locomotor activity of 6 week (A) and 3 week (B) old animals was assessed by consecutive horizontal beams breaks for a 2 hour testing period and the data shown in 5 minute increments. (Inset) The total number of beam breaks during the testing period shows that conditional HDAC1/2 DKO mice have significantly less total locomotor activity in comparison to littermate CTL mice at both 6 weeks (A) and 3 weeks (B) of age (n = 5 mice per group; *, $P < 0.05$ by t-test).

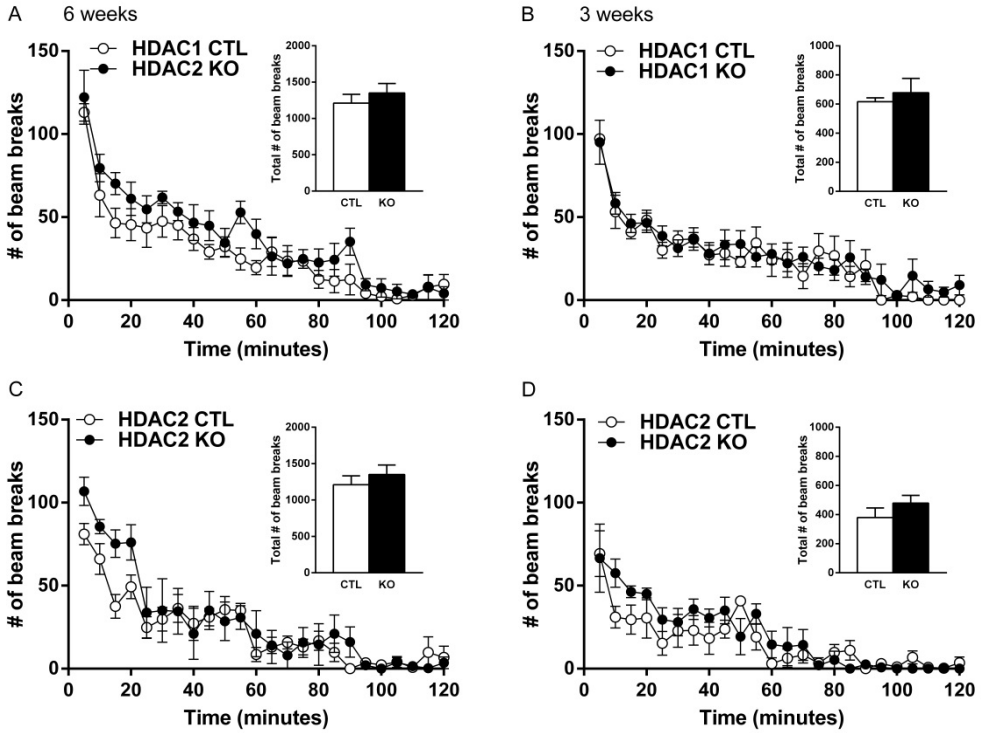

Figure 3-7. Conditional HDAC1 and HDAC2 KO mice do not have early impairments in locomotor activity. At 6 weeks of age conditional HDAC1 KO mice (A) and Conditional HDAC2 KO mice (C) do not display significant changes in locomotor ambulation or the total number of beam breaks (inset) over the 2 hour testing session compared to littermate control mice. When tested earlier at 3 weeks old both HDAC1 KOs (B) and HDAC2 KOs (D) have normal locomotion compared to CTLs.

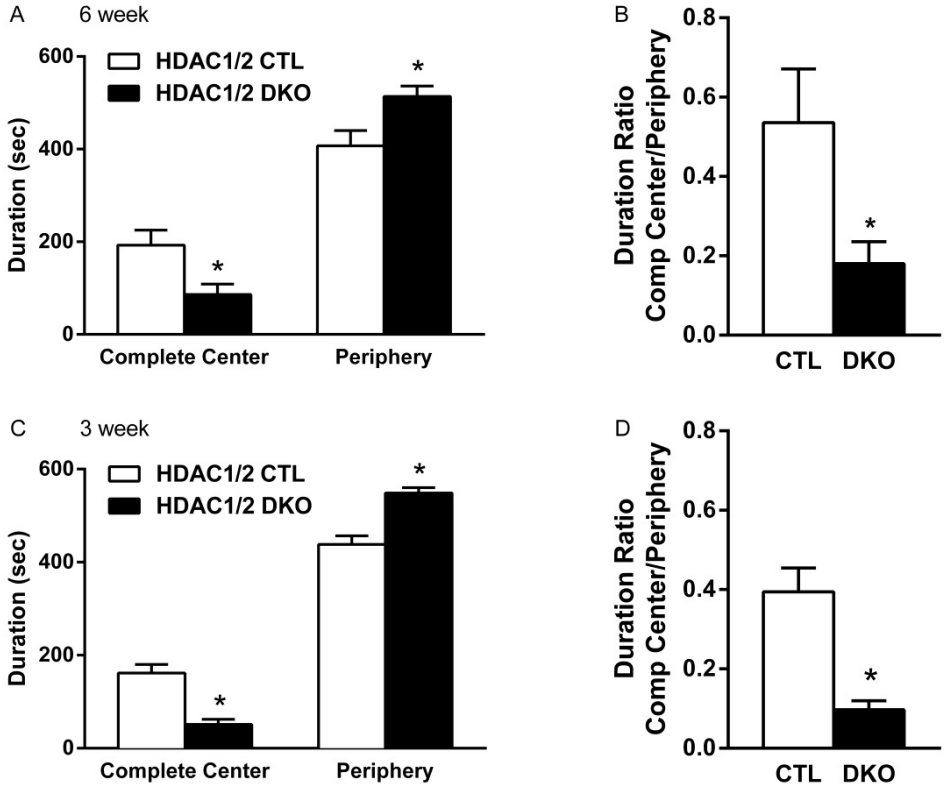

Figure 3-8. The loss of HDAC1 and HDAC2 in the forebrain results in increased anxiety-like behavior in the open field paradigm. 6 week old (A) and 3 week old (C) HDAC1/2 DKO mice spent significantly less time in the center field and more time in periphery of the arena, compared to respective littermate CTL mice. (B and D) At 6 and 3 weeks of age conditional HDAC1/2 DKOs mice have a significant decrease in the ratio of time spent in center over time spent in periphery compared to CTLs (3 week old animals CTL n=11; DKO n=9, 6 week old animals CTL n=7; DKO n=6; *P < 0.05 by t-test).

CHAPTER 4

NOVEL ROLE FOR HDAC1/2 IN REGULATING MECHANISMS UNDERLYING OBSESSIVE-COMPULSIVE-LIKE BEHAVIORS

Introduction

Histone deacetylases (HDACs) compress the chromatin structure, restricting access of transcription factors to the DNA and consequentially repressing gene expression. While individual HDAC genes are widely expressed throughout the body and possess differing deacetylase activity, their specific function in various tissues is only now starting to be examined. Two of the individual HDAC genes, HDAC1 and HDAC2, are class I HDACs that are ubiquitously expressed, localized to the nucleus, and generally found together in co-repressor complexes (Haberland et al., 2009). While recent work has shown that conditional brain-specific HDAC2 knockout mice have enhanced learning and memory, with no measurable effects observed following manipulation of HDAC1 expression (Guan et al., 2009) (Morris et al., 2013), it is unclear how the brain specific loss of both HDAC1 and HDAC2 impacts complex behavior. Our previous data has shown that conditional forebrain-specific HDAC1 and 2 double knockout (DKO) mice die at approximately 9 weeks of age, although survival is not impacted in the single knockouts. The DKO mice have behavioral abnormalities including hypoactivity and heightened anxiety shortly after the deletion of HDAC1 and 2.

In the current study we show that conditional HDAC1/2 DKO mice develop an unexpected and severe facial lesion as a result of increased grooming that resembles

obsessive-compulsive-like behavior. Obsessive-compulsive disorder (OCD) is a debilitating neuropsychiatric disease characterized by intrusive thoughts, compulsive behaviors, and heightened anxieties (Kessler et al., 2005a) (Ruscio et al., 2010). Current estimates reported by the National Institute of Mental Health (NIMH) show the 12-month prevalence of OCD is approximately 1% of the U.S. adult population with 50.6% of these affected individuals classified as severe cases (Kessler et al., 2005b). Currently, SSRIs are the most effective line of treatment to alleviate symptoms of OCD. Non-serotonergic antidepressants such as desipramine and anxiolytic drugs have not been shown improve OCD related symptoms suggesting that dysregulation of the serotonergic system contributes to the pathophysiology of the disease. However, SSRI treatment can take several weeks to take effect and a significant percentage of patients (roughly 30%) are resistant to treatment highlighting the need for more effective therapies.

Data has started to emerge identifying potential candidate genes as well as reliable mouse models of OCD-like behaviors. A behavioral paradigm consistently used to measure OCD behavior is testing for excessive grooming behavior. Compulsive grooming in mice can often lead to hair loss and facial lesions which is suggested to model the trichotillomania observed in some OCD patients. In early studies investigating genetic models of OCD it was found that mice with mutations in Hoxb8, a transcription factor that plays a role in providing positional information during embryonic development, display excessive grooming (Greer and Capecchi, 2002). Hoxb8 knockout mice develop deep facial lesions and fur loss which resulted from compulsive grooming behavior following the loss of the gene. However, studies were not done to test whether the excessive grooming could be alleviated with SSRI

treatment, thus this genetic mutation offers little validity as a mouse model of OCD-like behaviors. More recently, mutations in SLIT and NTRK-like protein-5 (Slitrk5) were found to produce a similar excessive grooming phenotype seen in Hoxb8KO mice. The SLITRK family of proteins has been linked to OCD like behavior and previous data demonstrated that variants in human SLITRK1 are associated with Tourette's syndrome, a disorder classified within the OCD spectrum (Abelson et al., 2005). Data from Shmelkov et al. later showed that a loss of SLITRK5 in mice leads to compulsive grooming behavior that results in a severe facial lesion (Shmelkov et al., 2010). Additional behavioral tasks which model OCD-like behavior in rodents include tests to measure anxiety as well as paradigms to assess repetitive behaviors. The marble burying task tests for obsessive and repetitive behavior seen OCD patients and an increased propensity to bury marbles is suggested to model obsessive-compulsive behavior. SLITRK5 KO mice have elevated anxiety in the open field and elevated plus maze paradigms, as well as an increase in marble burying behavior. Both the grooming and anxiety phenotypes were responsive to treatment with the SSRI fluoxetine indicating that the loss of SLITRK5 results in deficits specifically related to OCD-like behaviors.

More recently, human neuroimaging studies have implicated dysfunction of glutamatergic signaling within cortico-striatal circuitry in patients suffering from OCD (Saxena and Rauch, 2000) (Ting and Feng, 2008). Previous genetic analyses have linked the post-synaptic scaffolding protein SAP90/PSD-95-associated protein 3 (SAPAP3) to obsessive-compulsive behavior and grooming disorders in humans (Bienvenu et al., 2009) (Zuchner et al., 2009). A recent study found that mice lacking SAPAP3 leads to the development of OCD-like

behaviors (Welch et al., 2007). SAPAP3 is one of four isoforms of the SAPAP family of scaffolding proteins, which function to regulate the trafficking and targeting of neurotransmitters to the post-synaptic membrane during excitatory synaptic transmission. Interestingly, SAPAP3 is the only isoform highly expressed in the striatum (Welch et al., 2004), a brain region linked to the pathogenesis of OCD (Welch et al., 2007) (Wu et al., 2012). Mice with a genetic deletion of SAPAP3 display elevated anxiety and a severe compulsive grooming phenotype that results in a prominent facial lesion and fur loss. Moreover, SAPAP3 KO mice have impairments in excitatory neurotransmission at cortico-striatal synapses, and these behavioral and synaptic defects can be rescued with selective expression of SAPAP3 within the striatum, suggesting an important role for SAPAP3 in regulating obsessive-compulsive-like behaviors.

In addition to the increased grooming, we interestingly found that conditional HDAC1/2 DKO mice have a selective down regulation of SAPAP3 in the striatum and cortex. HDAC1/2 DKO mice also have elevated anxiety, a hallmark symptom of patients suffering from OCD, at time points coinciding with the development of the excessive grooming phenotype. The compulsive grooming in conditional HDAC1/2 DKO mice is rescued with chronic treatment with the selective serotonin reuptake inhibitor (SSRI), fluoxetine, providing validity of our model of obsessive-compulsive-like behavior. Moreover, a targeted deletion of HDAC1/2 in the striatum of adult mice recapitulates the excessive grooming and decrease in SAPAP3 expression seen in conditional DKO mice, suggesting HDAC1/2 function within the striatum is sufficient to mediate the observed deficits related to OCD-like behavior.

A well characterized target of HDAC1/2 is methyl-CpG binding protein 2 (MeCP2), a transcription factor that is ubiquitously expressed in neurons and known to play important roles in mediating complex behavior and synaptic function. A loss of function of MeCP2 leads to the development of Rett (RTT) syndrome, a neurological disorder that affects females and results in severe mental retardation and autistic-like behaviors (Amir et al., 1999) (Guy et al., 2001). In addition to social and cognitive deficits, RTT patients display repetitive behaviors such as stereotypical hand movements (Hagberg et al., 1983) (Zoghbi, 1988). Our lab has previously shown that a forebrain-specific postnatal loss of MeCP2 in mice is sufficient to recapitulate several behavioral aspects of RTT syndrome (Gemelli et al., 2006), however it is not known whether mutations in MeCP2 can affect obsessive-compulsive-like behaviors. MeCP2 is known to interact with HDAC1/2 and form a co-repressor complex to regulate gene expression (Nan et al., 1998), thus we were interested to examine its potential contribution to the deficits caused by the deletion of HDAC1/2. We find that conditional MeCP2 KO mice show similar excessive grooming behavior and decrease in SAPAP3 within the striatum as seen in conditional HDAC1/2 DKO mice. Moreover, we show that MeCP2 binds to the promoter region of SAPAP3, suggesting that HDAC1/2 mediates SAPAP3 function through its assembly with MeCP2. Collectively, our results demonstrate a unique role for HDAC1/2 and MeCP2 in the regulation of obsessive-compulsive like behaviors.

Materials and methods

Generation of the conditional KO and DKO mice

Separate cohorts of conditional HDAC1 knockout (HDAC1 KO), HDAC2 knockout (HDAC2 KO) and HDAC1/2 double knockout (DKO) mice were generated by breeding transgenic mice expressing Cre recombinase under the control of the Calcium/Calmodulin-dependent kinase II promoter (CaMKII-Cre93 line) with floxed HDAC1, floxed HDAC2, or double floxed HDAC1/2 mice as previously described. Two separate mouse lines were maintained for conditional HDAC1/2 DKO breeding. For the first cross, floxed HDAC1/2 female mice were crossed with CamKII-Cre male mice and male progeny carrying the Cre transgene were maintained (CamKII-Cre-flHDAC1/2). HDAC1/2 double knockouts were generated by crossing floxed HDAC1/2 females with CamKII-Cre-flHDAC1/2 male mice. Conditional MeCP2 KO mice are previously reported (Gemelli et al., 2006). Floxed Sin3a mice were generously provided by Dr. Ted Abel (University of Pennsylvania). Conditional Sin3a mice were generated by crossing floxed Sin3a mice with CamKII-Cre93 mice. Mice were maintained on a 12 hour light/dark cycle with *ad libitum* access to food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas Southwestern Medical Center.

Drug injections

All injections were delivered intraperitoneally (IP). Conditional HDAC1/2 DKO mice and control littermates received a once daily injection of either 0.9% saline or fluoxetine (Flx) at a concentration of 10 mg/kg in 0.9% saline, during the morning hours of the day for

7 or 21 days.

Adeno-associated virus injection

The adeno-associated virus-green fluorescent protein (AAV-GFP) or adeno-associated virus expressing Cre recombinase tagged with GFP (AAV-CreGFP) viruses were obtained from Penn Vector Core. Previous work demonstrated that GFP-tagged Cre recombinase possesses normal enzymatic activity (Adachi et al., 2009) (Berton et al., 2006). To inject the AAV-GFP or AAV-CreGFP in striatum, 3-5 month old floxed HDAC1/2 mice were subjected to stereotaxic surgery as previously described (Adachi et al., 2008). Briefly, adult floxed HDAC1/2 mice were anesthetized with a mixture of ketamine and xylazine (intraperitoneally, 100 mg/kg and 10 mg/kg respectively), and then placed onto the stereotaxic apparatus. The skull was exposed and holes were drilled bilaterally above the target injection sites. The coordinates relative to Bregma for dorsal striatum were, anteroposterior +1.2 mm, lateral +2.5 mm, dorsoventral −3.0 mm at a 10° angle. A total of 1 μL of virus was bilaterally infused over the course of 4 minutes using a Hamilton syringe with a 33-gauge needle. The syringe was left in place for an additional 5 minutes to ensure diffusion of the virus. Floxed HDAC1/2 mice were injected with either the control virus AAV-GFP (AAV2/1.CMV.PI.EGFP.WPRE.bGH) or AAV-CreGFP (AAV2/1.CMV.HI.GFP-Cre.SV40), which expresses a fusion construct of Cre recombinase and GFP to induce the localized deletion (Penn Vector Core). Previous work has demonstrated that the GFP did not interfere with Cre recombinase activity (Adachi et al., 2009) (Berton et al., 2006). Animals were allowed to recover for three weeks before being

subjected to behavioral testing.

RNA extraction and quantitative reverse transcription (qRT) PCR

To determine relative expression of *Hdac1* and *2* mRNA after stereotaxic AAV injection, we performed qRT-PCR as described previously (Adachi et al., 2009). Briefly, the animals were sacrificed by rapid decapitation and the brains were sectioned at 14 μm. The dorsal striatum expressing GFP or Cre-GFP was laser-micro dissected out from each section using the AS LMD (Leica) system. Eight sections were pooled to extract RNA using PicoPure RNA isolation kit (Arcturus). Each section was 140 μm apart, thus encompassing the majority of the AAV infusion site in the striatum. Conditions for construction of complementary DNA (cDNA) were described earlier (Adachi et al 2009). Using cDNA as a template, transcripts for *Hdac1* and *2*, *Sapap3*, *Cre* recombinase, and *Gapdh* were amplified using Power SYBR Green PCR master mix (Applied Biosystems) in a 7500 Real-Time PCR system (Applied Biosystems). The thermal cycling conditions for PCR amplification consisted of 1 cycle of 50°C for 2 min and 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 60 sec, and 1 dissociation cycle of 95°C for 15 sec, 60°C for 60 sec, 95°C for 15 sec, and 60°C for 15 sec. The primer sequences for Cre recombinase are listed in Adachi et al (2008). Others are 5'-AGG GCA CCA AGA GGA AAG TCT GTT-3' and 5'-GCA GCA AAT TGT GAG TCA TGC GGA-3' for *Hdac1*; 5'-GCG TAC AGT CAA GGA GGC GG-3' and 5'-GCT TCA TGG GAT GAC CCT GGC-3' for *Hdac2*; 5'-AGC AGT ACC TTC CCC AGG AT-3' and 5'-AAA CTG GTC CAG GAG TGT GG-3' for *Sapap3*; 5'-CAG TGC AGA TGG GAA CAG AGG TAA-3' AND 5'- TGC ACA GGA AAG GAC GAG TGT-3'

for *Slitrk5*; 5'-AGG TCG GTG TGA ACG GAT TTG-3' and 5'-TGT AGA CCA TGT AGT TGA GGT CA-3' for *Gapdh*.

For *Sapap3* expression analysis in HDAC1 KO mice, HDAC2 KO mice, and HDAC1/2 DKO mice, the following brain regions were dissected FC, HC, STR, and CBL and total RNA was extracted using Trizol reagent (Ambion) according to the manufacturer's instructions. Single stranded cDNA was synthesized by treating extracted RNA with random primers and SuperScript Reverse Transcriptase III (Invitrogen). Amplification was performed using Power SYBR Green PCR master mix in a 7500 Real Time PCR detection System (Applied Biosystems). The fold change in *Sapap3* expression relative to *Gapdh* was calculated as mean ± SEM.

For qRT-PCR using ChIP samples, 1uL of ChIP cDNA was used as a direct template and amplification was performed using Power SYBR Green PCR master mix in a 7500 Real Time PCR detection System (Applied Biosystems). The fold change in *Sapap3* expression relative to input DNA was calculated as mean ± SEM. Primer sequences for *Sapap3* are as follows; 5'- GGG ACT AGT GCG GAG AA -3' and 5'- TCT TAG GCT CCT GTC CTT AG -3' for *Sapap3* P-600; 5'- ACC CTG ACT CAG AAC ATC TC -3' and 5'- AGC TAC ATC CTC TAC CTT GTC-3' for *Sapap3* P-1200; 5'- CCC AAC AAT GAC TCC TAT TTC T -3' and 5'- CTC CAG GTT CTA GCT CCT TA -3' for *Sapap3* P-1300; 5'- GTT GGC TAG TCT GTT CAT CTC -3' and 5'- CCA CAC CCA TTC CCT AGA -3' for *Sapap3* P-1400; 5'- TTC CTG AAC CAA CTT TCT ATC T -3' and 5'- CCA CGA GAC CAC TTC AAT C -3' for *Sapap3* P-1600; 5'- CTT TGG TAA TGG CAA AGA AGA A -3' and 5'- TGG AGC ATA AGA ACA GTC ATC -3' for *Sapap3* P-1700.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described (Flavell et al., 2008) with modifications. Briefly, a pool of 3 dissected mouse striata were homogenized in crosslinking-buffer (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 25 mM Hepes-KOH, pH 8.0) containing 1% paraformaldehyde. Cross-linking was quenched by adding glycine (final concentration is 125 mM) for 5 min after a 10 min incubation at RT.) Cells were then rinsed 3 times in ice-cold PBS containing complete protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and lysed by Buffer I (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10 % Glycerol, 0.5 % NP-40, complete protease inhibitor cocktail) for 10 min at 4℃. Nuclei were then pelleted by centrifugation at 2000 g for 10 min at 4℃. The isolated nuclei were rinsed with IP buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 1% Triton X-100, 0.1% deoxycholic acid (sodium salt) and complete protease inhibitor cocktail). Samples were fragmented by sonication in IP buffer. The sizes of DNA fragments range from 200 bp to 2 kb. Insoluble materials were removed by centrifugation at 12,000 g for 10 min at 4℃. The supernatant was transferred to a new tube and the final volume of the resulting nuclear lysate was adjusted to 1 mL by adding additional IP buffer. 1/20 volume of the ChIP sample (50 ul from 1 mL lysate) was saved as input material. The rest of the lysate was incubated with the indicated antibody (MeCP2 (Abcam) or IgG (Abcam), 2 ug) overnight at 4℃ for immunoprecipitation.

The next day, 20 ul of pre-rinsed Protein A/G PLUS Agarose (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) was added to each ChIP reaction and further incubated for 2 hrs at 4℃. The beads bound by immune-complexes were pelleted and washed twice

with each of the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl) and LiCl buffer (0.25M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1). In each wash, the beads were incubated with wash buffer for 10 min at 4℃. The washed beads were then rinsed once with 1x TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunoprecipitated materials were eluted from the beads twice by adding 150 ul of elution buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1 % SDS) to each ChIP reaction and incubating the sample at 65℃ for 10 min with brief vortexing every 2 min. 250 ul of elution buffer was also added to the saved input material (50 ul) and this sample was processed together with the ChIP samples. The eluates were combined and crosslinking was reversed by incubation at 65℃ for 4~5 hrs. To each eluate (300 ul), 5 ul of diluted (1:50, 2 mg/mL) RNaseA (Qiagen) was added and incubated for 1 hr at 37℃. Then 7 ul of Proteinase K (20 mg/ml; New England Biolabs, Ipswich, MA) was added and incubated for 2 hrs at 50℃. The immunoprecipitated genomic DNA fragments were extracted with Phenol/Chloroform (Invitrogen). The extracted DNA fragments were then purified using the QIAquick PCR purification kit (Qiagen) and DNA fragments were eluted in 60 ul of 10 mM Tris-HCl, pH 8.5.

Behavioral Overview

For all behavior testing, mice were age-matched littermates and groups were balanced by genotype. All experiments were conducted and scored by an observer blind to group

assignments. Mice were habituated to testing facilities one hour prior to behavioral assessment. For all experiments, data was presented as mean ± SEM and significance was $P<0.05$.

Locomotor Activity

Mice were placed individually in a standard mouse cage (18 cm x 28 cm) with fresh bedding, and activity was monitored over 2 hrs by five horizontal photobeams linked to data acquisition software (Photobeam Activity System, San Diego Instruments, San Diego, CA). Ambulatory activity was measured by counting the number of consecutive beam breaks in 5-minute increments. Data were analyzed with the Student's T-test. Data are presented as mean ± SEM.

Open field

Mice were placed in the periphery of a novel open field environment (44 cm x 44 cm, walls 30 cm high) in a dimly lit room and allowed to explore for 10 minutes. The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision 3.0, Noldus, Leesburg, Virginia) to determine the total time spent in the periphery (5 cm from the walls) and the center (14 cm x 14cm). The open field arenas were cleaned between mice. Data were analyzed with the Student's T-test. Data are presented as mean ± SEM.

Grooming

Test sessions were recorded under red light by a video camera directly in front of the mice located inside of the testing room. Mice were placed individually into a fresh mouse cage and allowed to move freely for 30 minutes. An observer blind to group and genotype analyzed the videotape and scored self-grooming of any parts of the body. Duration and number of bouts were measured at 10-minute intervals. Continuous grooming for greater than one second was recorded as a grooming bout, and sessions separated by two or more seconds constituted a new bout. Data were analyzed with the Student's T-test and presented as mean ± SEM.

Rotorod

Each mouse was placed on the rotarod (IITC Life Science), which accelerated from 0 to 45 rpm in over the course of 60 seconds. Each session ended when the mouse fell off the rod and the total time spent on the rotorod before falling was measured. The mouse was returned to its original cage for 1 hour. The test was repeated for a total of 8 trials over the course of 2 days (4 trials per day). Data were analyzed by a two-way analysis of variance.

Statistical analysis

Data are reported as mean ± SEM. Statistical differences were assessed using the unpaired or paired two-tailed Student's t test, one-way ANOVA, or two-way ANOVA with multiple comparisons when appropriate. Tukey post hoc tests were used following two-way ANOVA. Statistical significance was defined as $P < 0.05$.

Results

Conditional HDAC1/2 DKO mice exhibit excessive grooming

Each conditional HDAC1/2 DKO mouse develops a facial lesion at approximately 7 weeks of age, just prior to death (Figure 4-1). We concluded this was not due to fighting amongst cage mates as the lesion still appeared in singly housed mice. Necropsy studies concluded that the lesion was not due to any form of dermatitis therefore we investigated whether the facial lesion was due to excessive grooming in the HDAC1/2 DKO mouse. We monitored the behavior of 6 week old mice for a 30 minute time period (between 8 am - 12 pm). Conditional HDAC1/2 DKO mice displayed a significant increase in the total time spent grooming, with a significant difference observed within the initial 10 minutes of the test, compared to littermate control mice (Figure 4-2A). Since the HDAC1/2 DKO mice show behavioral abnormalities at 3 weeks of age, we examined grooming behavior at this time point and found the HDAC1/2 DKO mice display excessive grooming compared to CTLs (Figure 4-2B). In contrast, individual conditional HDAC1 or HDAC2 KO mice do not develop a facial lesion nor do they display any differences in grooming behavior compared to their respective littermate controls (Figure 4-3A, B), demonstrating that this phenotype is due to the loss of both HDAC1 and HDAC2.

Chronic administration of fluoxetine attenuates excessive grooming

Excessive grooming in mice has been suggested to model aspects of obsessive-compulsive disorder (OCD), with previous data demonstrating that the serotonin selective reuptake inhibitor (SSRI), fluoxetine, can alleviate excessive grooming in mice (Shmelkov et

al., 2010; Welch et al., 2007) similar to the attenuation of symptoms of OCD in human patients (Fan et al., 2001; Jones et al., 1998; Shmelkov et al., 2010). We therefore investigated whether the excessive grooming in conditional HDAC1/2 DKO mice was responsive to chronic fluoxetine administration. We treated 3 week old HDAC1/2 DKO mice and littermate CTLs with fluoxetine (intraperitoneal injections, 10mg/kg/day) and assessed grooming behavior one week later at 4 weeks of age, and 3 weeks later at 6 weeks of age. We found that one week of fluoxetine treatment did not attenuate the increased grooming behavior of the HDAC1/2 DKO mice (Figure 4-4A). However, three weeks of fluoxetine administration significantly reduced the amount of time spent grooming to a comparable level seen in CTLs (Figure 4-4B) suggesting that the excessive grooming observed following the loss of HDAC1/2 mimics compulsive-related behavior. Fluoxetine treatment did not prevent the development of the facial lesion or increase longevity of conditional HDAC1/2 DKO mice.

Loss of HDAC1/2 leads to dysregulation of SAPAP3

Clinical studies have suggested a genetic link between the post-synaptic scaffolding protein SAP90/PSD-95-associated protein 3 (SAPAP3) and the development of obsessive-compulsive behavior and grooming disorders in humans (Bienvenu et al., 2009) (Zuchner et al., 2009). Furthermore, mice lacking SAPAP3 display an excessive grooming phenotype resulting in a facial lesion, similar to the HDAC1/2 DKO mice (Welch et al., 2007). We therefore assessed whether SAPAP3 expression was altered by the loss of HDAC1 and HDAC2. Quantitative PCR (Q-PCR) analysis revealed a significant decrease in SAPAP3

expression in the frontal cortex and striatum, with no alterations in hippocampus and cerebellum in HDAC1/2 DKO mice (Figure 4-5A). These results are in agreement with previous work implicating the cortical-striatal pathway for the grooming phenotype in the SAPAP3 knockout mice (Welch et al., 2007). SAPAP3 expression was not altered in conditional HDAC1 or HDAC2 single KO mice consistent with the premise that loss of both HDAC1 and 2 was necessary to dysregulate its expression (Figure 4-5B, C). We also examined the expression of SLIT and NTRK-like protein-5 (Slitrk5), another gene that has been linked to excessive grooming behavior (Shmelkov et al., 2010), and found no change in expression in the striatum or cortex, suggesting specificity to altered SAPAP3 expression in the HDAC1/2 DKO mice (Figure 4-6).

Striatal specific deletion of HDAC1/2 recapitulates excessive grooming seen in conditional DKO mice

Previous work showed that overexpression of SAPAP3 in the striatum of SAPAP3 knockout mice rescued the grooming phenotype (Welch et al., 2007). However, given the phenotypes of the HDAC1/2 DKO mice, including the early lethality, it was not feasible to perform a similar type surgery to overexpress SAPAP3 in these mice. We thus took the approach to selectively delete HDAC1/HDAC2 in the striatum and directly investigate the impact on SAPAP3 expression and grooming behavior. We used stereotaxic methods to bilaterally inject into the dorsal striatum of adult (3- 5 months old) floxed HDAC1/HDAC2 mice an adeno-associated virus (AAV) vector expressing Cre recombinase tagged with green fluorescent protein (CreGFP) or GFP as a control (Figure 4-7A). Mice were tested in

behavior three weeks after surgery, a time point sufficient for Cre mediated recombination with AAV-CreGFP (Berton et al., 2006) then sacrificed to confirm viral placements using laser microscopy, with off-target injected animals eliminated from the data (Figure 4-7B). Q-PCR showed an approximate 50% reduction in HDAC1 and HDAC2 expression in dorsal striatum of AAV-CreGFP compared to AAV-GFP injected mice (Figure 4-7C). Deletion of HDAC1 and HDAC2 in striatum has no effect on weight compared to control mice and there was no evidence of premature death that had been observed in the conditional HDAC1/2 DKO mice (data not shown). We found no differences between AAV-CreGFP and AAV-GFP injected floxed HDAC1/2 mice in locomotor activity, anxiety-like behavior, or motor coordination as assessed on the rotorod (Figure 4-8A-C). Moreover, the striatal specific deletion of HDAC1 and 2 did not result in any obvious structural alterations or apoptotic activity within the striatum (Figure 4-8D). However, deletion of HDAC1/2 in striatum resulted in a significant increase in the time spent grooming over a 30 minute testing session (Figure 4-9A) similar to that observed in the conditional HDAC1/2 DKO mice (Figure 4-5A). Additionally, Q-PCR analysis revealed a significant decrease in SAPAP3 expression in AAV-CreGFP mice compared to AAV-GFP control mice (Figure 4-9B).

Conditional MeCP2 KO mice exhibit excessive grooming

Our data so far demonstrates that loss of both HDAC1 and HDAC2 in the striatum results in a down regulation of SAPAP3 expression and excessive grooming. HDAC1 and HDAC2 do not bind DNA directly but rather can participate as part of co-repressor complexes to impact gene transcription. Methyl-CpG-binding protein 2 (MeCP2) is a

transcription factor that has been shown to interact with HDAC1 and HDAC2 in a co-repressor complex and impact gene expression (Jones et al., 1998; Nan et al., 1998). Previous data had shown that mice with loss of MeCP2 in inhibitory forebrain neurons display an excessive grooming phenotype (Chao et al., 2010). The CamKII-Cre93 line used to generate the conditional HDAC1/2 DKO mice expresses Cre recombinase in excitatory neurons in broad forebrain regions as well as striatal medium spiny neurons (Fan et al., 2001). In previous work we had noted that conditional MeCP2 knockout mice that were generated with the CaMKII-Cre93 line had increased grooming. To quantitate the increased grooming following the deletion of MeCP2, we crossed floxed MeCP2 mice with the CaMKII-Cre93 transgenic mice as previously described (Gemelli et al., 2006). We found that the conditional MeCP2 knockouts show a significant increase in time spent grooming (Figure 4-10A) similar to what was seen in conditional HDAC1/2 DKOs (Figure 4-5A) as well as the striatal specific HDAC1/2 deletion mice (Figure 4-9A). Moreover, conditional MeCP2 KO mice have a significant decrease in SAPAP3 expression in the striatum compared to control littermates, suggesting a role for MeCP2 in mediating the defects which occur following the loss of HDAC1 and HDAC2 (Figure 4-10B).

HDAC1/2 and MeCP2 can recruit other proteins to the co-repressor complex, including Sin3a, critical regulator of cellular proliferation and cell cycle maintenance (Nan et al., 1998) (Jones et al., 1998). We generated forebrain conditional Sin3a KO mice and assessed grooming behavior to test whether Sin3a functions with the HDAC1/2/MeCP2 complex to regulate SAPAP3. We found that conditional Sin3a KO mice do not display excessive

grooming, and spend similar time grooming as control littermate mice, suggesting specificity for the HDAC1/2/MeCP2 complex in regulating SAPAP3 (Figure 4-11).

MeCP2 interacts with SAPAP3 at the promoter region

Our results thus far suggest that HDAC1 and HDAC2 may be regulating SAPAP3 expression through their assembly with MeCP2. To investigate whether MeCP2 directly binds to SAPAP3, we performed chromatin immunoprecipitation (ChIP) analysis on several regions of the SAPAP3 promoter (Figure 4-12A). Previous data has shown that MeCP2 selectively binds to methyl CpG sites adjacent to A/T-rich sequences (Maren, 2001). No A/T-rich sequences are found within 600 base pairs of the transcriptional start site, therefore sites for analysis were chosen upstream of the distal promoter region (Figure 4-12A). Moreover, we chose one site on the promoter region that lacks CpG islands and therefore isn't expected to exhibit binding activity to serve as a negative control (P1200). Following ChIP analysis on striatal tissue from conditional MeCP2 KO mice, we found nonspecific binding of MeCP2 to P1600, P1400, and P1300 sites of the SAPAP3 promoter region when compared to P1200. Some binding activity was detected at P600, however values did not reach significance when analyzed against the P1200 control site. However, a significant binding preference of MeCP2 to the P1700 site was measured when compared to P1200, indicating that MeCP2 interacts with SAPAP3 at the promoter region (Figure 4-12B).

Discussion

In the present study we show that a forebrain specific postnatal loss of HDAC1 and HDAC2 leads to compulsive grooming and dysregulation of SAP90/PSD-95-associated protein 3 (SAPAP3), a gene previously linked to the development of obsessive-compulsive disorder (OCD). We find that MeCP2 interacts with SAPAP3 at the promoter region suggesting that HDAC1/2 regulates SAPAP3 through its association with MeCP2.

A striking feature of the conditional HDAC1/2 DKO mice was the occurrence of a prominent facial lesion around 7-8 weeks old. HDAC1/2 DKO and littermate control mice were separated after being weaned from the mother, and single housed to confirm the lesion is self inflicted and not due to fighting amongst cage mates. Human grooming disorders such as pathological nail biting, skin picking, and trichotillomania, are commonly found in persons suffering from OCD (Lenane et al., 1992) . More recent data linked has linked polymorphisms in the human *Sapap3* gene to the development of obsessive-compulsive disorders (Bienvenu et al., 2009) (Zuchner et al., 2009). It has additionally been demonstrated in rodents that Sapap3 knockout mice have excessive grooming resulting in severe facial hair and skin loss that can be attenuated by expressing SAPAP3 in the striatum (Welch et al., 2007). We therefore wanted to determine whether the facial lesion was due to excessive grooming and found conditional HDAC1/2 DKO mice indeed spend significantly longer grooming compared to control littermates. Moreover, the excessive grooming behavior started at 3 weeks of age, a time point preceding the formation of the facial lesion (~7 weeks of age) indicating that the lesion itself is not the cause of the grooming behavior. Interestingly, conditional HDAC1 and HDAC2 single KO mice have normal grooming

behavior indicating that the loss of both genes is necessary for the development of the obsessive compulsive-like behavior. To validate our model of obsessive compulsive-like behavior, conditional HDAC1/2 DKO mice were chronically treated with the SSRI, fluoxetine, to test whether excessive grooming behavior could be alleviated. Following 21 days of injections, grooming behavior in conditional HDAC1/2 DKO mice was normalized to levels comparable to control mice. Our finding that every conditional HDAC1/2 DKO mouse displays excessive grooming resulting in the development of a similar facial lesion led us to examine SAPAP3 expression. SAPAP3 was selectively down-regulated in the in the striatum and cortex of the HDAC1/2 DKO mice, but not in the hippocampus and cerebellum, in agreement with previous studies linking the cortical-striatal circuit for the development of this behavioral phenotype (Welch et al., 2007) (Shmelkov et al., 2010). Evidence has also shown that impairments in NMDA and AMPA mediated neurotransmission at cortico-striatal synapses result in an excitatory/inhibitory imbalance that can account for obsessive compulsive-like behavior (Welch et al., 2007) (Ting and Feng, 2008), however due to apoptotic activity in the cortex of conditional HDAC1/2 DKO mice we were unable to perform these studies. We found that the individual loss of HDAC1 or HDAC2 does not result in dysregulation of SAPAP3 suggesting HDAC1 and 2 together function in regulating SAPAP3 expression.

SAPAP3 is most highly expressed in the striatum (Welch et al., 2004) and while our previous data demonstrated neuronal apoptosis occurs in forebrain regions of conditional HDAC1/2 DKO mice, no cell death was detected in the striatum. We utilized a viral mediated approach to more directly test the potential involvement of HDAC1 and HDAC2 in

downregulating SAPAP3 expression in the striatum and mediating the excessive grooming. We locally deleted HDAC1 and HDAC2 by injecting AAV-CreGFP into the dorsal striatum of adult floxed mice, as this region of the striatum has been implicated in OCD-like behaviors (Welch et al., 2007). We found that this knockdown recapitulates the excessive grooming phenotype and decreases SAPAP3 in this brain region. No cell death occurred following the striatial specific deletion and other behaviors including locomotor activity and anxiety-like behaviors were not impacted, indicating a specific role for HDAC1/2 in the striatum in regulating SAPAP3. Classical learning and memory tasks were not tested in these mice, however we would not expect to see deficits as the deletion of HDAC1/2 was specific to the striatum and these types of memory formation are primarily dependent on the hippocampas and amygdala. These data suggested that HDAC1/2 may be upstream mediators of SAPAP3 expression and likely involves an indirect mechanism, as the loss of HDAC1/2 would be expected to upregulate direct gene targets.

HDAC1/2 and MeCP2 are known to interact (Nan et al., 1998), therefore we hypothesized that MeCP2 may be involved in regulating the excessive grooming and changes in SAPAP3 expression that occurs in conditional DKO mice. Interestingly we found that mice with a forebrain specific conditional deletion of MeCP2 also exhibit excessive grooming and have a decrease of SAPAP3 in the striatum. To determine whether MeCP2 interacts with SAPAP3, we used ChIP assays to test binding of MeCP2 at different binding sites on the SAPAP3 promoter. We found that MeCP2 has a binding preference at a region 1700 base pairs upstream of the transcriptional start site, suggesting that HDAC1/2 regulates SAPAP3 through its assembly with MeCP2.

Taken together, we show that a conditional forebrain specific deletion of HDAC1/2 leads to the unanticipated development of a severe excessive grooming phenotype and self-inflicted facial lesion, as well as dysregulation of the OCD-related gene, SAPAP3. Our data confirms previous findings that a down regulation of SAPAP3 recapitulates OCD-like behavior. To date, studies have focused on delineating how SAPAP3 mediates the mechanisms underlying obsessive-compulsive disorder however there is little data on what regulates SAPAP3. The excessive grooming is due to the loss of HDAC1 and HDAC2 together suggesting a role for MeCP2 in dysregulating SAPAP3 expression. A loss of Sin3A does not result in an excessive grooming phenotype indicating that there is specificity in the complex that regulates SAPAP3 expression. Our findings confirm that a complex of HDAC1/2MeCP2 can function to regulate gene expression and provide insight into the previous findings that MeCP2 loss in striatal inhibitory neurons mediates increased grooming behavior (Chao et al., 2010). Moreover we provide first evidence for HDAC1/2 as an upstream mediator of SAPAP3, and identify SAPAP3 as a novel target gene of MeCP2. As previously noted, a loss of function of MeCP2 leads to the development of Rett (RTT) syndrome; a neurological disorder characterized by repetitive behaviors and distinctive hand stereotypies. There is indeed overlap in the repetitive behaviors displayed by patients suffering from RTT and OCD, giving rise to the possibility that SAPAP3 contributes to the pathophysiology of autistic-like behaviors seen following the loss of MeCP2.

Thus far, selective serotonin reuptake inhibitors (SSRIs) are the only pharmacological treatment known to alleviate some OCD-like symptoms (Vaswani et al., 2003). SSRIs are also used to treat other psychiatric disorders including depression and generalized anxiety

disorder, and therefore offer little specificity in treating the debilitating symptoms directly associated with patients suffering from OCD. The identification of HDAC1/2 as a novel gene associated with obsessive-compulsive-like behavior provides an exciting avenue to explore potential new therapies in treating OCD. A deletion of HDAC1/2 in postmitotic neurons can trigger apoptosis is some neuronal subtypes, however we show that a selective deletion in the striatum mediates an increase in OCD-like behavior. This data is in agreement with previous clinical MRI studies and mouse models of obsessive-compulsive-like behavior implicating the striatum as a key brain region in mediating OCD-like phenotypes (Rosenberg and Keshavan, 1998) (Yang and Lu, 2011) (Wu et al., 2012). It is plausible that the use of HAT inhibitors to suppress acetylase activity within the striatum can serve as a potential therapeutic target in treating OCD-like symptoms. Effective HAT inhibitors have only started to emerge yet one compound, C646, has recently been developed as a specific and potent inhibitor of p300 HAT activity (Bowers et al., 2010). In future studies, it would be interesting to test whether an infusion of a HAT inhibitor in mice lacking HDAC1/2 in the striatum can alleviate excessive grooming and rescue SAPAP3 expression. Taken together current findings demonstrate that HDAC1 and HDAC2 through their association with MeCP2 regulate SAPAP3 expression in the cortico-striatal pathway identifying a novel role for HDAC1/2/MeCP2 in regulating the mechanisms underlying obsessive-compulsive-like behaviors.

Figure 4-1. Conditional HDAC1/2 mice develop a severe facial lesion. Representative images of the facial lesion which occurs in conditional HDAC1/2 DKO mice at approximately 7 weeks of age. Most lesions occur on the face and around the ear, however some lesions occur on top of the head or under the mouth. The phenotype is 100% and every conditional HDAC1/2 DKO mouse develops this lesion.

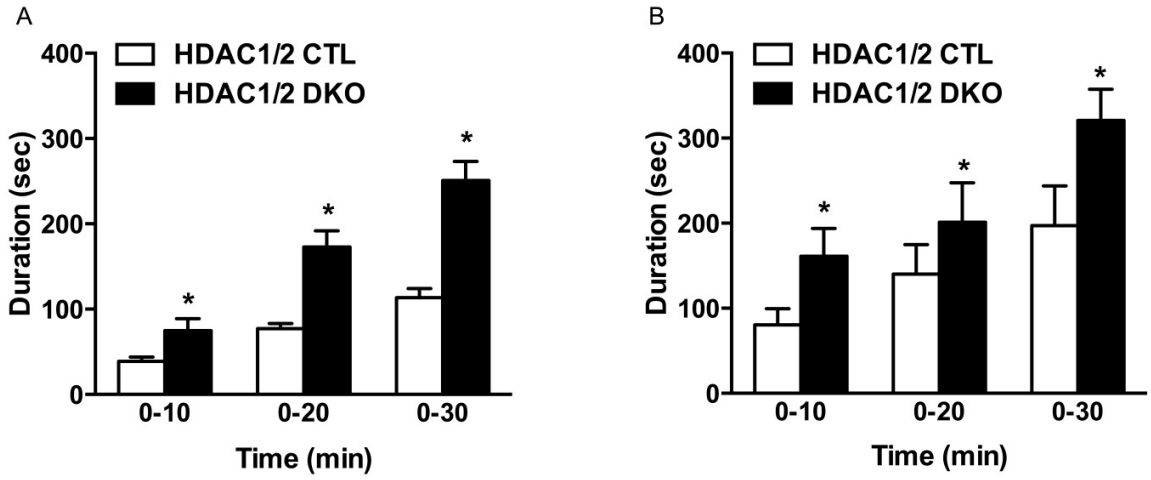

Figure 4-2. Conditional HDAC1/2 mice display excessive grooming behavior. Grooming behavior was assessed by quantifying the total time spent grooming over a 30 minute period. Conditional HDAC1/2 DKO mice spend significantly more time grooming at both 6 weeks (B) and 3 weeks (B) of age compared to littermate CTL mice (3 week old animals CTL n=11; DKO n=9, 6 week old animals CTL n=7; DKO n=6; *$P < 0.05$ by t-test).

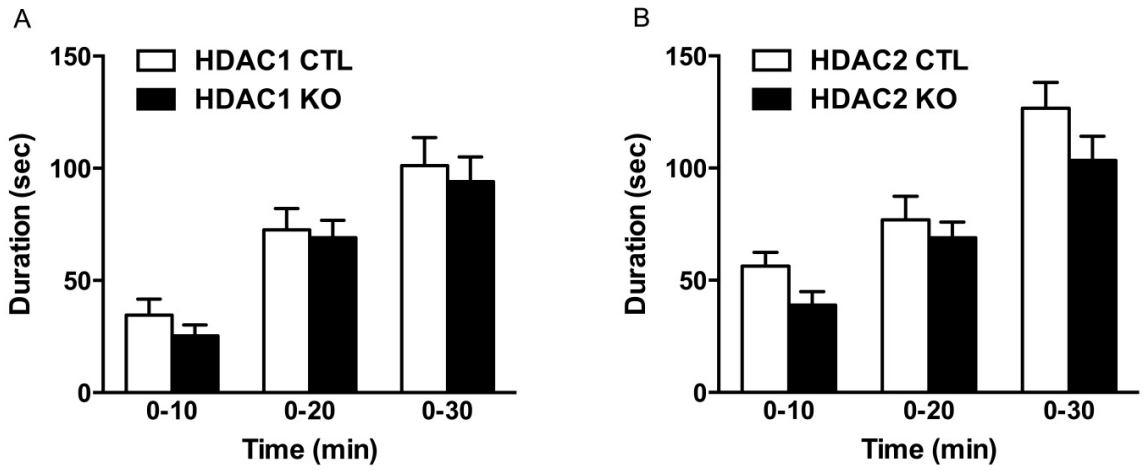

Figure 4-3. Conditional HDAC1 and HDAC2 single KO mice display normal grooming behavior. Grooming behavior was assessed in conditional HDAC1 and HDAC2 single KO mice and the total time spent grooming over a 30 minute testing period was recorded. (A) Conditional HDAC1 KO mice spent approximately the same amount of time grooming as control littermate mice ($n = 6$ mice per group; $^*P < 0.05$ by *t*-test). (B) No significant differences were found in the total amount of time spent grooming between the HDAC2 single KO mice and control littermates ($n = 6$ mice per group; $^*P < 0.05$ by *t*-test).

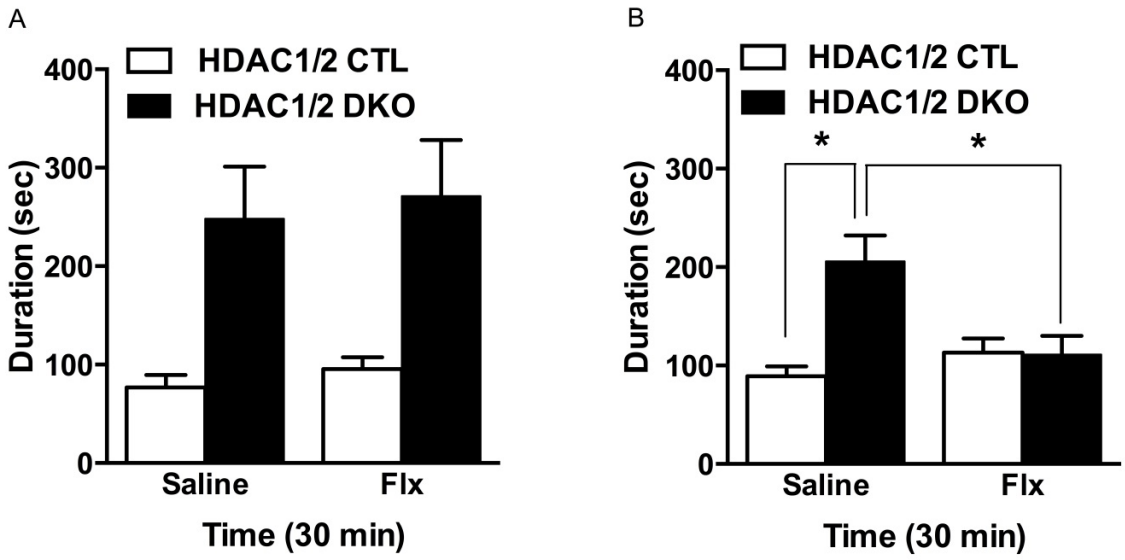

Figure 4-4. 21 days of fluoxetine administration attenuates excessive grooming in conditional HDAC1/2 DKO mice. Conditional HDAC1/2 DKO mice were treated daily with either saline or 10 mg/kg of fluoxetine for 7 or 21 days. (A) Seven day treatment of fluoxetine did not reduce the excessive grooming seen in the conditional HDAC1/2 DKO mice. (B) 21 days of Fluoxetine administration effectively attenuated the grooming phenotype in conditional HDAC1/2 DKO animals to levels comparable to littermate CTL mice (CTL Saline n=5; CTL Flx n=6; DKO Saline n=4; DKO Flx n=6; ANOVA $F_{(3,17)}$ = 7.078 for treatment; Tukey's post hoc analysis for CTL Saline versus DKO Saline P = 0.0024, DKO Saline versus DKO Flx P = 0.01).

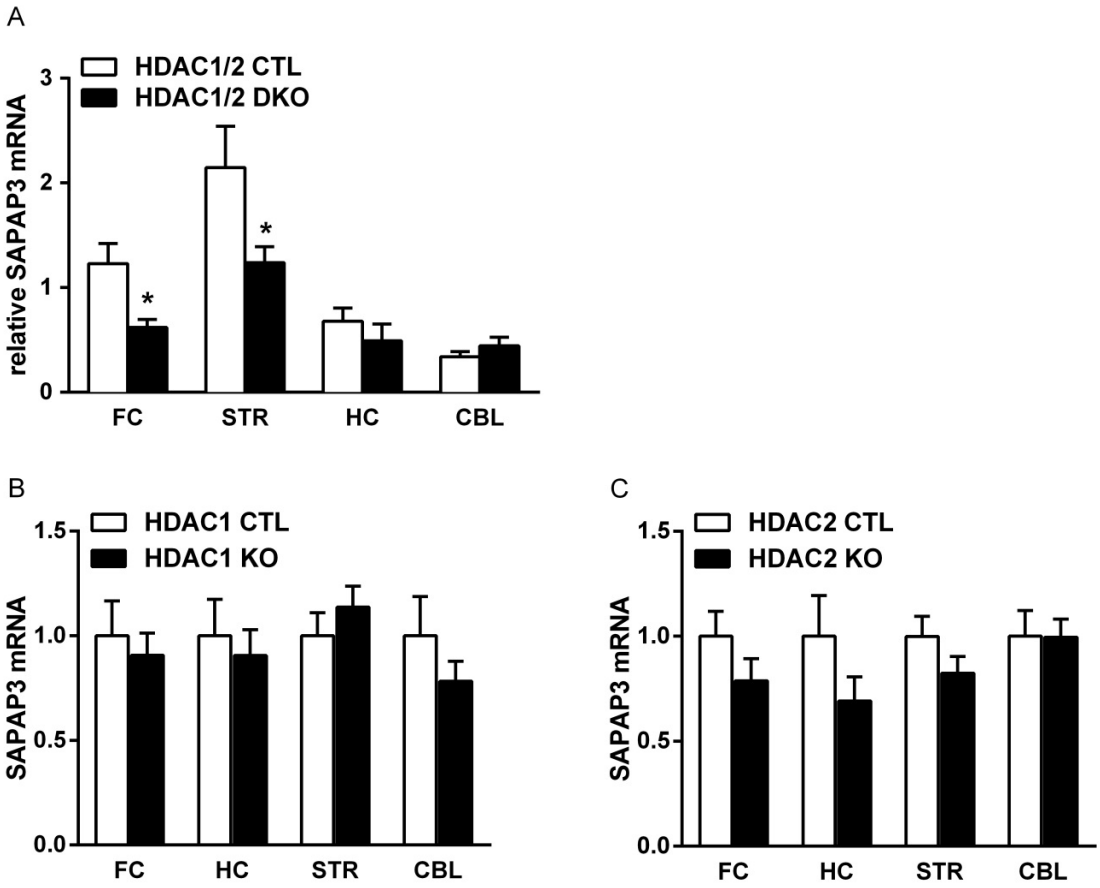

Figure 4-5. SAPAP3 expression is altered in conditional HDAC1/2 DKO mice. (A) Q-PCR analysis showed that mRNA levels of SAPAP3 was significantly down regulated in the frontal cortex (FC) and striatum (STR) of conditional HDAC1/2 DKO mice compared to littermate CTL mice, with no change in expression in the hippocampus (HC) and cerebellum (CBL) (FC CTL n=7; DKO n=6, STR CTL n=6; DKO=7; HC CTL n=8; DKO n=7, CBL CTL n=7; DKO=4; *P < 0.05 by t-test). For single KO mice, Q-PCR analysis showed that mRNA expression levels of SAPAP3 were unchanged in the frontal cortex (FC), hippocampus (HC), striatum (STR), and cerebellum (CBL) of both HDAC1 single KO mice (B) and HDAC2 single KO mice (C) compared to their respective control littermate mice (HDAC1 FC CTL n=6; KO n=6, STR CTL n=6; KO n=6; HC CTL n=5; KO n=6, CBL CTL n=5; KO n=6; HDAC2 FC CTL n=6; KO n=6, STR CTL n=6; KO n=6; HC CTL n=5; KO n=6, CBL CTL n=6; KO n=6; *P < 0.05 by t-test)

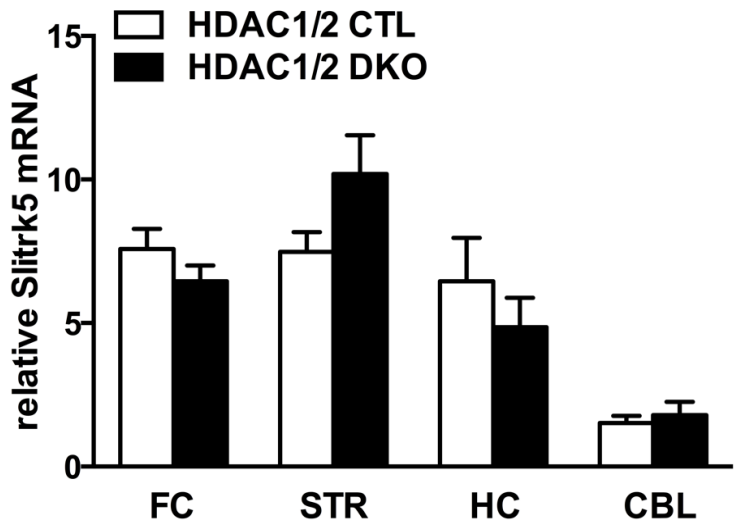

Figure 4-6. Slitrk5 expression is unaltered in conditional HDAC1/2 DKO mice. Q-PCR analysis showed that mRNA levels of Slitrk5 are similar between HDAC1/2 DKO and CTL mice in the frontal cortex (FC), striatum (STR), hippocampus (HC), and cerebellum (CBL) (FC CTL n=8; DKO n=7, STR CTL n=7; DKO=7; HC CTL n=6; DKO n=7, CBL CTL n=7; DKO=4; *$P < 0.05$ by t-test).

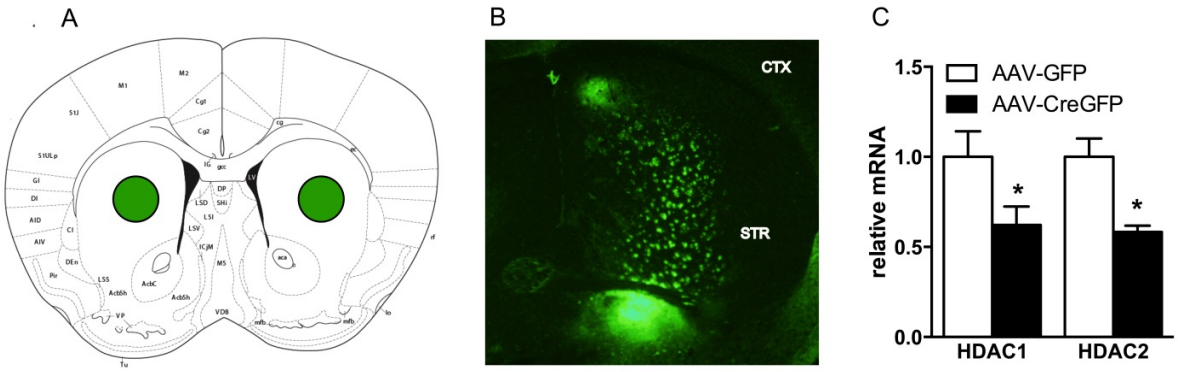

Figure 4-7. Striatal specific deletion of HDAC1/2. (A) Representative diagram of the approximate virus injection site in the dorsal striatum. (B) Striatal sections containing GFP epifluorescence were laser microdissected out and subjected to real-time PCR for quantitation of HDAC1 and HDAC2 mRNA levels. Presented is an example of the coronal section indicating the GFP infected neurons at the injection site. (C) Q-PCR analysis confirmed a significant knockdown of both of HDAC1 and HDAC2 mRNA levels in the striatum of mice which received AAV-CreGFP, in comparison to mice which received AAV-GFP (HDAC1 AAV-GFP *n*=7; AAV-CreGFP *n*=8; HDAC2 AAV-GFP n=*7*; AAV-CreGFP n=*7*; *P < 0.05 by *t*-test).

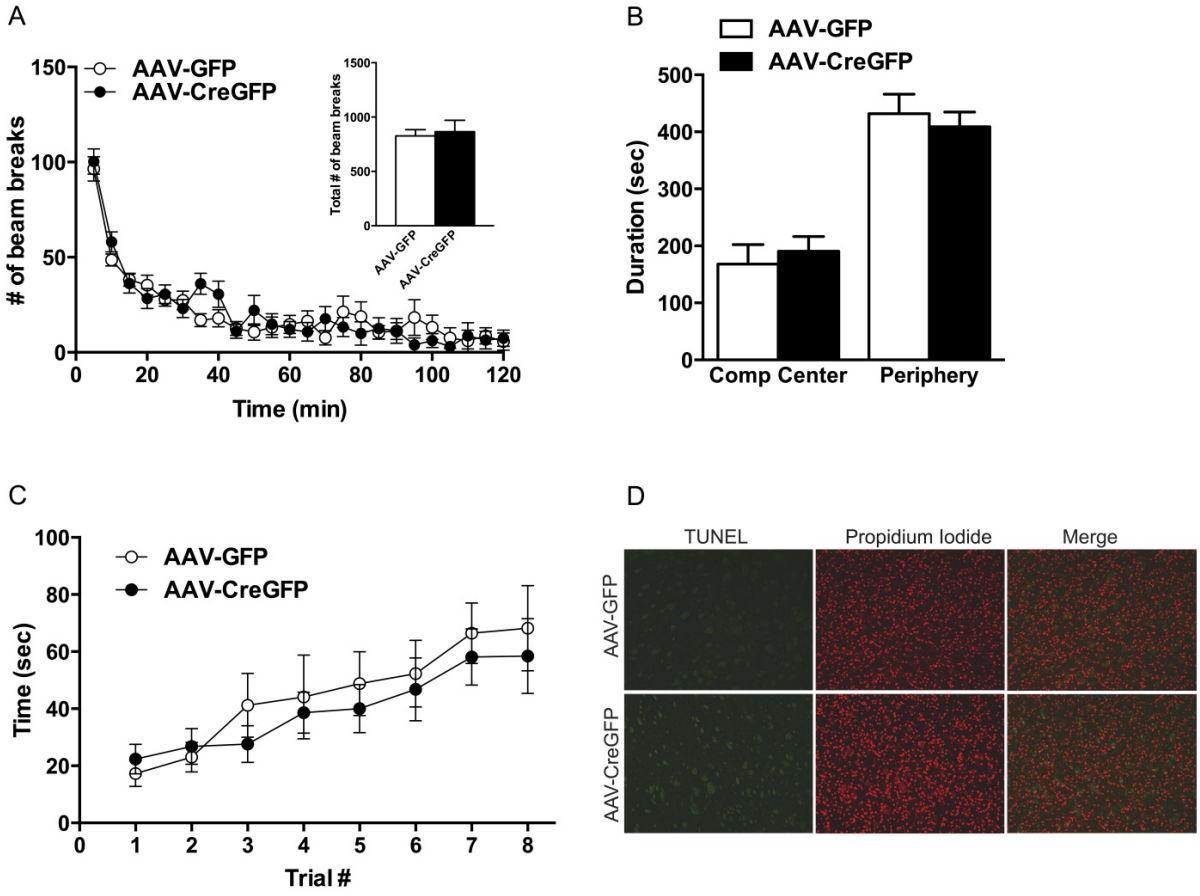

Figure 4-8. Selective loss of HDAC1 and HDAC2 in the striatum does not impact locomotor activity, anxiety, motor coordination, or cell death. (A) Locomotor activity of mice with a striatal specific deletion of HDAC1/2 was measured over a 2 hour testing period in 5 minute increments. The inset represents the total number of beam breaks in the 2 hour testing period. AAV-CreGFP mice showed no deficits in locomotor activity compared to AAV-GFP control mice (AAV-GFP n=9; AAV-CreGFP n=8; *P < 0.05 by t-test). (B) Mice with a striatal specific loss of HDAC1/2 were assessed for anxiety like behavior in open field paradigm. There were no significant differences in the total time spent in the complete center or periphery of the arena between the mice with AAV-CreGFP and AAV-GFP (AAV-GFP n=9; AAV-CreGFP n=8; *P < 0.05 by t-test). (C) Motor coordination was assessed using the rototod test. No significant differences were measured in the amount of time spent on the rod between AAV-CreGFP mice and AAV-GFP control mice, and AAV-CreGFP mice showed improvement over the 8 trials at a similar rate as AAV-GFP control mice (AAV-GFP n=9; AAV-CreGFP n=8; *P < 0.05 by t-test). (D) TUNEL did not reveal any cell death in the striatum following the AAV-mediated deletion of HDAC1 and HDAC2. Both AAV-CreGFP and AAV-GFP groups show no TUNEL positive cells indicating an absence of apoptotic activity.

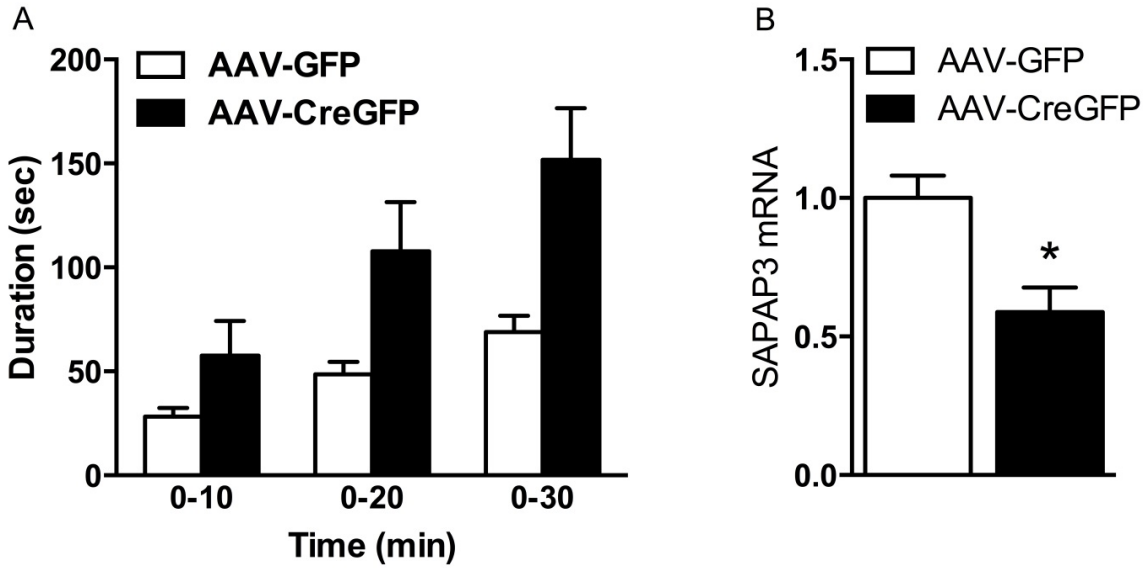

Figure 4-9. Deletion of HDAC1/2 in the striatum leads to excessive grooming and dysreguation of SAPAP3. (A) Mice which received AAV-CreGFP spent significantly more time grooming compared to mice injected with AAV-GFP, recapitulating a similar phenotype observed in conditional HDAC1/2 DKO mice (AAV-GFP n=6; AAV-CreGFP n=6; *P < 0.05 by t-test). (B) Striatal mRNA expression of SAPAP3 in the animals injected with AAV-CreGFP was significantly reduced compared to those with AAV-GFP (AAV-GFP n=6; AAV-CreGFP n=6; *P < 0.05 by t-test).

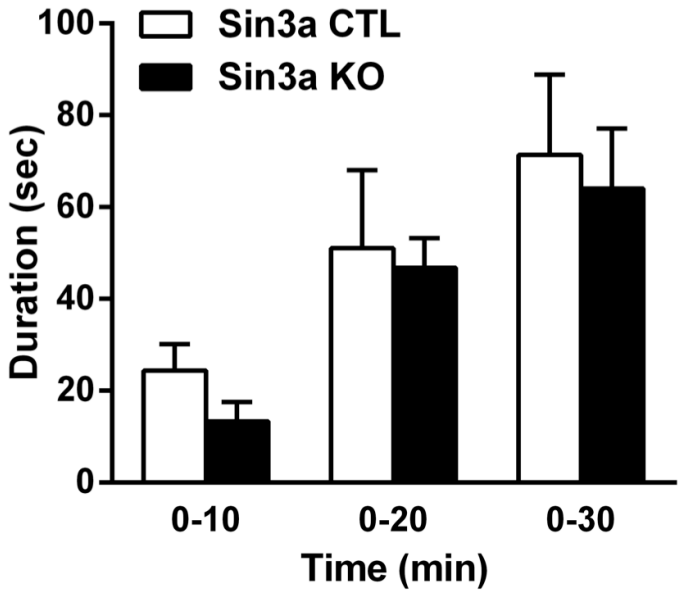

Figure 4-10 Conditional Sin3a KO mice display normal grooming behavior. Grooming behavior was assessed in conditional Sin3a mice and the total time spent grooming over a 30 minute testing period was recorded. Conditional Sin3a mice spent approximately the same amount of time grooming as control littermate mice (CTL n=3; DKO n=4; $*P < 0.05$ by *t*-test).

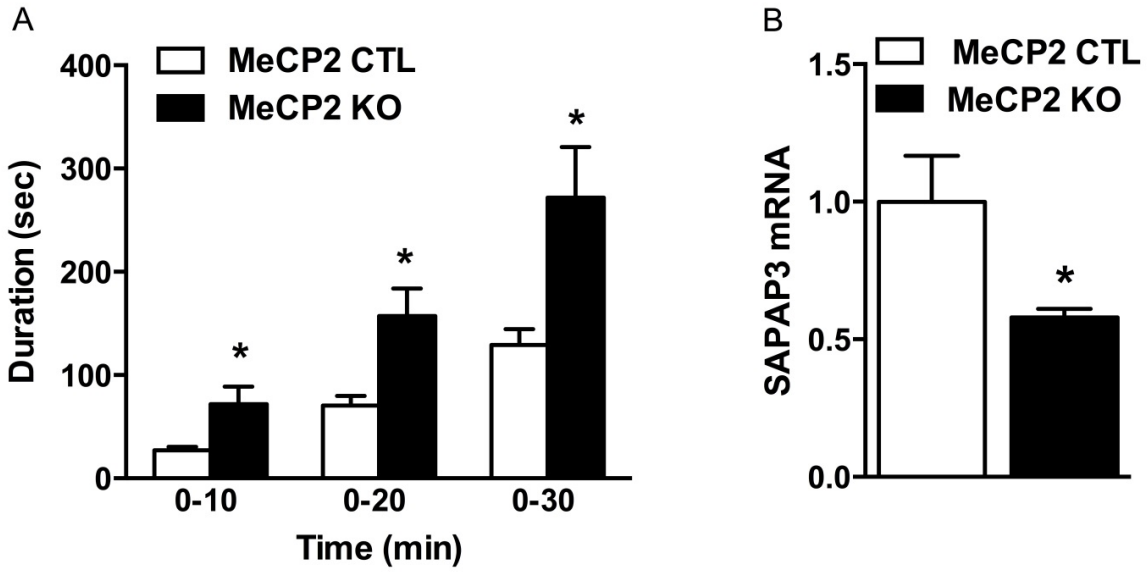

Figure 4-11. Conditional MeCP2 KO mice display excessive grooming and dysregulation of SAPAP3. (A) Conditional MeCP2 KO mice spend significantly more time grooming over the 30 minute testing session compared to littermate CTL mice (CTL n = 15; KO n = 12; *P < 0.05 by t-test). (B) Q-PCR analysis shows that SAPAP3 mRNA expression in the striatum of MeCP2 KO mice is significantly reduced compared to CTLs (n = 4 mice per group; *P < 0.05 by t-test).

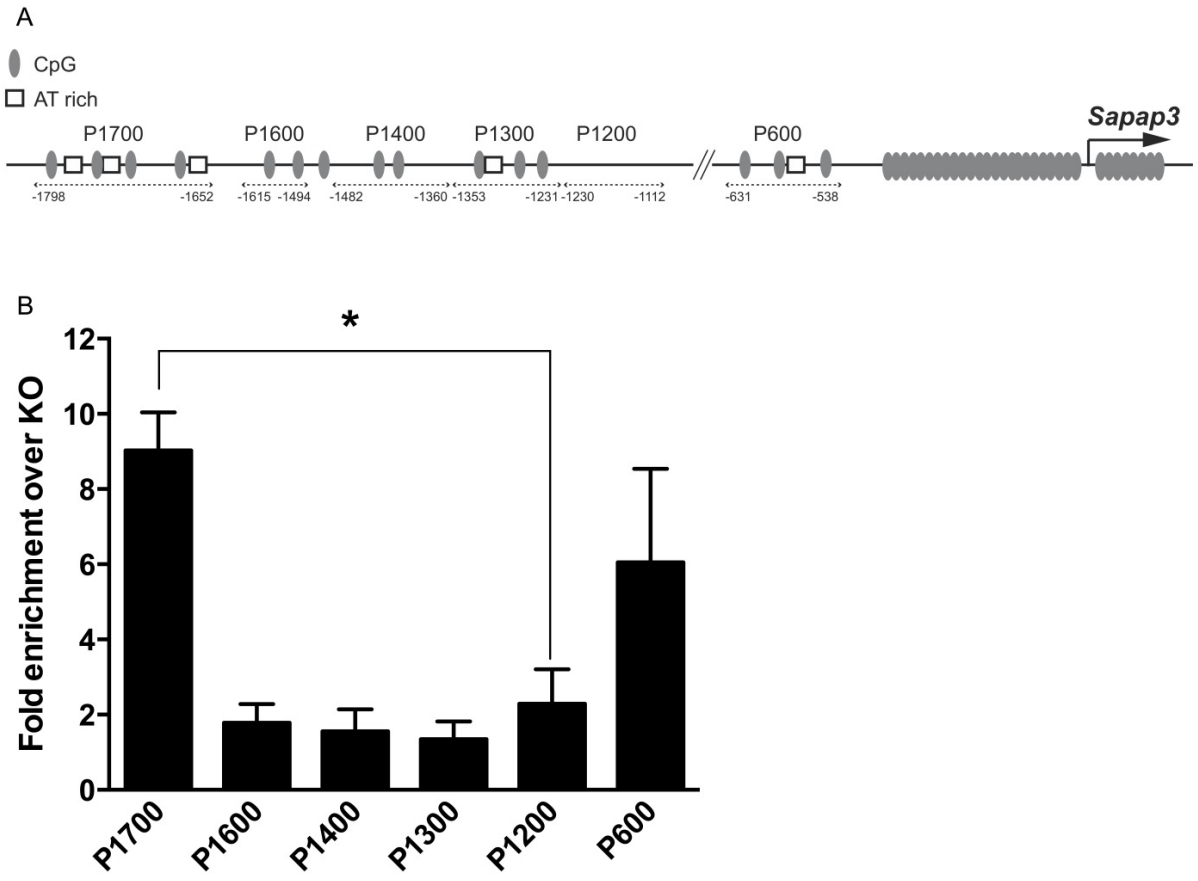

Figure 4-12. MeCP2 interacts with SAPAP3 at the promoter region. (A) Schematic of Sapap3 gene promoter. Dotted lines with arrow heads indicate the regions amplified for MeCP2 ChIP. Gray ovals represent CpG di-nucleotides. An AT-rich sequence depicted by a rectangle contains more than 4 A/T nucleotides in a row. (B) MeCP2 occupancy was examined in 6 regions of the Sapap3 promoter as well as Bdnf promoter IV, which is known to be bound by MeCP2. Note that P1200 region does not contain CpG sites; thus, serving as a negative control. The data was presented as fold enrichment in comparison to MeCP2 KO samples, showing significant MeCP2 occupancy in the P1700 region in comparison to that in the P1200 (n = 3 mice per group; *$P < 0.05$ by t-test).

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The preceding chapters describe our recent observations that define a role for the Class I histone deacetylases (HDACs), HDAC1 and HDAC2, in postnatal development and neuronal homeostasis. These data add to the growing knowledge about HDACs and their role as global epigenetic regulators but also demonstrate more specific roles for these individual HDACs. While our current findings extend our understanding of how HDAC1 and HDAC2 regulate learning and memory and provide insight into a novel role for HDAC1/2 in mediating obsessive-compulsive-like behaviors, there is much to delineate regarding the mechanisms underlying HDAC1/2 function as they pertain to these complex behaviors.

Class I HDACs are regulators of learning and memory mechanisms, however until recently, most previous data in support of this hypothesis stem from pharmacological studies which offer little insight to individual roles of different HDACs (Levenson et al., 2004) (Vecsey et al., 2007) (Barrett and Wood, 2008). We thus used a genetic strategy and began by investigating a role for HDAC1 in mature neurons to determine whether a postnatal deletion impacts behavior, specifically learning and memory. Mice with a constitutive deletion of HDAC1 are not viable postnatally (Lagger et al., 2002) (Montgomery et al., 2007) therefore we generated conditional forebrain specific HDAC1 knockout (KO) mice to carry out an extensive behavioral characterization. Conditional HDAC1 KO mice were viable with no differences in weight or locomotor activity compared to littermate controls. We found that the loss of HDAC1 alone does not impact any of the following types of behavior:

anxiety, motor coordination, social interaction, reflex tests, and depressive-like behavior. Moreover, HDAC1 KO mice have no impairments in learning and memory as assessed by the fear conditioning paradigm, in agreement with previous data that an embryonic deletion of HDAC1 does not result in any overt phenotypes and that overexpressing HDAC1 did not lead to changes in learning and memory behavior (Montgomery et al., 2009) (Guan et al., 2009). Taken together, our findings indicate a non-redundant role for HDAC1 in postnatal development and suggest that other HDACs within the Class I family regulate the learning and memory enhancements described in pharmacological studies.

It is important to note a recent study which showed that HDAC1 acts as a positive regulator of extinction learning in the fear conditioning paradigm (Bahari-Javan et al., 2012). The authors showed that a viral mediated overexpression of HDAC1 into the dorsal hippocampus facilitates extinction of contextual fear memories without affecting other cognitive abilities. Since we did not see any deficits in context or cue dependent fear conditioning in conditional HDAC1 KO mice, we did not perform detailed analysis on fear extinction. However, given the recent findings from Bahari-Javan et al., it would be interesting to test whether the loss of HDAC1 in mature neurons impairs extinction of fear memories in future experiments.

In contrast to the HDAC1 impact on behavior, we found that the postnatal deletion of HDAC2 in forebrain neurons impacts learning and memory behavior. Very recent data described a role for HDAC2 as a positive regulator of learning and memory, however these studies were carried out using mice lacking HDAC2 during the time of embryogenesis (Guan et al., 2009). Our lab was interested in looking outside the window of neuronal proliferation

to understand the role of HDAC2 in mature neurons. Conditional HDAC2 KO mice were generated using the same breeding strategy as HDAC1 KOs, and mice were viable with no gross impairments compared to littermate control mice. Conditional HDAC2 KO mice were tested in baseline behavioral measures, and no differences were detected in locomotion or anxiety-like behavior. However, as with mice with an embryonic deletion of HDAC2, conditional HDAC2 KO mice have enhancements in LTP as well as improved learning in context and cue dependent fear conditioning. Interestingly, conditional HDAC2 KO mice also have accelerated extinction of fear responses and conditioned taste aversion suggesting a more specific role for HDAC2 in mature neurons in regulating associative learning (Morris et al., 2013). Collectively with our previous results, we identify distinct roles for HDAC1 and HDAC2 in regulating mechanisms related to cognitive function.

Several downstream targets have been identified as potential genes which may contribute to the mechanism by which HDAC2 regulates learning and memory behaviors. HDACs do not bind directly to DNA but rather are recruited to target genes via their direct association with transcriptional activators and repressors and are typically incorporated into large transcriptional complexes to mediate gene expression. One such transcription factor is MeCP2, an important regulator of synaptic transmission that interacts with a co-repressor complex that contains HDAC1/2 (Nan et al., 1998). We examined protein levels of MeCP2 in both conditional HDAC1 and HDAC2 KO mice yet found no differences in expression in either line of mice. These data are in agreement with previous *in vitro* findings from our lab that following HDAC inhibition in mature hippocampal cultures, MeCP2 protein is not altered (Akhtar et al., 2009). The previously described study from Guan et al. additionally

found that HDAC2 binds to several genes implicated in memory formation and synaptic plasticity including *BDNF Egr1, Fos, Cpg15, Camk2a, Creb1, Crebbp, NRXN3* and the NMDA receptor subunits, suggesting HDAC2 suppresses the expression of synaptic remodeling and plasticity genes. In future studies, it would be interesting to examine whether expression of these genes are down regulated in conditional HDAC2 KO mice. BDNF is a known target of MeCP2-dependent transcription. Additionally, BDNF signaling can trigger S-nitrosylation of HDAC2 which induces its release from chromatin and results in an increase in transcription of genes associated with neuronal development (Nott et al., 2008), therefore it may have a critical role in HDAC2 mediated regulation of memory formation. Moreover, HDAC2 can come together with different co-repressors such as Sin3a, CoREST, and NuRD to control gene expression, therefore additional binding studies will be necessary to delineate the exact mechanism of how HDAC2 regulates signaling pathways associated with learning and memory in mature neurons.

Based on the findings detailed above that HDAC2 can act as a negative regulator of learning and memory mechanisms, attention has been focused on developing selective HDAC2 inhibitors as therapeutic drugs in treating diseases related to neurodegeneration and cognitive impairment (Fischer et al., 2010) (Graff and Tsai, 2013b). A critical concern is successful development of a compound selective for HDAC2 that doesn't also target HDAC1 due to the high degree of similarity of approximately 85% between HDAC1 and HDAC2. Previous work described redundant roles for HDAC1/2 in the brain when deleted embryonically in mice with death at postnatal day 7 (Montgomery et al., 2009), suggesting that targeting both HDAC1 and HDAC2 has detrimental effects. To test a more clinically

relevant model of HDAC inhibition, we examined whether a postnatal deletion of both HDAC1 and HDAC2 in mature neurons enhances learning and memory in the same manner as the deletion of HDAC2 alone. HDAC1 is most highly expressed early in development in progenitor cells and primarily limited to glial cells in mature neurons (MacDonald and Roskams, 2008). With no measured effects following a postnatal deletion of HDAC1 alone we hypothesized that postnatal loss of both HDAC1/2 would recapitulate the phenotypes observed in conditional HDAC2 KO. Interestingly we found that conditional HDAC1/2 double knockout (DKO) mice die are viable, yet die at approximately 9 weeks old. We concluded that early lethality was due to apoptosis in the cortex and hippocampus shortly after Cre induction, however the precise age when cell death is triggered remains unclear. A limitation in performing more detailed histological analysis to determine the precise timeframe of apoptosis is the difficulty in breeding double knockout mice with a shortened lifespan. Several cohorts of mice were necessary for molecular and behavioral studies however in future experiments it would be interesting to perform a time course of TUNEL analysis on conditional HDAC1/2 DKO to determine how quickly apoptosis occurs once the genes are deleted. Anxiety-like behavior and hypoactivity is detected as early as 3 weeks old in conditional HDAC1/2 DKO mice, therefore it is important to understand whether the behavioral deficits are truly due to the loss of the genes or consequences of early cell death. We confirmed a significant deletion of HDAC1/2 in amygdala by Western blot, a brain region associated with anxiety (Campeau and Davis, 1995) (Maren and Fanselow, 1995) (LeDoux, 2007), however did not detect cell death in the amygdala at the time point (8 weeks) we examined. We conclude that the heightened anxiety is due to disruption of

HDAC1/2 however additional TUNEL analysis at different time points would further strengthen this hypothesis. Moreover, other behavioral measures of anxiety such as the elevated plus maze and dark/light tests would provide further support for an anxiety-like phenotype in conditional HDAC1/2 DKO mice as more cohorts become available in the future.

A significant finding of our study is that the loss of HDAC1 and HDAC2 together leads to behavioral impairments and impacts neuronal survival, highlighting critical caveats associated with the use of HDAC2 inhibitors to treat neurological diseases associated with learning and memory impairments. No selective compounds currently exist, as the structure of HDAC2 is nearly identical to HDAC1 making it difficult to develop HDAC2-specific inhibitors (Graff and Tsai, 2013a). An embryonic or postnatal loss of HDAC1 alone does not impact behavior or cell survival, therefore the prevailing dogma has been that HDAC2 selective compounds which could also target HDAC1 would have negligible consequences. Our findings also provide critical insight to the potential problems associated with loss of both HDAC1 and HDAC2 and provide strong support for the importance of cautiously targeting subtype selective isoforms as possible off target effects may be harmful.

Due to early lethality of conditional HDAC1/2 DKO mice, we speculate that there are no compensatory effects of other HDACs within the Class I family, however in future studies it would be interesting to examine whether other Class I HDACs are dysregulated following the loss of HDAC1/2. We did show that there was no significant change in HDAC2 or HDAC1 in conditional HDAC1 and HDAC1 single KO mice, respectively. We did not examine expression of other Class I HDACs in the single KO mice, yet recent work has

demonstrated that selective inhibition of HDAC3 in the CA1 sub-region of the hippocampus is sufficient to enhance long-term memory in object recognition tasks (McQuown et al., 2011). We are currently breeding forebrain specific conditional HDAC3 KO mice in the lab for future studies to test whether these animals have enhancements in learning and memory behaviors and synaptic plasticity, as we were unable to perform these experiments in conditional HDAC1/2 DKO mice. Furthermore, our lab has previously shown that mice with a postnatal forebrain specific KO of HDAC4 impairs memory formation and LTP with no impact seen following a deletion of HDAC5 (Kim et al., 2012). Taken together, these data provide interesting insight on dissociable roles for different classes of HDACs in regulating learning and memory mechanisms, and further highlight potential limitations of the use of broad-acting HDAC inhibitors to treat cognitive impairment.

A significant and unexpected phenotype in conditional HDAC1/2 DKO mice was the formation of a prominent lesion on the face around 7 weeks of age, shortly before animals die. It closely resembled that of a previously published mouse model of obsessive-compulsive disorder (OCD) (Welch et al., 2007) leading us to investigate the nature of this lesion, which was 100% penetrant in all DKO mice. We concluded that the lesion was indeed due to compulsive grooming behavior measured in HDAC1/2 DKO mice, with littermate CTLs unaffected. An important consideration is that the excessive grooming could be due to impaired sensation or nociception. We were unable to test HDAC1/2 DKO mice for footshock sensitivity to rule out this possibility due to the stressful nature of the test and the young age of the animals, however future studies could be carried out using a hotplate test which may be more tolerable in younger mice. There are other measures of repetitive

behavior such as the marble burying task, which has previously been implicated in anxiety and obsessive-compulsive-like behavior (Deacon, 2006) (Chadman et al., 2009). However, marble burying is more reliable as a measure of anxiety like phenotypes and other mouse models of OCD do not necessarily have enhancements in this test. Conditional HDAC1/2 DKO mice show excessive grooming as early as 3 weeks old indicating a very robust obsessive-compulsive-like phenotype and therefore were not tested in marble burying behavior. Moreover, the excessive grooming phonotype was attenuated following chronic treatment with fluoxetine, providing validity for our model of OCD behavior.

Since we were able to confirm that the facial lesion was a result of excessive grooming, we next carried out molecular studies to further understand how HDAC1/2 functions to regulate obsessive-compulsive-like behavior. We found a selective down regulation of SAP90/PSD-95-associated protein 3 (SAPAP3), a key protein linked to the development of OCD (Welch et al., 2007), in the cortex and striatum. To ensure that changes in SAPAP3 expression were specific to the loss of HDAC1/2 we also measured expression of another gene lined to obsessive-compulsive-like behavior, SLIT and NTRK-like protein-5 (Slitrk5). Targeted inactivation of Slitrk5, a neuron-specific transmembrane protein, also results in a self-inflicted facial lesion due to compulsive grooming (Shmelkov et al., 2010), however we found no significant changes in Slitrk5 expression in conditional HDAC1/2 mice. The first known gene implicated in the pathogenesis of OCD was *Hoxb8,* a member of the mammalian *Hox* (Homeobox-containing) complex and an important transcription factor that functions during early development in pattern formations of the anterior-posterior axis (Capecchi, 1997) (Greer and Capecchi, 2002). Mice with a loss of function of *Hoxb8* were found to

have an excessive grooming phenotype that leads to facial skin loss, however it these mice were not tested for responsiveness to fluoxetine treatment (Greer and Capecchi, 2002). We therefore did not examine expression of this gene in conditional HDAC1/2 DKO mice as Hoxb8 mutant mice show less validity as a mouse model of ODC compared to SAPAP3 and Slitrk5 mutant mice. Due to the neuronal apoptosis observed in conditional HDAC1/2 DKO mice we hypothesized that dysregulation of genes related to cell cycle maintenance and cell death may occur. We therefore examined mRNA levels of Cyclin-dependent kinase 5 (Cdk5), a gene linked to cell toxicity in models of neurodegeneration (Ohshima et al., 1996) (Tsai et al., 2004) and surprisingly found no changes in Cdk5 expression in any brain regions, recapitulating the specificity of the changes we see in SAPAP3 expression following the loss of HDAC1/2. These results suggest that the selective decrease of SAPAP3 in the cortex and striatum is a result of targeted inhibition of HDAC1 and HDAC1 and not due to global transcriptional repression, and seems to occur independently of previously noted apoptosis.

The striatum has been heavily implicated in regulating obsessive-compulsive related behaviors (Ting and Feng, 2008) (Wu et al., 2012) and SAPAP3 in the only member of the SAPAP family that is highly expressed in the striatum (Welch et al., 2004). Interestingly we found no evidence of cell death in the striatum of HDAC1/2 DKO mice leading us to hypothesize that HDAC1 and HDAC2 specifically in the striatum may contribute to the excessive grooming phenotype observed in conditional DKOs. We used a viral mediated approach to generate mice with a striatal specific deletion of HDAC1 and HDAC2 and found that these mice recapitulate the compulsive grooming phenotype as well as the dysregulation

of SAPAP3 seen in conditional HDAC1/2 mice. No cell death occurred in the striatum following the deletion of HDAC1/2 and other behaviors were not impacted suggesting specificity for HDAC1 and HDAC2 in regulating the OCD-like behavioral and molecular changes observed in conditional HDAC1/2 DKO mice. In the future, it may be important to determine whether chronic treatment with fluoxetine can also alleviate the excessive grooming in mice with a striatal deletion of HDAC1/2 to understand whether differences exist between grooming behavior in young animals (conditional HDAC1/2 DKOs) versus adult animals (mice with a striatal deletion of HDAC1/2).

We were interested to further examine how HDAC1/2 indirectly regulates SAPAP3, and hypothesized that methyl-CpG binding protein 2 (MeCP2) may be involved since as previously noted, HDAC1/2 and MeCP2 are known to come together in a complex to control gene transcription (Nan et al., 1998). Our lab previously characterized conditional MeCP2 KO mice and found that a deletion of MeCP2 in the forebrain is sufficient to recapitulate many phenotypes seen in Rett syndrome patients (Gemelli et al., 2006). We showed that conditional MeCP2 KOs have heightened anxiety, however these mice were not tested for other OCD-related behaviors. Recent work has shown that loss of MeCP2 in GABAergic neurons leads to the development of repetitive behaviors (Chao et al., 2010). Interestingly we found that conditional MeCP2 KO mice self-groom excessively compared to littermate controls, and show a similar decrease in SAPAP3 as mice lacking HDAC1/2. We further concluded from ChIP studies that MeCP2 binds SAPAP3 at the promoter region, suggesting a novel role for the HDAC1/2/MeCP2 complex in regulating SAPAP3. Sin3a, a protein shown to be critical in cellular proliferation and cell cycle regulation, is also a known

component of the HDAC1/2/MeCP2 complex (Nan et al., 1998) (Jones et al., 1998). To determine whether Sin3a contributes to regulation of obsessive-compulsive behaviors, we generated conditional Sin3a KO mice and assessed grooming behavior. Sin3a KO mice spend equal amounts of time grooming compared to control littermate mice. Collectively these findings suggest that the obsessive-compulsive-like phenotypes and dysregulation of SAPAP3 in HDAC1/2 DKO mice are not due to the transcriptional repressor complex containing Sin3a but rather the assembly of HDAC1/2 and MeCP2 via a different mechanism. HDAC1/2 and MeCP2 have generally been known to form a repressor complex that silences gene transcription, therefore it remains unclear how a complex of HDAC1/2/MeCP2 can function to upregulate a downstream target gene. Studies have alluded to the idea that both HDACs and MeCP2 can function as transcriptional activators as well as repressors (Nusinzon and Horvath, 2005) (Chahrour et al., 2008), however additional experiments are necessary to further examine this possibility.

We were unable to perform overexpression studies with SAPAP3 as those described in Welch et al. in conditional HDAC1/2 DKO mice due to early lethality, however MeCP2 KO mice are viable through adulthood. Studies are currently underway to overexpress SAPAP3 in conditional MeCP2 KO mice to determine if compulsive grooming can be rescued. Another limitation we encountered with conditional HDAC1/2 DKO mice due to early apoptosis was inability to perform electrophysiological experiments to examine synaptic transmission. Accumulating evidence has suggested a role for glutamate signaling in the striatum as a critical regulator of mechanisms underlying obsessive-compulsive disorder (Saxena and Rauch, 2000) (Ting and Feng, 2008). Excitatory inputs from the cortex are

disrupted in the striatum of Slitrk5 and SAPAP3 mutant mice establishing an important role for the cortico-striatal circuit in mediating OCD-like behavior (Welch et al., 2007) (Shmelkov et al., 2010). More specifically, hyperactivity of the glutamatergic system in Slitrk5 and SAPAP3 knockout mice is specific to NMDA-mediated signaling. For future experiments it would be interesting to measure striatal neurotransmission in striatal specific HDAC1/2 DKO mice and conditional MeCP2 KO mice to test whether NMDA-mediated synaptic transmission is disrupted in order to further our understanding of how HDAC1/2 and MeCP2 contribute to the neurocircuitry related to obsessive-compulsive disorder.

In conclusion, the current research has furthered our understanding of the complex role of HDAC1 and HDAC2 in regulating postnatal development, learning and memory mechanisms, and obsessive-compulsive-like behaviors. These studies provide novel insight into mouse models of neuropsychiatric disorders and pose many exciting questions critical to the field of epigenetics. We hope that this research will challenge the field to further explore these questions and expand our knowledge regarding the role of HDAC1 and HDAC2 and behavioral and neuronal homeostasis.

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