

EFFECTS OF A NOVEL, NON-TOXIC HISTONE DEACETYLASE INHIBITOR ON  
HIPPOCAMPAL MEMORY FORMATION, HISTONE ACETYLATION, AND BDNF  
GENE EXPRESSION IN MALE MICE

by

Sarah Beamish

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## ABSTRACT

### EFFECTS OF A NOVEL, NON-TOXIC HISTONE DEACETYLASE INHIBITOR ON HIPPOCAMPAL MEMORY FORMATION, HISTONE ACETYLATION, AND BDNF GENE EXPRESSION IN MALE MICE

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The University of Wisconsin-Milwaukee, 2021  
Under the Supervision of Dr. Karyn Frick

Memory dysfunction is a common symptom of aging, neuropsychiatric disorders, and neurodegenerative disorders, yet truly effective treatments for memory loss do not exist. *De novo* gene transcription is a molecular requirement for long-term memory formation. The transcription of genes related to synaptic plasticity and learning is regulated in part by histone acetylation, an epigenetic mechanism that regulates chromatin accessibility. Pharmacological compounds that maintain histone acetylation, called histone deacetylase inhibitors (HDACi), enhance memory by preventing deacetylation of core histone proteins, which initiates binding of transcriptional machinery to open chromatin. Therefore, HDACi are potentially promising therapeutics that could be used to prevent or delay memory loss associated with aging and other disorders. However, existing HDACi have poor solubility and undesirable toxicity. We collaborated with Drs. Mahmum Hossain and Doug Steeber of the University of Wisconsin-Milwaukee to develop a novel brain-penetrant HDACi compound, MJM-1, that shows minimal toxicity and is capable of crossing the blood brain barrier. The goal of this study was to determine the extent to which MJM-1 enhances spatial and object recognition memory consolidation, alters hippocampal histone acetylation, and modifies

hippocampal *bdnf* expression in male mice. Here, we demonstrate that post-training i.p. administration of MJM-1 enhances hippocampal-dependent spatial, but not object recognition, memory consolidation in male mice. Interestingly, however, we observed no treatment effects of MJM-1 on hippocampal histone acetylation when administered alone or immediately following object training. Finally, we also demonstrated that MJM-1 did not alter hippocampal expression of *bdnf* exons I or IV. Our results suggest that MJM-1 likely enhances spatial memory consolidation by affecting acetylation states of nonhistone proteins. These results underscore the need to better understand the ways in which systemic HDACi administration affects numerous other nonhistone substrates that can also promote beneficial effects on cognition.

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## LIST OF ABBREVIATIONS

BDNF	Brain derived neurotrophic factor
DH	Dorsal hippocampus
DMSO	Dimethyl sulfoxide
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
I.p.	Intraperitoneal
NaBu	Sodium butyrate
OP	Object placement
OR	Object recognition

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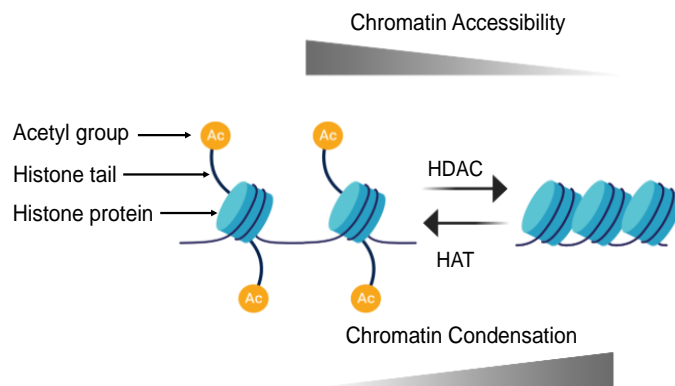
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## INTRODUCTION

### Chromatin and Histone Acetylation

The term epigenetics has historically been used to describe the ways in which genes interact with their environment to produce a particular phenotype (Waddington 1942). Epigenetic modifications serve to regulate experience-dependent changes in gene expression that are independent of the actual genome sequence itself (Bird 2007). Chromatin is composed of DNA that is wrapped around a core histone octamer which contains two copies each of the histone proteins H2A, H2B, H3, and H4. Epigenetic modifications serve to regulate the compaction and relaxation of chromatin by modifying the strength of interactions between DNA and core histone proteins (Kim & Kaang, 2017). One of the most well studied epigenetic alterations that alters learning and memory is histone acetylation, a process in which acetyl groups are added to lysine residues on the N-terminal tails of histone proteins (Figure 1). The positive charges of unmodified histone proteins promote a tight interaction with negatively charged DNA which promote a transcriptionally unfavorable state,

as transcriptional machinery is unable to bind (Bannister & Kouzarides, 2011). Histone acetylation is enzymatically controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). The addition of acetyl groups by



**Figure 1. Histone acetylation and deacetylation is mediated by HATs and HDACs, respectively.** In eukaryotic cells, DNA is wrapped around core histone proteins. The N-terminal tails of histone proteins can become acetylated at specific lysine residues by HATs, which increase chromatin accessibility. HDACs remove acetyl groups and increase compaction of chromatin.

HATs weakens the electrostatic affinity between histones and negatively charged DNA, effectively relaxing the chromatin structure so that it is more permissive for gene transcription. Conversely, the removal of acetyl groups by HDACs restores the strong electrostatic affinity between histone and DNA, effectively repressing gene transcription (Strahl & Allis, 2000). Therefore, chromatin is considered to be a highly plastic substrate that is capable of responding to changes in neuronal signaling that support cognition (Haggarty & Tsai, 2011).

### *Established Classes of Histone Deacetylases and Their Inhibitors*

The superfamily of HDACs consists of eighteen HDAC isoforms categorized into four classes: class I, II, IV, and the structurally distinct class III (Gregorette et al., 2004). The classes of HDACs differ among their catalytic sites, expression across cell types, as well as their subcellular localization (Penney & Tsai, 2014). Class I, II, and IV HDACs are zinc ( $Zn^{2+}$ )-dependent enzymes, whereas class III HDACs are nicotinamide adenine dinucleotide ( $NAD^+$ )-dependent enzymes. The  $Zn^{2+}$ - and  $NAD^+$ -dependent catalytic sites play a crucial role in targeting HDACi to the active site of HDACs, and thus, are central pieces of the scaffold in designing isoform-specific HDACis (Zhang et al., 2018). Class I HDACs (HDAC1, 2, 3, and 8) are primarily found within the nucleus and are ubiquitously expressed throughout cell types, with the exception of HDAC8, which is largely muscle-specific (Waltregny et al., 2005; Xu et al., 2011) Interestingly, class I HDACs are extensively expressed in neurons within the rat hippocampus, amygdala, and other cortical areas, all of which are all critical brain regions involved in learning and memory (Broide et al., 2007). As such, class I HDACs have been the most commonly investigated HDACs in the context of cognition (Society for the Study of Evolution., 1947). Class II

HDACs are larger and contain more regulatory domains. These HDACs are further subdivided into class IIa HDACs (HDAC4, 5, 7, and 9) and class IIb HDACs (HDAC6, and 10), which are predominantly capable of migrating between the cytoplasm and nucleus, and are expressed in a cell-specific manner (Morris & Monteggia, 2013). Class IV is represented by HDAC11, which is also found within the nucleus and is expressed in the brain primarily within oligodendrocytes (Gräff & Tsai, 2013; Singh et al., 2018)

There are four major classes of small-molecule HDACi that include: 1) carboxylic acids (i.e., sodium butyrate, valproic acid), 2) hydroxamic acids (i.e., trichostatin A, SAHA), 3) benzamides (i.e., MS-275), and 4) natural products (i.e., FK228) (Haggarty & Tsai, 2011). The classes of small-molecule HDACis all vary in their selectivity towards the aforementioned classes of HDACs. Sodium butyrate, valproic acid, MS-275, and FK228 are all selective towards inhibiting class I HDACs, whereas trichostatin A and SAHA inhibit both class I and II HDACs (Yoshida et al., 1990; Furumai et al., 2002; Ryan et al., 2005; Duvic et al., 2007; Kim & Bae, 2011). Only the first three classes of HDACi have been studied in animal models of the context of learning and memory (Alarcón et al., 2004; Korzus et al., 2004; Lattal et al., 2007; Levenson et al., 2004; Guan et al., 2009; Stefanko et al., 2009). As such, the experiments in this thesis will use sodium butyrate (NaBu) as our positive control because NaBu is capable of crossing the blood brain barrier when administered i.p. and selectively inhibits class I HDACs.

### *Memory Formation Accompanies Increased Histone Acetylation*

Although scientists have long studied how memories are formed, the neurobiological nature of the memory trace remains a subject of intense inquiry. The mysterious nature of memory formation stems from the fact that the proteins supporting

the formation of long-term memories eventually turnover, which begs the question of how memories can persist for years (Crick 1984; Roberson & Sweatt, 1999). The biochemical blueprint for long-term memory is now considered to be regulated, in part, by experience-dependent changes to the epigenome (Zovkic et al., 2013; Marshall & Bredy, 2016). In particular, histone acetylation is a molecular driver of successful long-term memory formation (Federman et al., 2009). Seminal work demonstrated that long-term facilitation in the sea slug, *Aplysia californica*, preferentially increases levels of H3 and H4 acetylation of the CAAT enhancer binding protein (C/EBP) promoter, whereas long-term depression prevented H3 and H4 acetylation of C/EBP (Guan et al., 2002). These findings were among the first to demonstrate that the opposing actions of histone acetylation and deacetylation are a critical force underlying synaptic plasticity. This work led to another seminal study conducted in male rats that demonstrated H3, but not H4, hippocampal acetylation was increased 1h, but not 24h, after contextual fear conditioning, whereas latent inhibition (another form of associative learning) preferentially increased hippocampal H4, but not H3, acetylation (Levenson et al., 2004). This work not only demonstrated that histone acetylation is associated with long-term memory formation in male rats, but also raised questions about how different forms of learning elicit different histone acetylation patterns in the brain. Since then, other work has demonstrated that histone acetylation in the hippocampus is associated with memory enhancement in a number of behavioral tasks including contextual fear conditioning in male rodents (Chwang et al., 2007; Vecsey et al., 2007) and fear extinction in male rats (Lattal et al., 2007; Stafford et al., 2012), as well as object recognition in male (Korzus et al., 2004;

Fontán-Lozano et al., 2008) and ovariectomized female mice (Zhao et al., 2010; 2012; Fortress et al., 2014).

Furthermore, other work has demonstrated that increases in histone acetylation do not necessarily occur on a genome-wide level, but rather, targets the promoters of numerous genes deemed critical for memory formation (Gräff & Tsai, 2013). For instance, contextual fear conditioning in male rats has been shown to specifically increase H3 acetylation in the promoter IV region of the brain-derived neurotrophic factor (*bdnf*) gene (Lubin et al., 2008), whereas behavioral tasks assessing fear extinction trigger increases in H4 acetylation of promoter IV *bdnf* in the prefrontal cortex (Bredy & Barad, 2008). Work from our lab has demonstrated that the memory enhancing effects of 17 $\beta$ -estradiol, which require H3 acetylation, specifically increase levels of H3 acetylation at *bdnf* promoters II and IV in young and middle-aged ovariectomized female mice (Zhao et al., 2010; 2012; Fortress et al., 2014).

Thus, histone acetylation is an epigenetic mechanism that is associated with successful forms of long-term memory formation by acting in part as a transcriptional activator for memory-promoting genes.

#### Targeting Histone Deacetylases To Assess Memory Formation

The data indicating that histone acetylation is involved in synaptic plasticity and learning behavior led to questions about which specific HDAC isoforms are responsible for long-term memory formation. In particular, the role of class I HDACs in modulating learning and memory were of specific interest given recent evidence showing that class I HDAC inhibitors (such as NaBu) could reinstate learning in mice with massive neuronal loss (Fischer et al., 2007), and that class I HDACs were predominately expressed in areas

responsible for memory formation itself (Broide et al., 2007). In a landmark study, Guan et al. (2009) systematically assessed the involvement of HDAC1 and HDAC2 in associative learning and synaptic plasticity by developing four different strains of mice who either overexpressed (OE) or contained a knockout (KO) of HDAC1 or HDAC2. They observed decreased H4K5 and H4K12 acetylation in HDAC2OE mice, a molecular signature that was accompanied with reduced freezing during context- and tone-dependent fear learning, as well as impaired spatial navigation in the Morris water maze, both of which were behavioral impairments not observed in HDAC1OE mice. Conversely, the authors observed that HDAC2KO mice exhibited reinstated H4K5 and H4K12 acetylation, as well as increased freezing during the context- and tone-dependent fear learning tasks. Furthermore, HDAC2KO mice had increased H3 and H4 acetylation of memory-promoting genes (i.e. *Bdnf*, *Egr1*, *Fos*, *Creb*) in the hippocampus, which was accompanied by increased density of dendritic spines on CA1 pyramidal neurons (Guan et al., 2009). Additionally, a subsequent study reported that focal HDAC3 deletions within the mouse hippocampus resulted in spatial memory enhancement in an object location task, an effect that was accompanied by increased acetylation of the immediate early gene *c-fos* (McQuown et al., 2011a). Collectively, these studies demonstrated that HDAC2 and HDAC3, but not HDAC1, are negative regulators of long-term memory formation in mice. Perhaps more importantly, these studies highlight the enormous therapeutic potential in bidirectionally modifying activity of individual HDACs to preserve long-term memory formation.

Although overexpression, knockouts, and focal deletions of particular HDACs are useful tools for examining the role of HDACs in learning and memory, their therapeutic

utility in humans is limited because deletion of HDACs would have long-term effects on other biological roles of HDACs outside of the central nervous system, including cell-cycle progression, differentiation, and apoptosis (Ropero & Esteller, 2007). Although extant HDACi compounds cannot discern amongst specific isoforms of HDACs, they have nonetheless provided the field of behavioral neuroscience with useful insights into how histone acetylation regulates spatial and object recognition memory in rodents. For instance, systemic pre-training injection of NaBu facilitates the formation of long-term object recognition memory in male mice (Stefanko et al., 2009b). Our lab has also demonstrated that immediate post-training dorsal hippocampal infusion of HDACi trichostatin A (TSA) enhances object recognition memory consolidation in ovariectomized female mice (Zhao et al., 2010). Furthermore, pre-training dorsal hippocampal infusion of TSA enhances long-term object placement memory in male mice (Hawk et al., 2011). Together, these studies indicate that HDACi treatment can enhance long-term spatial and object recognition memory.

#### *Developing Selective HDACi to Treat Disorders of Cognition*

The balance between histone acetylation and deacetylation goes awry in neurodegenerative disease, such that cells start to overexpress HDACs, ultimately reducing gene transcription (Saha & Pahan, 2006). Based on the promising neuroprotective effects of non-selective HDACi (pan-HDACi) in animal models of neurodegenerative disease, it was thought that these benefits would translate well the clinical setting (Kazantsev & Thompson, 2008; Yang et al., 2017). A handful of studies have assessed the efficacy of pan-HDACi compounds for ameliorating symptoms in cognitive disorders, including Alzheimer's disease (clinicaltrials.gov, NCT03056495),

Huntington's disease (NCT00212316), and schizophrenia (NCT03010865). However, this work has been largely unsuccessful because none of the trials resulted in the approval of pan-HDACi for the treatment of neurodegenerative disease or cognitive disorders (Shukla & Tekwani, 2020).

Our understanding of the shortcomings of pan-HDACi compounds (i.e., SAHA, FK228, vorinostat) as treatment options for neurodegenerative disease has largely come from studies assessing the efficacy of these same compounds in cancer patients. Consensus in the field is that the non-selective nature of pan-HDACi compounds contributes to the cytotoxicity and negative side effects (i.e., fatigue, nausea, dehydration, reduced blood platelet count) observed in clinical trials (Hamze 2020). Additionally, pan-HDACi have poor blood-brain barrier permeability, which precludes their use in treating disorders of the central nervous system (Hiranaka et al., 2018). Therefore, developing isoform-selective HDACi that retain neuroprotective and memory-enhancing properties is a challenging obstacle for researchers due to the high sequence similarity in both the active site structure and catalytic mechanism of individual HDACs (Bieliauskas & Pflum, 2008).

Furthermore, developing HDACi that target particular HDACs is challenging because HDAC proteins do not function as independent enzymes. HDACs are known to form heterodimers and homodimers within different families of large multiprotein repressor complexes (i.e., Sin3, NuRD, CoREST) (Delcuve & Davie, 2012). HDACs are capable of repressing gene transcription only when they are bound to their larger co-repressor complex and recruited to regulatory stretches of DNA by transcription factors (i.e., p53, NF- $\kappa$ B, YY1). For example, HDAC1 and HDAC2 are known to dimerize with

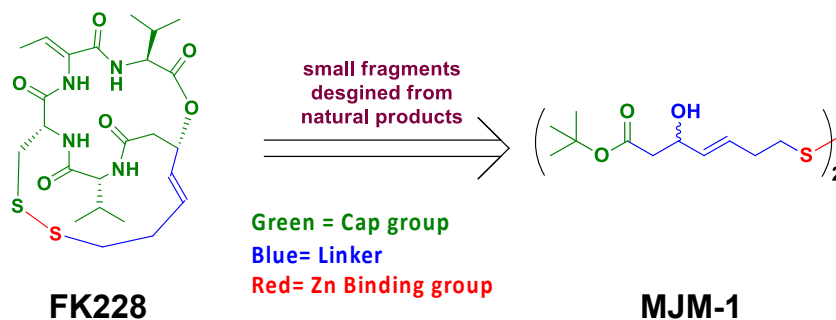
one another in all three types of repressor complexes such that the HDACs themselves form heterodimers (HDAC1/2) or homodimers (HDAC1/1 or HDAC2/2) to carry out their repressive effects on transcription (De Ruijter et al., 2003; Yang & Seto, 2008). Therefore, it is challenging to develop isoform-selective HDACi that target a single HDAC without affecting the activity of other HDACs.

Pan-HDACi are known to target non-histone proteins that can alter transcriptional activity in cells (Delcuve & Davie, 2012). A majority of the behavioral work utilizing pan-HDACi has suggested that the beneficial effects of HDACi treatment are a direct consequence of inhibiting HDACs. However, HDACis can also indirectly increase gene expression by preventing the deacetylation of other non-histone proteins, such as transcription factors and nuclear transport proteins (Delcuve & Davie, 2012; Seto & Yoshida, 2014). Although challenging, developing isoform-selective HDACi would be a major step in reducing unwanted side effects of pan-HDACi that alter the activity of HDACs responsible for cell-cycle progression, differentiation, and apoptosis (Ropero & Esteller, 2007). Therefore, there is a clear need to use isoform-selective HDACi in behavioral studies assessing long-term memory formation because these would reduce the sheer volume of possible non-histone targets and provide a more detailed view of the role of individual HDACs in learning and memory.

#### *Design and Efficacy of MJM-1 In Vitro*

Development of small-molecule HDACi typically follows a pharmacophore model that contains a cap, linker, and zinc binding group (Figure 2). The cap and linker groups

are responsible for binding within the HDAC pocket and are thought to contribute to the overall selectivity of HDACi. The zinc binding group binds to the zinc



**Figure 2. Scaffold of MJM-1.** The composition of MJM-1 is modeled after the scaffold of the FDA-approved HDACi FK228.

ion at the bottom of the HDAC pocket in class I, II, and IV HDACs and is considered to be a region that contributes to the overall activity of HDACi (Manal et al., 2016; Zhang et al., 2018). MJM-1 contains a noticeably smaller polar cap group (Figure 2), which gives MJM-1 the advantage of being more soluble and being permeable to the blood-brain barrier. The linker region of MJM-1 contains a six-carbon chain, approximately 11 Å, which is the reported length of all class I HDAC pockets (Wang 2009). Lastly, MJM-1 contains a disulfide bond as its zinc binding group, which is known to have a strong affinity for the active site in all zinc-dependent HDACs (Manal et al., 2016). Thus, the chemical and structural properties of MJM-1 address a major limitation of most HDACi in that it is highly soluble and capable of crossing the blood brain barrier, indicating that the current scaffold of MJM-1 is an ideal compound for observing effects on long-term memory formation.

Our collaborators were first interested in examining the extent to which MJM-1 modifies histone acetylation *in vitro* relative to a potent dose of HDACi FK228. They first treated DU145 cells with DMSO, 10 nM FK228, or one of three separate doses of MJM-1 (25, 50, or 100 µM) for 24 h before immunolabeling for acetyl-H3 (Lys 9/Lys 14). These

cells were chosen because they overexpress class I HDACs that inhibit the expression of tumor suppressor genes that ultimately permit prostate cancer cell proliferation and invasion (Weichert et al., 2008). Their findings indicate that MJM-1 dose-dependently increases acetyl-H3 (Lys 9/Lys 14) relative to DMSO-treated controls (Appendix Figure 1A-E). The observed levels of histone acetylation after MJM-1 treatment were qualitatively lower than those after treatment of FK228, suggesting that MJM-1 has a more favorable safety profile than FK228. They next determined the extent to which MJM-1 alters activity of recombinant purified HDACs relative to FK228. They demonstrated that despite being less potent than FK228, MJM-1 similarly inhibited both class I (HDAC1, 2, 3, and 8) and class IIb HDACs (HDAC6) (Appendix Figure 1F).

We were then interested in examining the extent to which two different doses of MJM-1 cross the blood brain barrier and be detected in the DH following a single i.p. injection. Male mice received an i.p. injection of either 20 or 40 mg/kg MJM-1, and DH tissue collected 10 or 30 min later (Appendix Figure 2A). Here, utilizing mass spectrometry (Shimadzu LCMS-8040), our group demonstrated that 20 mg/kg MJM-1 can be detected in the DH 10 min following i.p. administration (Appendix Figure 2B), whereas 40 mg/g MJM-1 can be detected at both 10 and 30 min (Appendix Figure C-D). These data suggest that MJM-1 penetrates the blood brain barrier and diffuses rapidly into the hippocampus after i.p. administration. Given that both 20 and 40 mg/g doses were detected in the brain, the present study examined the effects of 20, 30, and 40 mg/g MJM-1 on memory consolidation.

As such, the goals of this thesis were to determine the extent to which post-training administration of MJM-1 enhances spatial and object recognition memory and alters

hippocampal histone acetylation and *bdnf* gene expression in male mice. The preliminary data gathered by our collaborators collectively suggest that MJM-1 increases H3 acetylation *in vitro*, inhibits class I and IIb HDACs, and quickly penetrates the blood-brain barrier into the mouse hippocampus following i.p. administration. These findings helped form our hypotheses that MJM-1 would enhance spatial and object recognition memory consolidation in part by increasing global hippocampal histone acetylation or *bdnf* expression levels. Our results indicate that MJM-1 enhances spatial, but not object recognition, memory consolidation in male mice. Surprisingly, however, we observed no treatment effects of MJM-1 on hippocampal histone acetylation or *bdnf* expression patterns. Although the molecular mechanisms underlying MJM-1's beneficial effect on spatial memory consolidation remain unclear at the present time, this work provides a useful framework for understanding how to better develop small-molecule HDACis that can potentially be used to improve memory decline.

## METHODS

### Subjects

Male C57BL/6 mice were obtained from Taconic Biosciences (Germantown, NY) at 10 weeks of age and housed individually in shoebox cages in a room (22-23°C) with a 12/12-h light-dark cycle. Food and water were provided *ad libitum*. Mice were handled 30 s/day for 3 days before behavioral testing. All procedures were conducted from 10:00 to 17:00 h in a quiet room, and experimenters conducting behavioral testing were blind to the treatment each mouse received. All procedures were approved by the University of

Wisconsin-Milwaukee Institutional Animal Care and Use Committee and are consistent with the National Institutes of Health Guide for the Care and Use of Animals.

### General Experimental Design

The purpose of this study was to determine the extent to which MJM-1 can enhance memory consolidation and hippocampal histone acetylation and *bdnf* expression in male mice. Four separate cohorts were used in total for the behavioral and molecular experiments (Table 1). Cohorts 1 and 2 (n=60 each; 12/treatment) were used for OP and OR behavioral testing, where mice received an immediate post-training i.p. injection of one of five possible treatments. Negative control mice received injections of 100% dimethyl sulfoxide (DMSO). Positive control mice received sodium butyrate (0.6 g/kg NaBu), an HDACi that has been previously shown to enhance object recognition memory in male mice (Stefanko et al., 2009). Experimental mice received one of three doses of MJM-1 (20, 30, or 40 mg/kg) that our group shown to be present in the DH following i.p. administration (Appendix Figure 2). Upon completion of behavioral testing, all mice received a final i.p. injection of DMSO, NaBu, or one of three doses of MJM-1 (20, 30, 40 mg/kg), and DH tissue was collected 60 min later to assay histone acetylation alterations.

To determine the extent to which histone acetylation was altered by post-training MJM-1 treatment, Cohort 3 (n=36; 12/treatment) underwent object training and received an immediate post-training i.p. injection of DMSO, NaBu, or 40 mg/kg MJM-1, and DH was collected 30 min later to assay histone acetylation alterations.

To determine the extent to which MJM-1 treatment modifies hippocampal expression levels of *bdnf* exons I and IV, Cohort 4 (n=24; 8/treatment) received a single

i.p. injection of DMSO, NaBu, or 40 mg/kg MJM-1, and DH tissue was collected 60 min later to assay *bdnf* expression.

**Table 1: General Experimental Design.**

Cohort	Treatment Received	Behavior	Tissue Collection	Assays
1	DMSO, NaBu, or MJM-1 (20, 30, 40 mg/kg)	OP and OR	60 min following final i.p.	OR/OP; WB DH histone acetylation
2	DMSO, NaBu, or MJM-1 (20, 30, 40 mg/kg)	OP and OR	60 min following final i.p.	OR/OP; WB DH histone acetylation
3	DMSO, NaBu, MJM-1 (40 mg/kg)	Object Training	30 min following post-training i.p.	WB DH histone acetylation
4	DMSO, NaBu, MJM-1 (40 mg/kg)	-	60 min following single i.p.	qPCR <i>bdnf</i> exons I and IV

### Drugs and Injections

All drugs were prepared the day of use and administered by a single i.p. injection at a volume of 5 m/kg. NaBu was dissolved in sterile saline to a concentration of 0.6 g/kg (Stefanko et al., 2009). Our group's novel brain-penetrant HDACi compound, MJM-1, was dissolved in 100% DMSO to a concentration of 40 mg/kg, and then serially diluted to concentrations of 30 and 20 mg/kg. These doses were chosen because our data indicate that a 40 mg/kg dose of MJM-1 can be detected in the DH of male C57BL/6 mice by Shimadzu LCMS-8040 mass spectrometry both 10 and 30 min following a single i.p. injection (Appendix Figure 2C-D). Our data also suggest that a 20 mg/g dose of MJM-1 can be detected in the DH 10 min following a single i.p. injection (Appendix Figure 2B).

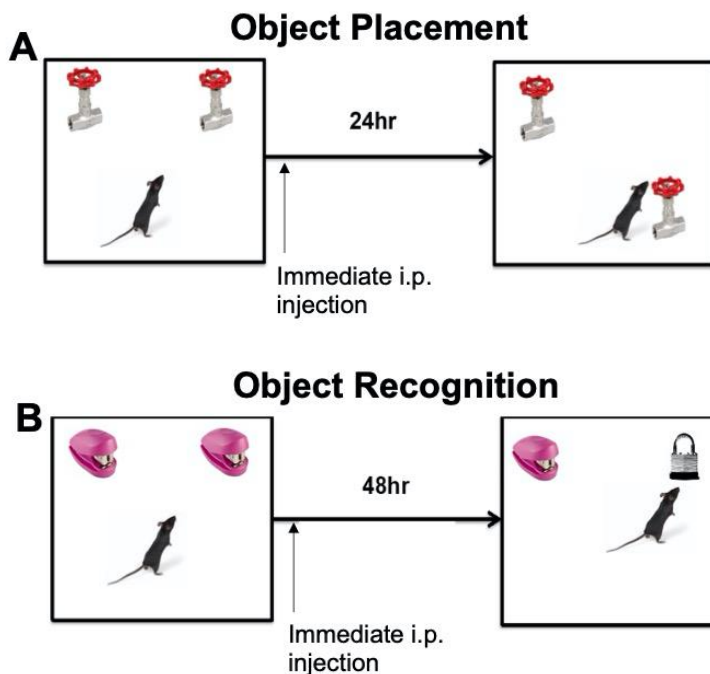
### Behavioral Tasks

The memory-enhancing effects of MJM-1 were examined in Cohorts 1 and 2 through use of object placement (OP) and object recognition (OR) behavior paradigms which assess spatial and object recognition memory, respectively (Boulware et al., 2013; Fernandez et al., 2008; Tuscher et al., 2016). Cohort 3 mice were first assigned to either

Homecage or Trained groups and received the same handling, habituation, and object training procedure described below without behavioral testing.

OP and OR were conducted in a white open field box (width, 60 cm; length, 60 cm; height, 47 cm). Prior to behavioral training, mice were handled for 30 s/day for 3 days. On the second day of handling, a single Lego Duplo block was placed in the home cage to acclimate mice to objects. Following handling, mice underwent a single habituation session for 5 min/day for 2 days. During habituation, mice were allowed to move about freely in the apparatus without objects

present. Following habituation, mice underwent OP and OR training, during which mice were given up to 20 min to accumulate 30 s exploring two identical objects placed in the upper right- and left-hand corners of the box. Experimenters manually scored (in real time) the amount of object exploration using ANYmaze tracking software (Stoelting). Object exploration was defined as when the mouse's nose and/or front paws were directed towards and/or touching the objects. Different objects were used for OP and OR,



**Figure 3. Overview of behavioral testing protocols.** A) In object placement, mice accumulate 30 s exploring 2 identical objects in an open arena. Mice then receive a post-training i.p. injection of DMSO, NaBu, or MJM-1 (20, 30, or 40 mg/kg). Retention is tested 24h later by moving one training object to a lower corner of the box. Mice who remember the familiar object spend more time than chance (15 s) exploring the moved object. B) Object recognition uses the same apparatus and general procedure, except during testing, mice are presented with a novel object. Testing is conducted 48 h after i.p. injection of compounds because OR memory persists longer than OP memory.

and all objects used were counterbalanced across mice to account for any potential effects of object preference. Immediately following training, mice received an i.p. injection of one of the compounds described above. All treatments in OP and OR were administered immediately post-training to pinpoint effects of MJM-1 on the consolidation phase of spatial and object recognition memory formation, respectively. If mice did not accumulate 30 s of exploration time during training, then were then retrained 4-7 days later with different objects. OP and OR were tested 24- and 48-hours post-training, respectively (Figure 3A-B). These time points were chosen based on previous evidence that vehicle-treated gonadally-intact male mice no longer remember the location or identity of the objects at these time points (Koss et al., 2018). Similarly, gonadally-intact male mice can remember object identity 24 hr after OR training (Frick & Gresack, 2003). OP testing consisted of moving the least-explored training object to either the lower right- or left-hand corner of the box (Figure 3A). OR testing consisted of replacing the least-explored training object with a novel object that mice had not previously encountered before (Figure 3B). Mice were given 20 min to accumulate 30 s of exploration time during OP and OR testing. Chance is designated at 15 s because this value represents equal exploration of both objects (Frick & Gresack, 2003). If they did not accumulate 30 s of exploration time then the mouse was given up to three separate chances to successfully complete the task. All mice were given at least one week between bouts of testing to ensure that any acute effects of previous drug injections dissipated before the next treatment.

### Tissue Collection

At the conclusion of behavioral testing, Cohorts 1 and 2 received a final i.p. injection of DMSO, NaBu, or one of three doses of MJM-1 (20, 30, or 40 mg/kg), and DH tissue was collected on 60 min later on wet ice and immediately frozen at -80°C. The 60 min timepoint was chosen because work from our lab assessing object recognition memory in ovariectomized mice has demonstrated that DH infusion of non-selective HDACi TSA increases H3 acetylation in the DH at 30 min (Zhao et al., 2010). We predicted that any histone acetylation changes from i.p. administration of MJM-1 would take longer than an intrahippocampal infusion, and as such, were expected to be detected in the DH by 60 min. Cohort 3 received a post-training i.p. injection of DMSO, NaBu, or 40 mg/kg MJM-1, and DH tissue was collected 30 min later on wet ice and immediately frozen at -80°C. The 30 min timepoint was chosen because previous studies found that administering a single i.p. injection of a 1.2 g/kg dose of NaBu following training significantly altered H3 and H4 acetylation levels in the DH at 30 min (Fischer et al., 2007; Gundersen & Blendy, 2009a; Takuma et al., 2014). Cohort 4 received a single i.p. injection of DMSO, NaBu, or 40 mg/kg MJM-1, and DH tissue was collected 60 min later. Samples were placed in RNAlater solution (ThermoFisher Scientific), and stored at 4°C overnight, and then transferred to -20°C for storage.

### Histone Extractions

All tissue samples were individually processed using the EpiQuik Total Histone Extraction Kit (EpiGentek, Farmingdale, NY) to maximally isolate histone proteins and preserve post-translational modifications. Briefly, tissue samples were homogenized in 1X pre-lysis buffer with a pellet-tissue grinder (Kimble) for 45 s and then centrifuged at 10,000 rpm for 1 min at 4°C. The remaining tissue pellet was resuspended in 3 volumes

of lysis buffer, incubated on ice for 30 min, and centrifuged at 12,000 rpm for 5 min at 4°C. Following centrifugation, the supernatant containing acid soluble histone proteins was transferred to a new 1.5 mL tube and dithiothreitol (DTT) buffer (0.3 volumes) were added to the histone fraction. The protein concentration of the eluted histone fraction was then estimated using a Bradford protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard. Samples were stored in -80°C until future preparation of Western blot aliquots.

### Western Blotting

Histone fractionated samples were normalized to 2 µg/µl by adding corresponding amount of sample buffer, lysis buffer, and homogenate in preparation for Western blotting. Samples were boiled for 5 min to denature proteins. Proteins were electrophoresed in 10 µl aliquots on 4-15% Tris-HCl polyacrylamide gels (Bio-Rad) and transferred to PVDF Midi membranes using a TransBlot Turbo transfer system (Bio-Rad). Membranes were blocked in 5% dried non-fat milk/TTBS and incubated overnight at 4°C with the following primary antibodies: acetyl-H2B (Lys 12), acetyl-H3 (Pan), acetyl-H3 (Lys 14), acetyl-H3 (Lys 9, Lys 14), and acetyl-H4 (Lys 12) (1:1000, Cell Signaling Technology). These acetylation sites were chosen to include all core histone proteins across a range of different lysine sites to assess which, if any, sites MJM-1 may preferentially acetylate. In particular, modifications to these lysine sites have been previously shown responsible for memory formation and synaptic plasticity (Alarcón et al., 2004; Levenson et al., 2004; Guan et al., 2009; Zhao et al., 2010; Valor et al., 2011). Blots were incubated the next day for 1 h at room temperature with rabbit HRP-conjugated secondary antibody (anti-rabbit HRP, 1:5000, Cell Signaling Technology). Membranes were then developed using

Clarity Max chemiluminescent substrate (Bio-Rad), and imaged using a ChemiDoc MP gel imager (Bio-Rad). Blots were then stripped with 0.2 M NaOH and incubated with the following antibodies for protein normalization: total-H2B (1:1000, Cell Signaling Technology), total-H3 (1:1000, Cell Signaling Technology), total-H4 (1:2000, Cell Signaling Technology). Dilutions of the aforementioned antibodies are based on previously published work from our lab (Zhao et al., 2012; Fortress et al., 2014). Densitometry analysis were conducted using Image Lab software (Bio-Rad, Image Lab version 6.0.1) and data are represented as percent of immunoreactivity relative to vehicle controls.

#### Real-Time Quantitative PCR

RNA was prepared and quantified as described previously (Zhao et al., 2010; Fortress et al., 2014; Gross et al., 2021). Briefly, RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Total RNA concentration was determined by reading absorbance at 260 and 280 nm using an Agilent Epoch Microplate Spectrophotometer (BioTek, Winooski, VT) and cDNA samples were prepared from 1 µg of extracted RNA using a Bio-Rad iScript cDNA Synthesis kit (Bio-Rad). Real-time quantitative PCR (RT-qPCR) was performed using SYBR Green Master Mix (Bio-Rad) on an Eppendorf Realplex 2 PCR System (Eppendorf). Primers for *bdnf* I and IV transcripts (Table 2) were obtained from Integrated DNA Technologies (Coralville, IA). Predesigned and optimized RT<sup>2</sup> qPCR Primer Assays (Qiagen) were used for analysis of *Gapdh* (#PPM02946E) as an expression control. RT-qPCR reactions were run at 95°C for 10 min and 40 cycles of 95°C for 15 s, followed by 60°C for 60 s, 95°C for 15 s and 60°C for 15 s, and then held at 95°C for 15 s. All samples were run in triplicate

and normalized to corresponding GAPDH values, and the  $\Delta\Delta\text{CT}$  method was used to calculate relative expression of each gene of interest (Zhao et al., 2010; Fortress et al., 2014; Gross et al., 2021).

**Table 2: Primers Used for qPCR Analysis of *bdnf* Exons I and IV**

Gene names	Primer sequences
<i>bdnf</i> Exon I	F: 5' GGA AAC GTC TCT CTC AGA ATG A -3'
	R: 5' TCA TCC ACC TTG GCG ATT AC -3'
<i>bdnf</i> Exon IV	F: 5' CTC AAG CGC TGC GAG TAT TA -3'
	R: 5' AGT CCT TGG CCG ATA TG -3'

### Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 8 (La Jolla, CA). All data were analyzed for outliers, defined by  $\pm 2$  standard deviations from the mean, which were removed prior to additional analysis. For each behavioral experiment, separate one-sample *t*-tests were performed for each group to determine if the time spent with the novel object differed from chance (15 s; Boulware et al., 2013; Frick & Gresack, 2003; Tuscher et al., 2016). To assess between-group treatment effects within each behavioral task, one-way analyses of variance (ANOVAs) were conducted followed by Fisher's LSD post hoc tests. For Western blotting analyses, one-way ANOVAs were conducted followed by Tukey's multiple comparison post hoc tests for between-group comparisons. For qPCR analyses, the  $\Delta\Delta\text{CT}$  method was used to obtain normalized values to vehicle-treated mice. One-way ANOVAs were conducted followed by Tukey's multiple comparison post hoc tests for between-group comparisons. Significance was determined as  $p \leq 0.05$ .

## RESULTS

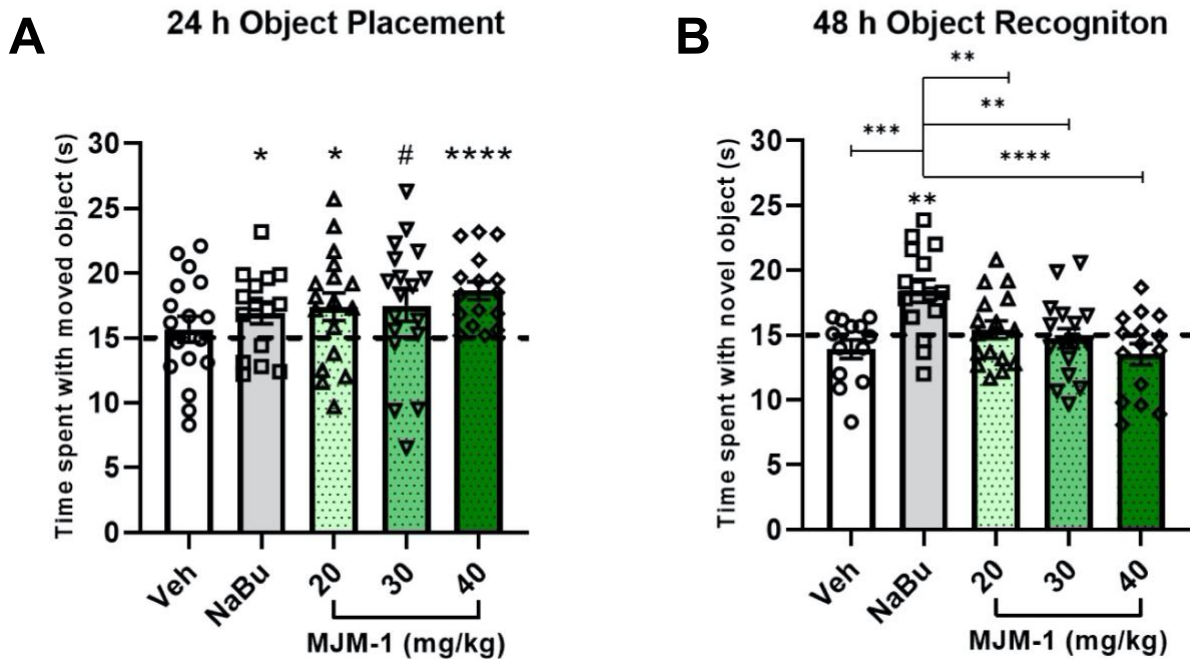
### MJM-1 Enhances Spatial, but not Object Recognition, Memory Consolidation

We were first interested in assessing the extent to which MJM-1 can enhance memory consolidation in male mice. As described in the Methods Section, the OP and OR tasks were used to assess spatial and object recognition memory consolidation, respectively. Briefly, each task consists of a training phase in which mice must accumulate 30 s exploring two identical objects in an open field (Figure 3A-B). Immediately after training, mice were injected i.p. with DMSO, NaBu, or one of three doses of MJM-1 (20, 30, 40 mg/kg).

Multiple doses of MJM-1 enhanced spatial memory consolidation (Figure 4A). Although a one-way ANOVA did not indicate significant between-group differences ( $F_{(4, 78)} = 1.134$ ;  $p = 0.3467$ ), one-sample  $t$ -tests that assess memory within each group revealed a differential pattern of spatial memory consolidation among the groups. Mice receiving DMSO did not spend significantly more time with the moved object than the chance value of 15 s ( $t(16) = 0.6857$ ,  $p > 0.05$ ;  $n=17$ ), suggesting that this group exhibited impaired memory for object location. In contrast, mice receiving NaBu spent significantly more time than chance with the moved object ( $t(14) = 2.265$ ,  $p = 0.0399$ ;  $n=15$ ), indicating enhanced memory for object location. Similarly, mice receiving 20 mg/kg MJM-1 ( $t(16) = 2.244$ ,  $p = 0.0393$ ;  $n=17$ ) or 40 mg/kg MJM-1 ( $t(15) = 5.248$ ,  $p < 0.0001$ ;  $n=16$ ) also spent significantly more time with the moved object than chance, indicating that these two doses of MJM-1 enhanced spatial memory consolidation. Although not quite statistically significant, mice receiving 30 mg/kg MJM-1 also tended to spend more time than chance

with the moved object ( $t(17) = 2.054$ ,  $p = 0.0556$ ;  $n=18$ ). These results suggest that acute i.p. administration of MJM-1 can enhance spatial memory consolidation in male mice.

In contrast to its effects on spatial memory, MJM-1 did not influence object recognition memory consolidation (Figure 4B). One-sample  $t$ -tests indicated that mice receiving vehicle did not spend significantly more time than chance with the novel object ( $t(12) = 1.533$ ,  $p > 0.05$ ;  $n=13$ ). Although mice receiving NaBu spent significantly more time than chance with the novel object, ( $t(14) = 3.915$ ,  $p = 0.0016$ ;  $n=15$ ), those receiving the 20, 30 or 40 mg/kg MJM-1 did not ( $t(14-15) = 0.6089$ ,  $0.3235$ , and  $1.747$ , respectively,  $p > 0.05$ ;  $n=15-16$ ). The main effect of treatment was significant ( $F_{(4, 70)} = 6.027$ ;  $p = 0.0003$ ), an effect driven by the fact that the NaBu-treated group differed significantly from every other treatment group (NaBu vs. Veh:  $p = 0.0002$ ; NaBu vs. 20:  $p = 0.0077$ ; NaBu vs. 30:  $p = 0.0013$ ; NaBu vs. 40:  $p < 0.0001$ ). Taken together, these results indicate MJM-1 can enhance spatial, but not object recognition, memory consolidation to a similar extent as the known memory-enhancing HDAC inhibitor NaBu.



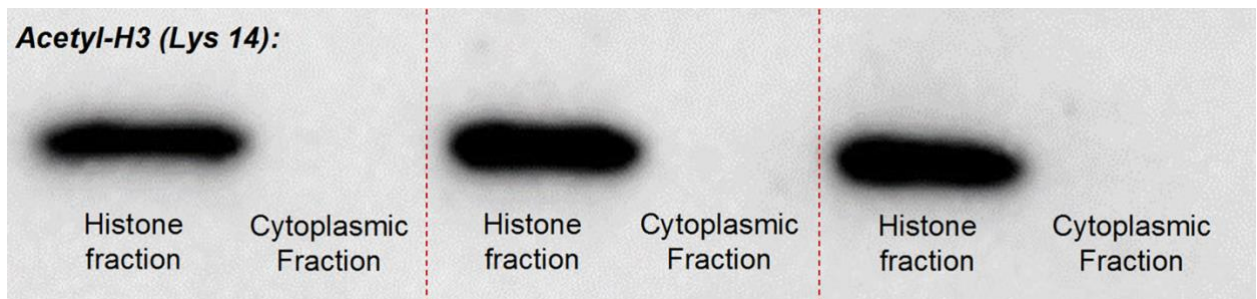
**Figure 4. Post-training i.p. administration of MJM-1 enhances spatial, but not object recognition, memory in male mice.** A) Male mice receiving an i.p. injection of the HDACi sodium butyrate (NaBu), or 20 or 40 mg/kg of MJM-1 spent significantly more time than chance (dashed line at 15 sec, \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ ) with the moved object 24 h after training ( $n=15-18$ /group). Although not statistically significant, mice receiving the 30 mg/kg dose of MJM-1 displayed a trend towards spending more time than chance with the moved object ( $\#p = 0.0556$ ). B) Only mice receiving NaBu treatment spent significantly more time than chance with the novel object 48 hr after training (\*\* $p < 0.01$ ). Mice treated with NaBu also spent significantly more time with the novel object than every other treatment group ( $n=13-15$ /group). Bars represent the mean  $\pm$  (SEM).

### MJM-1 Treatment Did Not Increase Hippocampal Histone Acetylation

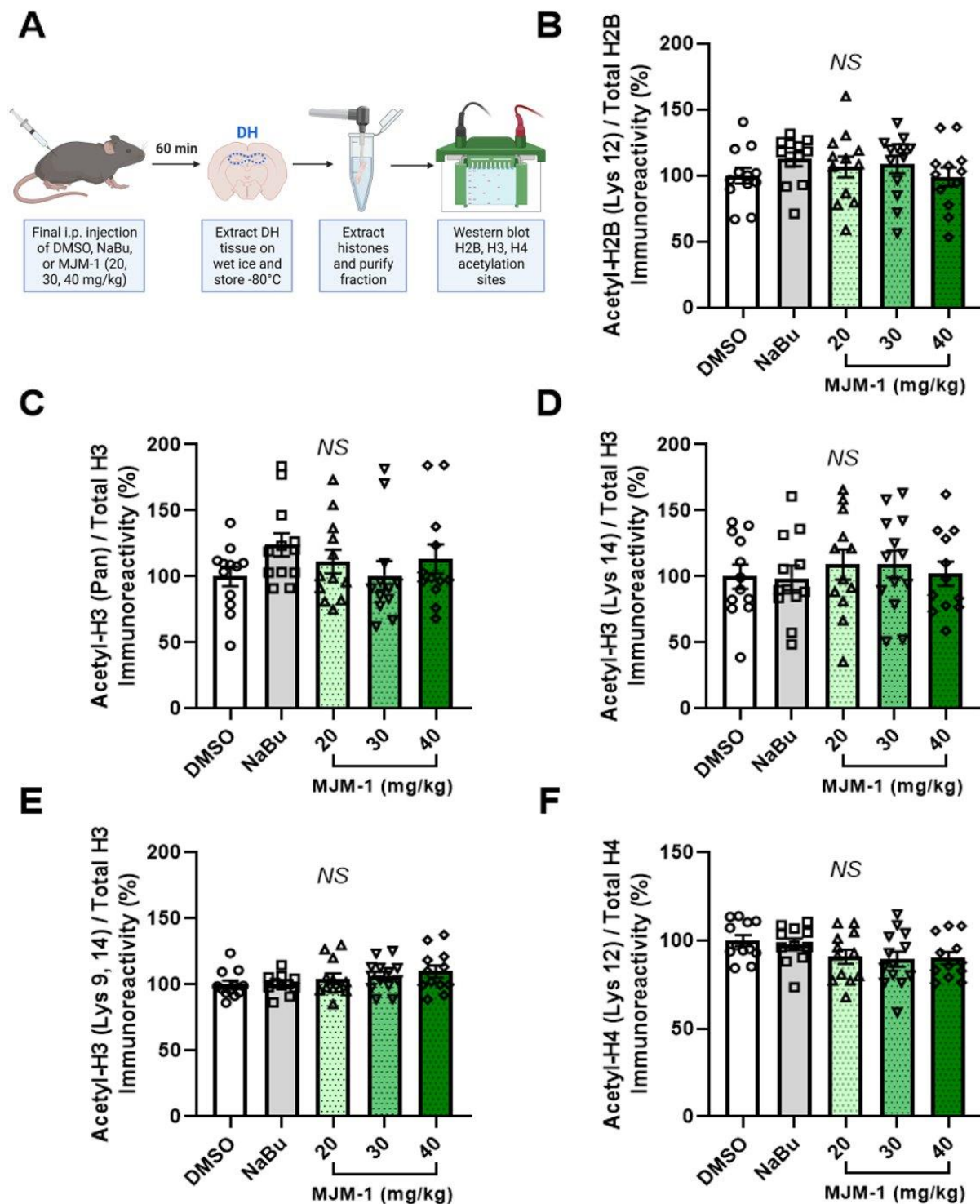
We next wanted to determine the extent to which the memory enhancing doses of MJM-1 alter hippocampal histone acetylation. The mice used for OP and OR testing were given two weeks before they received a final i.p. injection of DMSO, NaBu, or MJM-1 (20, 30, or 40 mg/kg), and the DH was collected 60 min later (Figure 6A). We first verified that we achieved sufficient separation of histone and cytoplasmic fractions and that the histone fraction contained detectable amounts of histone protein that could be assayed via Western blot (Figure 5). Despite observing excellent separation, there was no

significant main effect of treatment in the DH for altering histone acetylation for the sites acetyl-H2B (Lys 12) ( $F_{(4, 56)} = 0.7194$ ,  $p = 0.5823$ , Figure 6B), acetyl-H3 (Pan) ( $F_{(4, 55)} = 1.094$ ,  $p = 0.3686$ , Figure 6C), acetyl-H3 (Lys 14) ( $F_{(4, 56)} = 0.2665$ ,  $p = 0.8983$ , Figure 6D), acetyl-H3 (Lys 9, Lys 14) ( $F_{(4, 55)} = 1.387$ ,  $p = 0.2504$ , Figure 6E), or acetyl-H4 (Lys 12) ( $F_{(4, 55)} = 1.724$ ,  $p = 0.1579$ , Figure 6F).

The lack of an effect for MJM-1 in the DH was surprising considering our group found a dose-dependent increase in acetyl-H3 (Lys 9/Lys 14) immunofluorescence levels in DU145 tumor cells relative to DMSO-treated cells (Appendix Figure 1) and because we demonstrated that MJM-1 localizes to DH following systemic administration (Appendix Figure 2). Moreover, the lack of an effect for NaBu is particularly surprising because this compound is widely reported to increase bulk histone acetylation and because we found that NaBu enhances spatial and object recognition memory consolidation. These data alone suggest that a single i.p. injection of NaBu or MJM-1 does not alter global levels of histone acetylation 60 min later within the DH.



**Figure 5. Separation of histone and cytoplasmic fractions following histone extraction protocol.** DH tissue was homogenized and fractionated following the manufacturer's protocol for EpiQuik Total Histone Extraction Kit, and both fractions were prepared for Western blot and probed for acetyl-H3 (Lys 14). The histone fraction contains acetyl-H3 (Lys 14) protein, whereas the cytoplasmic fraction does not.

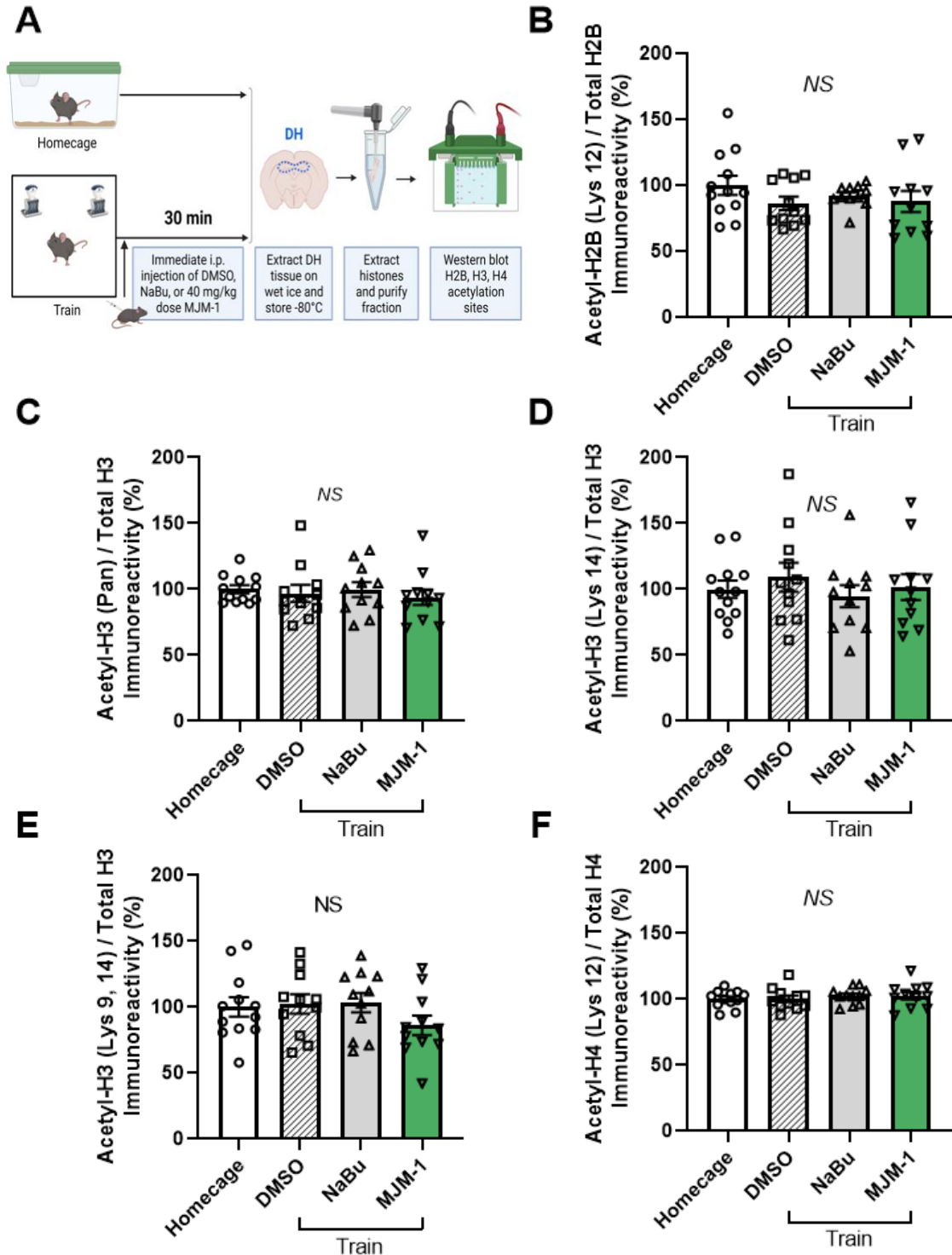


**Figure 6. MJM-1 treatment did not alter histone acetylation levels in the dorsal hippocampus.** A) Schematic of tissue collection procedure. Two weeks after completion of behavioral training, mice received a final i.p. injection of 100% DMSO, 0.6 g/kg NaBu, or one of three doses of MJM-1 (20, 30, or 40 mg/kg) ( $n=12-13/\text{group}$ ). DH tissue was collected 60 min later for Western blot analysis of B) acetyl-H2B (Lys 12), C) acetyl-H3 (Pan), D) acetyl-H3 (Lys 14), E) acetyl-H3 (Lys 9/Lys 14), and F) acetyl-H4 (Lys 12) modifications. Error bars represent mean  $\pm$  SEM. All  $p > 0.05$ .

## Post-Training MJM-1 Treatment Did Not Increase Hippocampal Histone Acetylation

Although HDACi have been used to enhance memory formation and rescue cognitive impairments, it remains unclear how they are able to have targeted effects on histone acetylation in brain regions that support cognition when administered systemically. It is thought that HDACi are capable of enhancing memory formation because they are administered around the time of learning, where task-relevant stimuli are already promoting expression of genes relevant to synaptic plasticity (Gräff et al., 2014). As such, we next sought to determine whether the lack of an effect on histone acetylation alterations in the DH for our NaBu- and MJM-1-treated mice could be explained by the following possibilities: that 1) we missed the timepoint in which these transient histone acetylation alterations occur, or 2) our behaviorally effective doses of NaBu and MJM-1, when administered alone, were not sufficient to drive global hippocampal histone acetylation changes without learning involved (Stafford et al., 2012; Raybuck et al., 2013).

We hypothesized that relative to Homecage controls, object training would increase H3 or H4 acetylation levels, whereas mice who receive a post-training i.p. injection of 0.6 g/kg NaBu or 40 mg/kg MJM-1 would have potentiated histone alterations relative to both Homecage and DMSO-trained mice. However, we found that there was no significant main effect of treatment for levels of acetyl-H2B (Lys 12) ( $F_{(3, 41)} = 1.041$ ,  $p = 0.3844$ , Figure 7B), acetyl-H3 (Pan) ( $F_{(3, 41)} = 0.2735$ ,  $p = 0.8442$ , Figure 7C), acetyl-H3 (Lys 14) ( $F_{(3, 41)} = 0.4276$ ,  $p = 0.7343$ , Figure 7D), acetyl-H3 (Lys 9/Lys 14) ( $F_{(3, 41)} = 1.140$ ,  $p = 0.3441$ , Figure 7E), or acetyl-H4 (Lys 12) ( $F_{(3, 41)} = 0.3220$ ,  $p = 0.8094$ , Figure 7F).



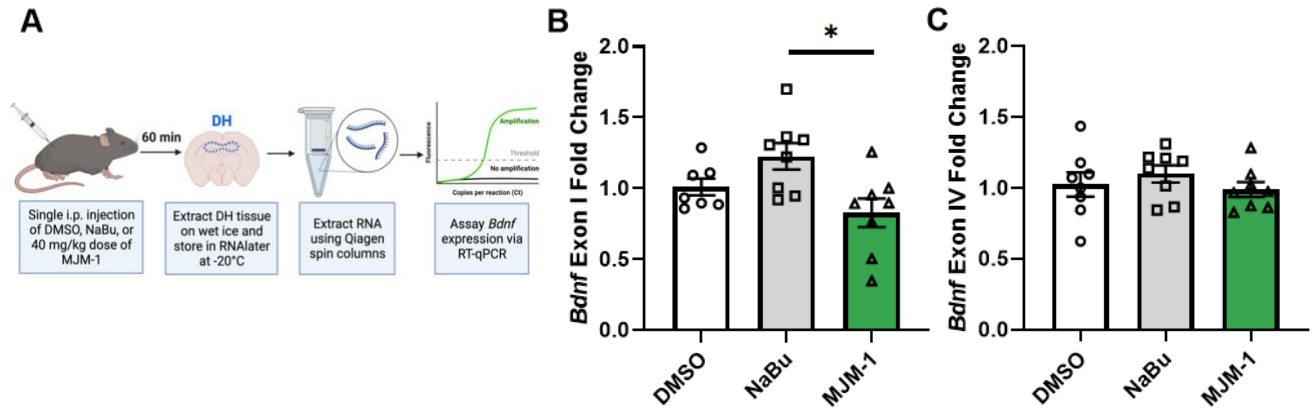
**Figure 7. Post-training *i.p.* administration of MJM-1 did not alter histone acetylation levels in the dorsal hippocampus.** A) Schematic of object training, post-training injection, and tissue collection schedule. Following successful completion of training, mice were given an immediate post-training *i.p.* injection of 100% DMSO, 0.6 g/kg NaBu, or 40 mg/kg MJM-1 ( $n=11-12/\text{group}$ ). Homecage controls were sacrificed in a counterbalanced fashion. DH tissue was collected 30 min later and samples were prepared for Western blot analysis of B) acetyl-H2B (Lys 12), C) acetyl-H3 (Pan), D) acetyl-H3 (Lys 14), E) acetyl-H3 (Lys 9/Lys 14), and F) acetyl-H4 (Lys 12) modifications. Error bars represent mean  $\pm$  SEM. All  $p > 0.05$ .

### MJM-1 Treatment Did Not Increase Hippocampal *bdnf* Expression

We finally examined the extent to which the behaviorally effective doses of NaBu and MJM-1 might alter expression of *bdnf*. Although our data suggest that NaBu and MJM-1 do not enhance memory formation by altering hippocampal histone acetylation when administered alone or following object training, we wanted to examine the possibility that these compounds enhance memory by promoting the transcription of genes that support synaptic plasticity. As such, we chose to examine *bdnf* because this neurotrophic factor has been widely demonstrated to be critical for enhancing synaptic plasticity by inducing structural and functional changes at synapses (Lu 2003; Leal et al., 2015; Miranda et al., 2019). Previous work in male mice demonstrated that a single i.p. injection of 1.2 g/kg NaBu immediately following object training increased mRNA expression levels of *bdnf* exons I and IV relative to vehicle-trained controls in the hippocampus collected 60 min following object testing (Intlekofer et al., 2013). As such, we hypothesized that the memory enhancing effects of NaBu and MJM-1 could arise in part by increasing hippocampal expression of *bdnf* exons I and IV relative to DMSO-treated controls.

A new cohort of behaviorally naïve male mice received a single i.p. injection of DMSO, NaBu, or 40 mg/kg MJM-1. DH tissue was collected 60 min later and stored in RNAlater solution prior to RNA extraction and qPCR analysis of *bdnf* exon I and IV transcripts (Figure 8A). A significant main effect of treatment was found for *bdnf* exon I expression ( $F_{(2, 20)} = 5.249$ ,  $p = 0.0147$ , Figure 8B), such that levels of *bdnf* exon I were significantly higher among NaBu-treated mice relative to MJM-1 treated mice ( $p < 0.05$ ).

However, there was no significant main effect of treatment found for *bdnf* exon IV expression ( $F_{(2, 21)} = 0.6951, p = 0.5101$ , Figure 8C).



**Figure 8. MJM-1 treatment did not alter expression of *bdnf* exons I or IV in the dorsal hippocampus.** A) Schematic of injection and tissue collection procedure. Behaviorally naïve mice were given a single i.p. injection of either 100% DMSO, 0.6 g/kg NaBu, or 40 mg/kg MJM-1 and total RNA was extracted for analysis of *bdnf* exons I and IV were assessed 60 min later in the DH (n=7-8/group). B) MJM-1 treatment did not alter *bdnf* exon I expression levels ( $p > 0.05$ ). However, mice injected with 0.6 g/kg NaBu had increased *bdnf* exon I expression relative to MJM-1 treated mice ( $*p < 0.05$ ). C) None of the treatments altered *bdnf* exon IV expression levels ( $p > 0.05$ ). Error bars represent mean  $\pm$  SEM.

## DISCUSSION

### Effects on Spatial and Object Recognition Memory Consolidation

We first demonstrated that MJM-1 can enhance spatial memory consolidation in male mice. A single post-training i.p. injection of 20 or 40 mg/kg MJM-1, and to a lesser extent 30 mg/kg MJM-1, enhanced spatial memory consolidation in the object placement task. These findings also indicate that MJM-1 enhances spatial memory consolidation in a similar manner to the established HDACi NaBu. The spatial memory enhancing effects of MJM-1 are consistent with a number of previously published studies utilizing extant HDACi in object placement tasks. For instance, post-training i.p. administration of NaBu has been previously shown to enhance spatial memory in male mice using a comparable object placement task (Haettig et al., 2011). Similarly, post-training infusion of NaBu or

other HDACi, including MS-275 and TSA, directly into the dorsal hippocampus enhances object placement memory consolidation in male mice (Hawk et al., 2011; Roozendaal et al., 2010). As such, the ability of MJM-1 to facilitate spatial memory formation is consistent with those of other well established HDACi.

In contrast, our data also indicate that MJM-1 does not enhance object recognition memory consolidation. The lack of an effect on object recognition memory is inconsistent with other studies in which post-training i.p. injection of NaBu given to young or aged male rodents or dorsal hippocampal infusion of TSA given to young ovariectomized female mice enhances object recognition memory consolidation (Stefanko, et al., 2009; Zhao et al., 2010; Haettig et al., 2011; Reolon et al., 2011). The reasons for this discrepancy are unclear at the present time. The object placement and recognition tasks are frequently used in the field of learning and memory because they are non-aversive, one-trial learning tasks that are amenable to repeated use of pharmacological interventions (Tuscher et al., 2015). However, the distinct molecular mechanisms that support the formation of spatial and object recognition memories remain unclear, let alone how HDACi interact within the existing molecular framework to enhance one form of learning over another.

The inconsistent effects of MJM-1 on spatial vs. recognition memory may reflect distinct contributions of individual HDACs that support memory formation in a task-specific manner. For instance, mice that overexpress HDAC2, but not HDAC1, exhibited impaired spatial memory formation (Guan et al., 2009), whereas focal deletion of HDAC3 in the dorsal hippocampus enhanced spatial, but not object recognition, memory in male mice (McQuown et al., 2011). Therefore, MJM-1 may preferentially influence spatial memory by partially inhibiting the activity of class I HDAC2 and HDAC3. Alternatively,

differential regulation by MJM-1 of the histone acetyltransferase transcription factor/co-activator complex CREB (cAMP response element binding protein) binding protein (CBP) may play a role, as post-training i.p. injection of NaBu enhances both spatial and object recognition memory in wild-type mice, but only facilitates object recognition in CBP mutant mice (Haettig et al., 2011). Thus, spatial and object recognition memory may differ in their requirement for interactions with CREB:CBP proteins.

Finally, the differential effects of MJM-1 on these two types of memory may relate to the brain circuitry that mediates them. Whereas spatial memory in rodents predominately relies on the hippocampus (Broadbent et al., 2004), object recognition memory is involving the hippocampus, insular cortex, and multiple regions of the temporal lobe (Hammond et al., 2004; Wilson et al., 2013). Interestingly, NaBu enhanced object recognition memory consolidation when it was infused post-training into the insular cortex, but not hippocampus, of male rats (Roozendaal et al., 2010). Although our mass spectrometry analyses indicated MJM-1 penetration into the hippocampus, it remains unclear whether MJM-1 localizes to and/or activates other brain regions that are relevant for object recognition memory formation.

#### *Effect of Isolated Treatment on Hippocampal Histone Acetylation*

We next wanted to examine the extent to which MJM-1 alters hippocampal histone acetylation relative to NaBu. We expected to see that MJM-1 treatment alone would increase histone acetylation in the DH at sites on H2B, H3, and H4 proteins, which have been previously shown to be altered by long-term memory formation and synaptic plasticity (Peixoto & Abel, 2012). However, we found that the 20, 30, and 40 mg/kg doses of MJM-1 did not alter global H2B, H3, or H4 acetylation levels in DH tissue assayed 60

min following injection. These findings are surprising considering multiple doses of MJM-1 enhanced spatial memory consolidation in the object placement task. Moreover, they are inconsistent with our immunofluorescence data demonstrating there is a dose-dependent effect of MJM-1 on H3 acetylation, such that higher doses of MJM-1 increase overall H3 (Lys 9, 14) immunofluorescence relative to DMSO-treated cells. However, it is possible that MJM-1 increases global histone acetylation in this context because the DU145 cell lines naturally overexpress HDACs (Abbas & Gupta, 2008). Nonetheless, these findings suggest that MJM-1 treatment alone is not sufficient to alter hippocampal histone acetylation and likely acts through a different mechanism to enhance spatial memory formation in mice.

We also did not find any treatment effects of NaBu on H2B, H3, or H4 acetylation. We were surprised to find that 0.6 g/kg dose of NaBu did not alter histone acetylation when this dose was sufficient to enhance both spatial and object recognition memory consolidation in our object placement and recognition tasks. Although numerous studies have examined the beneficial effects of NaBu on long-term memory formation and in ameliorating behavioral deficits caused by neurodegenerative disease, very few of those studies examined the degree to which a single systemic dose of NaBu alters histone acetylation levels in the brain. For example, i.p. administration of 0.6 g/kg NaBu was previously found to enhance object recognition memory consolidation (Stefanko et al., 2009), but this group did not examine the extent to which this dose enhances object recognition memory by increasing hippocampal histone acetylation. There have been no studies that directly examined the extent to which a single i.p. injection of 0.6 g/kg NaBu modifies hippocampal histone acetylation levels 60 min following administration.

However, the majority of studies that examined effects of acute systemic NaBu administration predominately used a higher 1.2 g/kg dose and found that it increases H3 and H4 acetylation levels 30 min later in whole hippocampal tissue (Ferrante et al., 2003; Fischer et al., 2007; Gundersen & Blendy, 2009; Takuma et al., 2014).

There is evidence, however, that suggests NaBu alters global histone acetylation in a time- and dose-dependent manner. For example, Schroeder et al. (2007) found that a 1.2 g/kg dose of NaBu increased hippocampal H3 and H4 acetylation at 30 min, but this increase returned to baseline levels 60 min following injection in mice. They also demonstrated that NaBu dose-dependently increases H3 acetylation in the hippocampus, such that a single 0.4 g/kg and 1.2 g/kg, but not 0.2 g/kg, dose of NaBu was required to alter H3 and H4 acetylation levels 30 min following injection (Schroeder et al., 2007). Together, these findings suggest that we potentially missed the timepoint in which any histone alterations occurred by administering a lower dose of NaBu alone and by assaying hippocampal tissue at a later timepoint. Nonetheless, our findings suggest that a single dose of 0.6 g/kg NaBu is not sufficient to alter H2B, H3, or H4 acetylation levels in DH.

#### *Effect of Post-Training Treatment on Hippocampal Histone Acetylation*

Although dosing and timing are important considerations for maximally assaying global histone acetylation changes, another critical aspect that gives rise to HDACi's ability to alter cognition and histone acetylation is the presence of the stimuli itself. As such, we next wanted to explore the possibility that our behaviorally effective doses of MJM-1 and NaBu affect global histone acetylation patterns in the DH when administered following successful completion of object training. This idea is supported previous studies

demonstrating that when paired with an inefficient extinction protocol, i.p. administration of the class I HDACi CI-994 enhances extinction learning at remote timepoints and increases H3 acetylation in the hippocampus, but the effect on global acetylation changes is seen only when the inhibitor is paired with extinction training (Gräff et al., 2014). These data lend support to the possibility that HDACi such as NaBu and MJM-1 enhance long-term memory formation in a stimulus-dependent manner.

We expected to find an effect of object training alone, such that mice receiving a post-training injection of DMSO would have increased histone acetylation relative to Homecage controls. Furthermore, we also expected to see that mice receiving post-training injections of either NaBu or 40 mg/kg MJM-1 would have potentiated levels of histone acetylation relative to both Homecage control and DMSO-trained mice. However, to our surprise, we did not find effects of object training or HDACi treatment on global histone acetylation levels in the DH. These findings are largely inconsistent with previous work from our lab demonstrating hippocampal histone acetyltransferase activity is necessary for successful consolidation of object recognition memories (Zhao et al., 2012). Moreover, work from other groups has demonstrated spatial and object memories in rodents globally increase H2B, H3, and H4 acetylation in the hippocampus (Fischer et al., 2007; Bousiges et al., 2010; Zhao et al., 2010; 2012; Fortress et al., 2014). The reason for these discrepant effects remains unclear at the present time. Nonetheless, our findings suggest that NaBu and MJM-1 do not enhance memory consolidation by altering global histone acetylation levels within the DH.

### Effect on Hippocampal *bdnf* Expression

Finally, we examined the possibility that NaBu and MJM-1 enhance memory consolidation by promoting the transcription of *bdnf*. We found that there is no effect of MJM-1 treatment on DH expression of *bdnf* exons I and IV. However, we did find that mice receiving NaBu treatment had increased *bdnf* exon I, but not exon IV, transcripts relative to MJM-1 treated mice.

The lack of a treatment effect for MJM-1 on hippocampal *bdnf* expression is not surprising considering this compound does not alter histone acetylation patterns in the DH when administered alone or following object training. Nevertheless, previous reports have demonstrated that numerous different HDACi promote *bdnf* transcription in the rodent brain. For example, a single i.p. injection of NaBu immediately following object training increased mRNA expression levels of *bdnf* exons I and IV relative to vehicle-trained controls in the hippocampus (Intlekofer et al., 2013). Similarly, chronic subcutaneous injections of NaBu increased BDNF immunoreactivity in hippocampus (Kim et al., 2009). Another study compared the effects of class I-selective HDACi MS-275 against the class II-selective HDACi MC1568 in rat primary neurons and found that both increased *bdnf* expression (Koppel & Timmusk, 2013). Although we only measured *bdnf* exons I and IV in the hippocampus, alterations in expression levels of these transcripts are regulated in part by chromatin remodeling through alterations to histone acetylation (Cunha et al., 2010; Leal et al., 2015). As such, these transcripts serve as a reliable proxy for examining how MJM-1 affects gene expression patterns relative to extant HDACi.

### Conclusions and Future Directions

The current study demonstrates that MJM-1 enhances spatial, but not object recognition, memory consolidation, but does not affect hippocampal histone acetylation levels or alter expression of *bdnf* exons I or IV. There are a number of future directions to expand upon our behavioral and molecular findings. Future studies should examine whether MJM-1 enhances spatial and object recognition memory when administered chronically prior to object training. The present study administered MJM-1 immediately following training in order to pinpoint the beneficial effects of MJM-1 to the consolidation phase of memory formation. However, more translationally relevant results can be obtained by examining whether lower doses of MJM-1 administered more frequently can have similar beneficial effects on memory formation. Future behavioral work should also assess the extent to which MJM-1's memory-enhancing effects generalize to other spatial memory tasks (i.e., Morris water maze).

It remains unclear at the present time how MJM-1 enhances spatial memory consolidation in mice. However, our findings indicate that MJM-1 does not regulate hippocampal histone acetylation or *bdnf* expression levels to promote spatial memory formation. It is possible that MJM-1 has more localized effects on histone acetylation levels rather than affecting bulk histone acetylation. Future studies should examine the extent to which MJM-1 regulates synaptic plasticity gene expression and alters histone acetylation at promoter regions of upregulated genes. It would also be interesting to further gauge the extent to which MJM-1 affects chromatin remodeling by assaying expression of the "writers" and "readers" of histone acetylation, including histone acetyltransferases and bromodomains, respectively (Marmorstein & Zhou, 2014).

Moreover, it is also possible that MJM-1 regulates acetylation of non-histone substrates such as transcription factors, nuclear receptors, and enzymes (Yang & Seto, 2007). In this regard, future experiments could examine the extent to which MJM-1 modifies expression transcription factors known to regulate memory formation (i.e., *Creb*, *Egr-1*, *AP-1*). Although the molecular mechanisms underlying MJM-1's beneficial effect on spatial memory consolidation remain unclear at the present time, this work provides a useful framework for how to better develop small-molecule HDACis that can be used to improve cognition.

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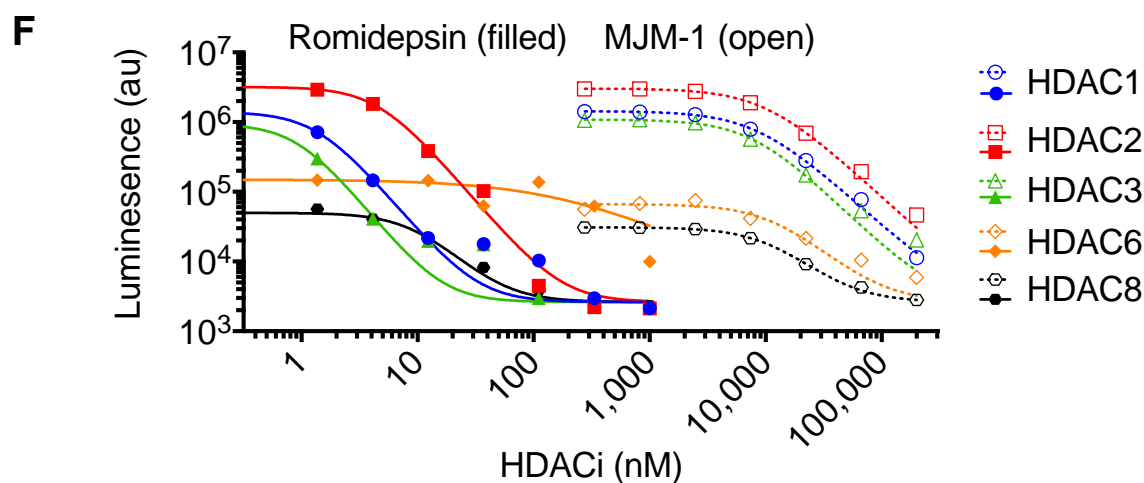
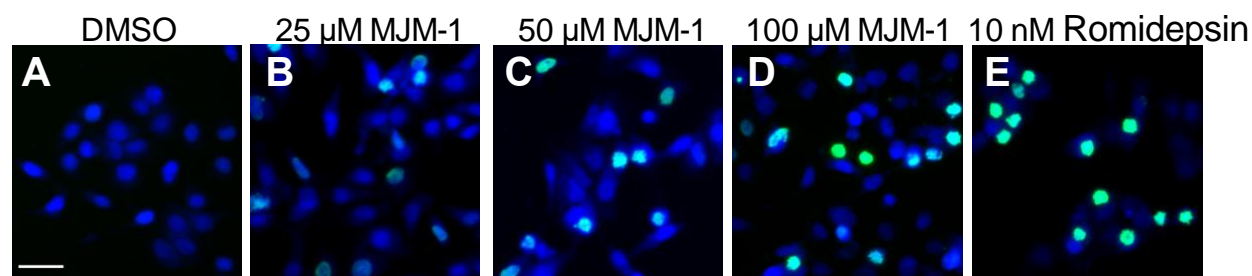
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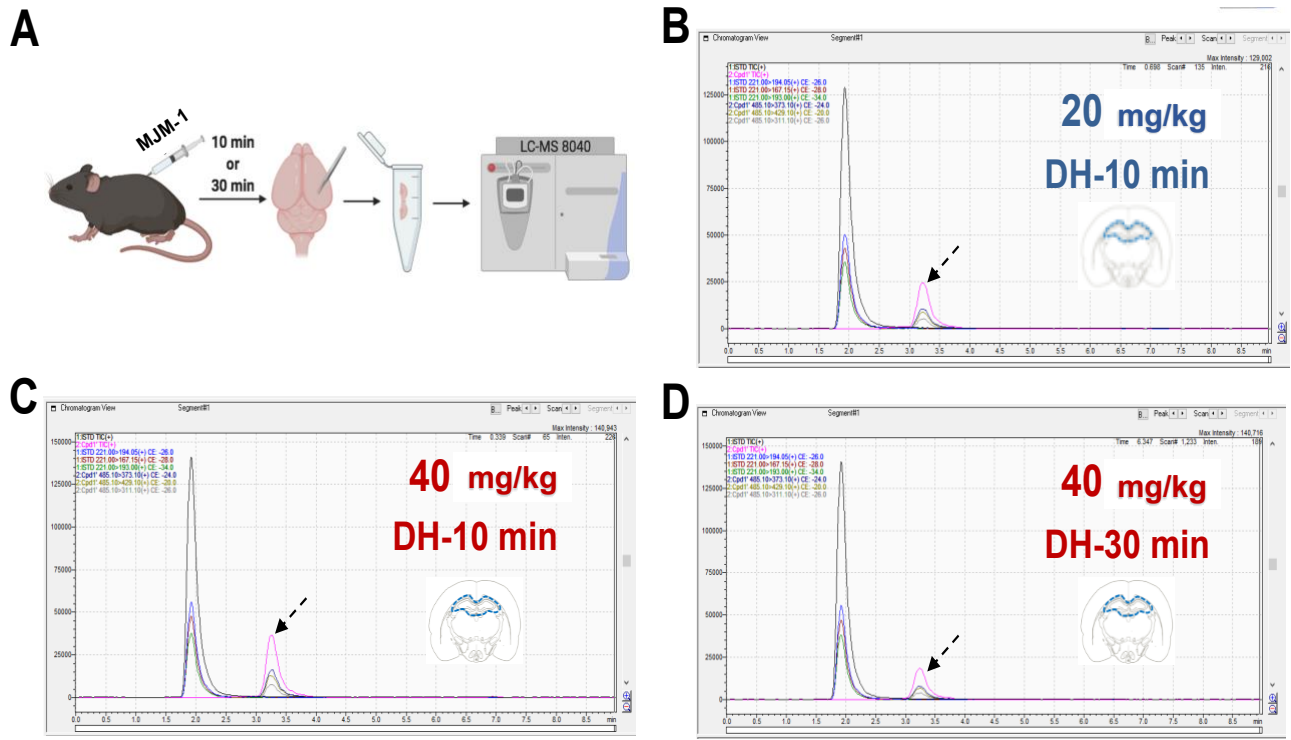
## APPENDIX



	Class	Romidepsin (nM)	MJM-1 (μM)	
■	HDAC1	I	$1.4 \pm 0.1$	$8.7 \pm 0.5$
■	HDAC2	I	$4.7 \pm 0.2$	$10 \pm 0.7$
■	HDAC3	I	$0.9 \pm 0.1$	$8.0 \pm 0.4$
■	HDAC6	IIb	$180 \pm 160$	$12 \pm 0.4$
■	HDAC8	I	$10 \pm 2.1$	$11 \pm 1.0$

**Appendix Figure 1. Ability of MJM-1 to increase histone acetylation and inhibit HDAC activity.**

(A-E) Representative images of histone H3 acetylation after treatment with MJM-1 or romidepsin. DU145 tumor cells were treated with the indicated concentrations of MJM-1 or romidepsin for 24 hrs. Cells were labeled with DAPI (blue) to identify nuclei and stained for acetylated H3 (green). Panel (A) shows cells treated with DMSO; (B) treated with 25 μM MJM-1; (C) treated with 50 μM MJM-1; (D) treated with 100 μM MJM-1; and (E) treated with 10nM FK228. Scale bar = 50 μm. (F) Ability of HDAC1 (circles), HDAC2 (squares), HDAC3 (triangles), HDAC6 (diamonds), or HDAC8 (hexagons) to deacetylate peptides in the presence of either FK228 (filled symbols) or MJM-1 (open symbols). All reactions were performed in triplicate with averages plotted and fitted to a concentration response equation using non-linear regression (GraphPad Prism). The table lists the concentration of either FK228 or MJM-1 needed to inhibit HDAC-catalyzed reactions by 50% (IC<sub>50</sub>). Deacetylation was coupled to aminoluciferin and luciferase such that luminescence (arbitrary units, au) linearly reflects deacetylase activity. Each reaction contained the same HDAC concentration (0.5 nM) and indicated amounts of a HDACi. Uncertainties reflect 95% confidence intervals of the curve fits.



**Appendix Figure 2. MJM-1 detected in the DH by LCMS-8040 mass spectrometry.** A) Schematic of i.p. administration and tissue collection procedure. The chromatograms (B-D) each depict a control internal standard (black peak) on left and MJM-1 (pink peak) on right. B,C) The 20 and 40  $\mu\text{g/g}$  dose of MJM-1 (dashed arrow) is detected in the DH at 10 min following i.p. injection. D) The 40  $\mu\text{g/g}$  dose can be detected in the DH to a lesser extent at 30 min.