

Inhibitors of Class I Histone Deacetylases Reverse Contextual Memory Deficits in a Mouse Model of Alzheimer's Disease

Mark Kilgore^{1,4}, Courtney A Miller^{1,4}, Daniel M Fass^{2,3}, Krista M Hennig^{2,3}, Stephen J Haggarty^{2,3}, J David Sweatt¹ and Gavin Rumbaugh^{*,1}

¹Department of Neurobiology, Evelyn F McKnight Brain Institute, University of Alabama at Birmingham School of Medicine, Birmingham, AL, USA;

²Stanley Center for Psychiatric Research, Broad Institute of Harvard University and Massachusetts Institute of Technology, Cambridge, MA, USA;

³Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by cognitive impairments that progress to dementia and death. The earliest symptoms of AD present as a relatively pure deficit in memory retrieval. Therefore, drug treatments that intervene in the early stages of AD by rescuing memory deficits could be promising therapies to slow, or even reverse progression of the disease. In this study, we tested the potential of systemic histone deacetylase inhibitor (HDACi) treatment to rescue cognitive deficits in a mouse model of AD. APPswe/PS1dE9 mice showed pronounced contextual memory impairments beginning at 6 months of age. Chronic HDACi injections (2–3 weeks) did not alter contextual memory formation in normal mice, but had profound effects in transgenic animals. Injections of sodium valproate, sodium butyrate, or vorinostat (suberoylanilide hydroxamic acid; Zolinza®) completely restored contextual memory in these mutant mice. Further behavioral testing of the HDACi-treated transgenic mice showed that the newly consolidated memories were stably maintained over a 2-week period. Measurement of the HDAC isoform selectivity profile of sodium valproate, sodium butyrate, and vorinostat revealed the common inhibition of class I HDACs (HDAC1, 2, 3, 8) with little effect on the class IIa HDAC family members (HDAC4, 5, 7, 9) and inhibition of HDAC6 only by vorinostat. These preclinical results indicate that targeted inhibition of class I HDAC isoforms is a promising avenue for treating the cognitive deficits associated with early stage AD.

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INTRODUCTION

Alzheimer's disease (AD) is characterized by debilitating memory impairments and significant neural degeneration. The earliest stages of AD are characterized by a relatively pure impairment of episodic memory (American Psychiatric Association, Task Force on DSM-IV, 1994). As the disease progresses, neurodegeneration in the temporal and frontal lobes becomes more profound and symptoms worsen (Braak *et al*, 1998b). These memory impairments result in a poor quality of life for the patient and hardship for the patient's family. As a result, developing therapeutic strategies to enhance the encoding, maintenance, and retrieval of memories is critical for improved AD patient outcomes.

The brains of AD patients have extensive extracellular β -amyloid (A β) plaques and intracellular neurofibrillary tangles (Braak and Braak, 1998a; Dickson, 1997). The extracellular plaques are formed through deposits of insoluble A β aggregates secreted from neurons. Recently, several studies have shown that soluble oligomers of A β are sufficient to cause structural and functional changes to neurons (Haass and Selkoe, 2007; Walsh *et al*, 2002). As a result, elevated levels of A β are believed to contribute to the cognitive impairments associated with AD (Lambert *et al*, 1998). A certain fraction of AD cases are early onset, and arise through autosomal inheritance of one of several causative genetic mutations (Selkoe, 2001). Several transgenic mouse lines have been developed that model the amyloidosis thought to underlie the onset of familial AD (Gotz *et al*, 2004; Wong *et al*, 2002). Overexpression of these human mutations in mice induces amyloidosis and these animals have significant cognitive impairments. In particular, mice that overexpress the delta exon 9 variant of presenilin-1 (PS1), in combination with the Swedish mutation of amyloid precursor protein (APP), have considerable levels of the aggregating form of A β , A β -42 (Jankowsky *et al*, 2004). Plaque deposition in this line is very aggressive

*Correspondence: Dr G Rumbaugh, Department of Neurobiology, Evelyn F McKnight Brain Institute, University of Alabama at Birmingham, 972 Shelby Building, 1825 University Blvd, Birmingham, AL 35294, USA, Tel: +1 205 996 6412, Fax: +1 205 975 5097, E-mail: grumbaugh@nrc.uab.edu

⁴These authors contributed equally to this work.

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and occurs as early as 6 months of age (Jankowsky *et al*, 2004), though cognitive impairments are not detected until much later in the cogenic line (Savonenko *et al*, 2005; but see also Jankowsky *et al*, 2005). Therefore, if early memory deficits were uncovered in this mouse line, it could serve as a particularly useful model to study early stage memory impairments associated with AD.

Episodic memory is severely disrupted in patients with AD. Studies over the past 50 years have shown that the medial temporal lobe, particularly the hippocampal formation and associated cortical areas, are directly involved in the encoding and consolidation of these memories (Eichenbaum, 2000; Squire *et al*, 2004). Associative learning activates biochemical signaling pathways that trigger the synthesis of new proteins necessary for the consolidation of hippocampus-dependent memories (Kandel, 2001; McGaugh, 2000). Because memory consolidation in the hippocampus is particularly susceptible to dysfunction during the early stages of AD, we hypothesized that the molecular mechanisms required for the synthesis of memory-promoting proteins are dysfunctional in AD. Consistent with this idea, pharmacologically stimulating gene transcription regulators that participate in memory formation may enhance cognitive performance in patients with this disease.

Histone acetyltransferases (HATs) catalyze the acetylation of lysine residues on core histone proteins, relaxing chromatin structure and facilitating gene transcription (Berger, 2007; Jenuwein and Allis, 2001; Kouzarides, 2007). This post-translational modification is reversed by histone deacetylases (HDACs), which remove the acetyl groups, promoting compaction of chromatin and a reduction in the transcription of corresponding genes. Histone acetylation occurs in the hippocampus in response to contextual learning (Levenson *et al*, 2004), and reducing the levels of histone acetylation by preventing HAT activity promotes amnesia and interferes with the consolidation of hippocampus-dependent memories (Alarcon *et al*, 2004; Korzus *et al*, 2004; Wood *et al*, 2006). In contrast, inhibiting HDACs, which artificially increases the acetylation and transcription of some genes, enhances hippocampus-dependent memory formation (Guan *et al*, 2009; Levenson *et al*, 2004; Vecsey *et al*, 2007; Wood *et al*, 2006). Recently, inhibitors of histone deacetylases (HDACi's) have been shown to restore cognitive function in an inducible model of neurodegeneration (Fischer *et al*, 2007). Therefore, we investigated the potential for HDACi treatment to restore memory consolidation and facilitate the storage of information in a mouse model of AD.

MATERIALS AND METHODS

Animals

All mice used in this study were APP^{swe}/PS1^{dE9} double-transgenic mice (APP/PS1) originally described as Line 85 (Jankowsky *et al*, 2004), and were obtained from the Jackson Laboratory (Bar Harbor, ME) with strain name B6C3-Tg (APP^{swe}, PSEN1^{dE9})85Dbo/J; stock number 004462). These mice express a chimeric mouse/human APP transgene that contains the Swedish mutations (K595N/M596L) as well as a mutant human PS1 transgene

carrying the deleted exon 9 variant under control of mouse prion promoter elements. The two transgenes co-segregate in this mouse line (Jankowsky *et al*, 2004). Mice were maintained as double hemizygotes by crossing transgene-positive males with wild-type (WT) females on a B6C3F1/J background strain (Jackson Laboratories: stock number 100010). Similar numbers of male and female subjects were used in each experiment. All mice were genotyped through tail clips and subsequent PCR analysis of genomic DNA. Mice were housed under 12:12 light/dark cycles, with food and water available *ad libitum*. All procedures were performed in accordance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee and with national regulations and policies.

Drugs

Sodium valproate (VPA; Sigma; P-4543) was dissolved in 0.1 M PBS and mice were given 100 mg/kg doses. Sodium butyrate (NaB; Sigma; 19364) was dissolved in Milli-Q water and mice received 1.2 g/kg doses. Suberoylanilide hydroxamic acid (SAHA) was synthesized by Medicilon/MPI Preclinical Research as described previously in WO 93/07148 PTC/US92/08454 (Gediya *et al*, 2005) and was given to mice as 50 mg/kg doses. SAHA was dissolved in 100% DMSO at a concentration of 50 mg/ml and then further diluted to 5 mg/ml in 0.9% saline. Injections (10 ml/kg) were given intraperitoneally for all drugs and were alternated daily between left and right sides of the abdomen.

Behavioral Procedures

For contextual fear conditioning, mice were placed into the training chamber and allowed to explore for 2.5 min, after which they received an electric footshock (2 s, 0.75 mA). After the shock, the mice remained in the chamber for an additional 28 s before being returned to their home cage. To test for long-term memory (LTM), all mice were returned to the training chamber 24 h later, and allowed to explore the context for 5 min. For reexposure experiments, mice were injected for an additional 14 days after training, before a second, 5 min reexposure to the context. Injections were given 2 h before training and testing. Activity was determined using an algorithm (Video Freeze; Med Associates) that subtracts differences between successive video frames. Freezing was defined as raw activity counts that fall below a preset threshold for more than 1 s. The algorithm was tested for accuracy by manually scoring at least three randomly chosen videos in each experiment. In all cases, the algorithm performed as accurately as a human observer.

Isolation of Hippocampus and Histone Extraction

Brains were first immersed in oxygenated (95%/5% O₂/CO₂) ice-cold cutting saline (in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH₂PO₄, 28 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 5 glucose, 0.6 ascorbate) immediately after rapid decapitation. The hippocampus was then dissected, frozen on dry ice, and stored at -80°C overnight. For histone extractions, all procedures were performed on ice and all solutions were chilled to 4°C before use unless otherwise indicated. All centrifugation steps were performed at 4°C. Tissue

homogenates were centrifuged at 7700 g for 1 min. The supernatant (cytoplasmic fraction) was aspirated and the pellet (nuclear fraction) was resuspended in 1 ml 0.4 N H₂SO₄. Histones were acid extracted from the nuclear fraction for 30 min, followed by centrifugation at 14 000 g for 10 min. The supernatant was transferred to a fresh tube, and proteins were precipitated with 100% trichloroacetic acid containing deoxycholic acid (Na+ salt; Sigma) for 30 min. Precipitated proteins were collected by centrifugation at 14 000 g for 30 min. The supernatant was discarded and the protein pellet was washed with 1 ml of acidified acetone (0.1% HCl) followed by 1 ml acetone, for 5 min each. Protein precipitates were collected between washes by centrifugation (14 000 g, 5 min). The resulting purified proteins were resuspended in 50 mM Tris (pH 8.0) and stored at -80°C.

Western Blotting

Acetylation levels of histone extracts were assayed by western blotting. Loading buffer was added (final concentration: 6.25 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 1.25% 2-mercaptoethanol, 0.1% bromophenol blue) and protein extracts (1 µg) were separated by SDS-PAGE on a 15% resolving gel and transferred to PVDF membranes for immunoblotting. Membranes were briefly rinsed with 100% methanol, air-dried for 15 min, and washed with TBST (in mM: 150 NaCl, 20 Tris (pH 7.5), 0.05% Tween 20) for 5 min at room temperature. The membranes were then blocked in 3% bovine serum albumin (BSA) in TBST for 45 min at room temperature and then incubated in primary antibody (1:5000 for all primary antibodies used) containing TBST and 5% BSA for 1 h at room temperature. The following anti-rabbit primary antibodies were used: anti-histone H3, anti-acetyl histone H3 (Lys-14), anti-histone H4, anti-acetyl-H4 (Lys-5/Lys-8/Lys-12/Lys-16) (all supplied by Upstate). This was followed by three washes in TBST and incubation in horseradish-peroxidase-conjugated anti-rabbit secondary antibody (1:10 000; Vector Labs) for 45 min at room temperature. The membranes were again rinsed in TBST and immunolabeling of membranes was detected by chemiluminescence (ECL; Pierce). Films were scanned and images were analyzed by densitometry.

HDAC Biochemical Assays

The *in vitro* activities of recombinant human HDACs 1-9 (BPS Biosciences) were measured with a 384-well-plate-based fluorometric deacetylase assay making use of acetylated tripeptide substrates that are amide-coupled to 7-amino-4-methylcoumarin that can detect either class I/IIb (substrate MAZ1600) or class IIa/HDAC8 (substrate MAZ1675) HDAC activity as described in detail in Bradner *et al* (2009), with the following modifications: HDAC1 (4.5 ng per reaction; MAZ1600 K_m = 6 µM); HDAC2 (4 ng per reaction; MAZ1600 K_m = 4.5 µM); HDAC3 (2 ng per reaction; MAZ1600 K_m = 9.5 µM); HDAC4 (0.1 ng per reaction; MAZ1675 K_m = 8 µM); HDAC5 (1 ng per reaction; MAZ1675 K_m = 57 µM); HDAC6 (50 ng per reaction; MAZ1600 K_m = 7 µM); HDAC7 (0.3 ng per reaction; MAZ1675 K_m = 20 µM); HDAC8 (3 ng per reaction; MAZ1675 K_m = 530 µM); HDAC9 (1.5 ng per reaction; MAZ1675 K_m = 37 µM); TCEP

was omitted from the assay buffer. Rates of reactions (slopes) were normalized to the mean of DMSO control treatments for each enzyme on each plate.

Statistical Analysis

One- or two-way univariate or repeated-measures analysis of variance was applied to all data. Details on the specific statistical model used in each experiment can be found in the figure legends. Student's *t*-tests and the Scheffe's *post hoc* test were used when necessary. Significance was set at $P \leq 0.05$ for all tests.

RESULTS

To test the hypothesis that systemic injections of HDACi's are able to rescue early cognitive deficits in an AD model, we first performed a series of experiments to establish when hippocampus-dependent memory deficits first appear in APP/PS1 mice. We chose to use contextual fear conditioning as a memory assay for two reasons. This associative learning paradigm is hippocampus dependent (Phillips and LeDoux, 1992), requires only a single trial, and results in a robust, long-lasting memory for a context. Thus, fear conditioning allows for the analysis of multiple stages of memory in a brain region thought to be severely affected in early stage AD. WT and aged-matched double-transgenic mice were trained at either 4 or 6 months of age with a mild training paradigm. The specific protocol was designed to yield approximately 40-50% freezing in a retrieval test, enabling us to detect both enhancements and deficits in memory strength. Transgenic mice (4-month old) showed levels of freezing identical to aged-matched WT littermates during the LTM test (Figure 1a; $F_{(1,21)} = 0.07$, $P > 0.05$). In contrast, a pronounced 24-h memory deficit was observed in 6-month-old transgenic mice ($F_{(1,12)} = 6.53$, $P < 0.05$), indicating that contextual memory deficits are present in these mice as early as 6 months of age. Importantly, there were no differences between WT and APP/PS1 mice in the amount of exploratory activity during the 150 s period before the footshock at the time of training (4-month-old WT: 147.2 ± 19.9 , APP/PS1: 129.2 ± 18.8 , $F_{(1,20)} = 0.43$, $P > 0.05$; 6-month-old WT: 42.6 ± 8.4 , APP/PS1: 57.2 ± 15.2 , $F_{(1,12)} = 0.64$, $P > 0.05$). Nor were there differences in the amount of post-shock freezing during training (4-month-old WT: 23.7 ± 5.5 , APP/PS1: 21.4 ± 5.3 , $F_{(1,20)} = 0.10$, $P > 0.05$; 6-month-old WT: 31.4 ± 8.3 , APP/PS1: 31.8 ± 11.5 , $F_{(1,12)} = 0.001$, $P > 0.05$). Given the memory deficit in the 6-month-old transgenic mice, we next tested their ability to acquire context-shock associations. WT and transgenic mice were given a retrieval test 1 h after training. Interestingly, APP/PS1 mice had normal freezing at this time point (Figure 1b; $F_{(1,13)} = 0.17$, $P > 0.05$). This intact short-term memory indicates that APP/PS1 mice are able to acquire contextual associations successfully at this age. Taken together, these data indicate that 6-month-old APP/PS1 mice have deficits in memory formation arising from abnormal consolidation and/or retrieval processes.

We next tested the possibility that HDACi's ameliorate the memory deficits in these mice. WT and APP/PS1 mice were chronically treated with either vehicle or valproic acid

before training with the mild fear conditioning protocol. At 24 h after training, all mice were given a 5 min retrieval test. Similar to our finding (see Figure 1), we detected an interaction between genotypes ($F_{(3,23)} = 19.10$, $P < 0.001$).

Vehicle-treated APP/PS1 mice had significantly lower freezing compared to vehicle-treated WT littermates during the LTM test (Figure 2a; $t_{(12)} = 5.69$, $P < 0.005$). We also

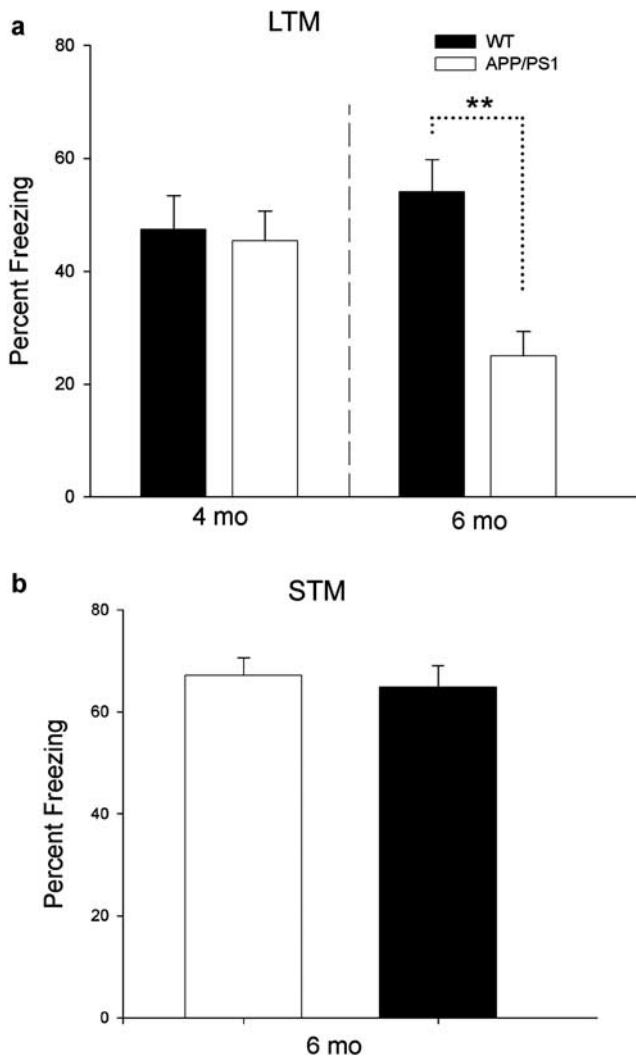
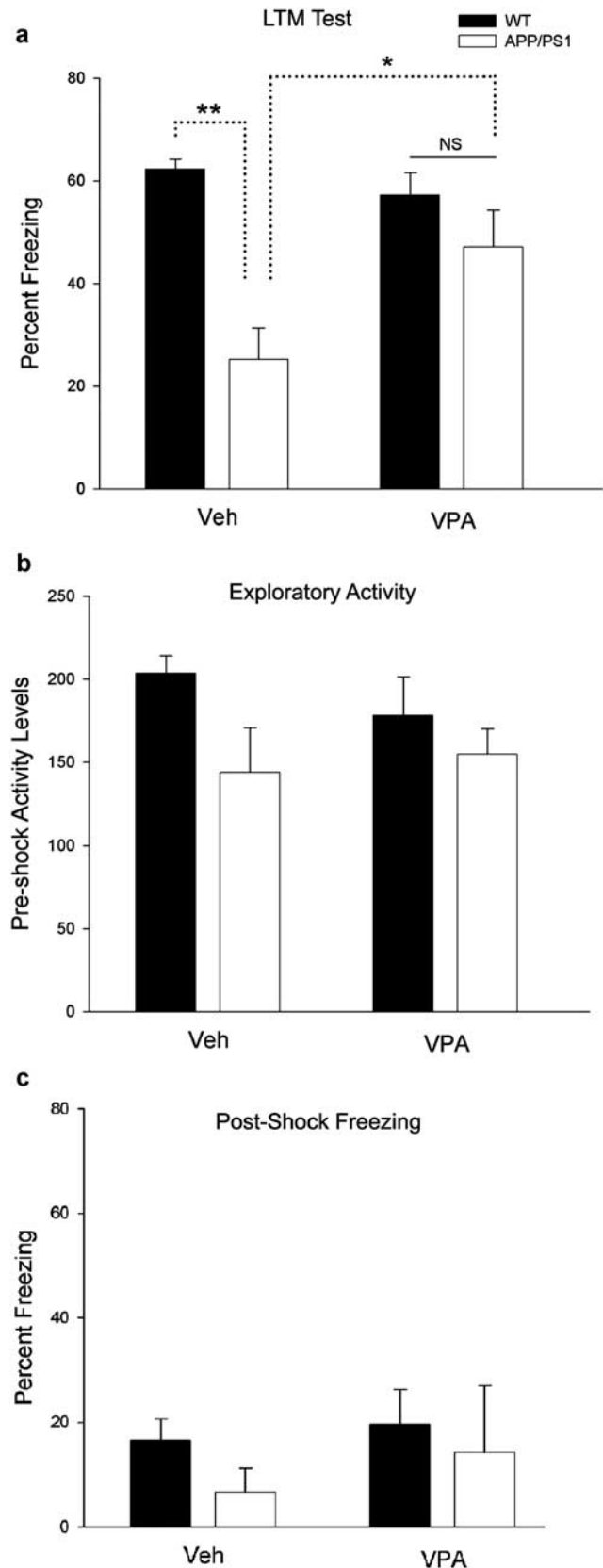


Figure 1 APPswe/PS1dE9 mice develop contextual memory deficits with age. (a) Wild-type (WT) and APPswe/PS1dE9 (4- or 6-month-old) mice were trained in a contextual fear conditioning paradigm (4 months: WT $n = 11$, APP/PS1 $n = 11$; 6 months: WT $n = 6$, APP/PS1 $n = 7$). After 24 h, all mice were reexposed to the conditioning context. Percent freezing was calculated for each animal. Analysis of variance (ANOVA), $**P < 0.01$. (b) WT and APP/PS1 (6-month-old) mice were trained in a contextual fear conditioning paradigm (WT $n = 7$, APP/PS1 $n = 7$). After 1 h, all mice were reexposed to the context to assess short-term memory. Percent freezing was calculated for each animal. ANOVA, $P > 0.05$.

Figure 2 Sodium valproate treatment rescues contextual memory formation in APPswe/PS1dE9 mutants. (a) Wild-type (WT) and APP/PS1 mice were injected with either vehicle (VEH) or 100 mg/kg sodium valproate (VPA) daily for 2 weeks (VEH: WT $n = 7$, APP/PS1 $n = 7$; VPA: WT $n = 6$, APP/PS1 $n = 7$). All mice were then trained in a contextual fear conditioning paradigm and tested for long-term memory 24 h later by reexposure to the conditioning context. Freezing was measured for each animal. Two-way univariate analyses with Scheffe's *post hoc* tests were performed. $*P < 0.05$, $**P < 0.01$. (b) Exploratory activity was measured for each animal during 150 s period before the footshock. (c) Percent freezing was calculated for each animal during the 28 s period immediately after the footshock.



detected a drug by genotype interaction ($F_{(3,23)} = 6.32$, $P < 0.05$). VPA treatments did not alter freezing behavior in WT mice ($t_{(11)} = 1.12$, $P > 0.05$). In contrast, VPA-treated APP/PS1 mice had significantly higher freezing levels during the 24 h retrieval test when compared to vehicle-treated mutant littermates ($t_{(12)} = -2.33$, $P < 0.05$; Figure 2a). In addition, mutants treated with valproic acid were not significantly different from either WT group, indicating that HDACi treatments restored performance to normal levels for a 6-month-old mouse (Figure 2a; P 's > 0.05). We detected no significant differences among groups in exploratory activity or immediate, post-shock freezing during the training session (Figure 2b and c; see legend for statistics), indicating that there were no state-dependent effects associated with VPA treatment and that APP/PS1 mice do not differ from WT controls in their behavior during training.

In a separate series of experiments, we tested the hypothesis that other HDACi's would rescue freezing deficits in this AD model. Indeed, chronic treatments of SAHA (Figure 3a; see legend for statistics) or NaB (Figure 4a, see legend for statistics) completely restored performance deficits in APP/PS1 mice. We did not detect an effect of NaB on WT mice. Similar to VPA treatments, these compounds had no effect on exploratory activity or post-shock freezing levels (Figures 3b and c, and 4b and c). Taken together, our data strongly suggest that chronically treating APPswe/PS1dE9 double-transgenic mice with broad-spectrum HDACi's rescues freezing deficits by enhancing memory formation.

Contextual memories are initially consolidated in the hippocampus and amygdala, but are eventually stabilized over the course of days to weeks through increasing involvement of neocortical circuits (Dudai, 2004; Frankland and Bontempi, 2005; Medina *et al*, 2008). Because the mechanisms of memory formation and memory storage are distinct, we were curious if memories formed in HDACi-treated APP/PS1 mice successfully stabilized over time. To test this idea, mice from the previous NaB experiment continued to receive daily injections for an additional 14 days. All mice were again reexposed to the conditioning context and freezing behavior was measured. Overall, results from the 15-day retrieval tests were qualitatively similar to those observed during the 24 h retrieval test (Figures 4a and 5a; Genotype: $F_{(3,34)} = 7.92$, $P < 0.01$; Drug: $F_{(3,34)} = 23.45$, $P > 0.001$). Untreated APP/PS1 mice had freezing levels that were statistically different from all other groups (Figure 5a; Veh APP/PS1 vs Veh WT: $t_{(16)} = 4.42$, $P < 0.001$; Veh APP/PS1 vs NaB APP/PS1: $t_{(17)} = -4.43$,

$P < 0.001$), whereas freezing performance in the HDACi-treated APP/PS1 mice was not significantly different from either WT group (Drug by Genotype: $F_{(3,34)} = 2.71$, $P > 0.05$). This indicates that newly consolidated contextual

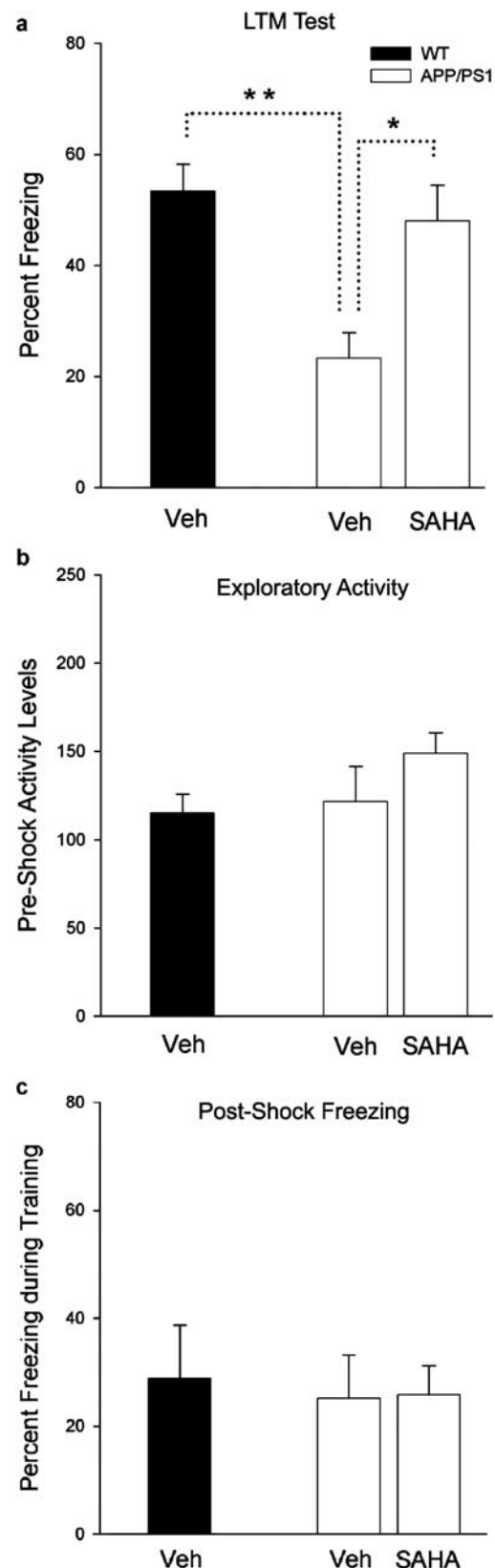


Figure 3 Suberoylanilide hydroxamic acid (SAHA) rescues memory deficits in APPswe/PS1dE9 mice. (a) Wild-type (WT) and APP/PS1 (6-month-old) mice were injected with either vehicle (VEH) or 50 mg/kg SAHA daily for 19 days (VEH: WT $n = 7$, APP/PS1 $n = 7$; NaB: APP/PS1 $n = 7$). All mice were then trained in a contextual fear conditioning paradigm and tested for long-term memory 24 h later. Freezing was measured for each animal. One-way analysis of variance (ANOVA) tests were performed, $F_{(2,20)} = 8.93$, $P < 0.005$. * $P < 0.05$, ** $P < 0.01$. (b) Exploratory activity was measured for each animal during 150 s period before the footshock ($F_{(2,18)} = 0.06$, $P > 0.05$). (c) Percent freezing was calculated for each animal during the 28 s period immediately after the footshock ($F_{(2,18)} = 1.50$, $P > 0.05$).

memories successfully achieve stability over time in mutants treated with HDACi's. Interestingly, we also detected a significant difference between WT mice treated with NaB relative to the WT vehicle group (Figure 5a; $t_{(17)} = -2.35$, $P < 0.05$), accounting for the lack of a drug by genotype interaction. This could be due to an increase

over time in the performance of the HDACi-treated animals or a decrease in the vehicle-treated groups. Therefore, we compared freezing responses between test days 1 and 15 within each group (Figure 5b). In both WT and APP/PS1 animals treated with vehicle, there was a significant reduction in freezing levels during the remote test relative to the recent test (Veh WT: $t_{(8)} = 2.54$, $P < 0.05$; Veh APP/PS1: $t_{(8)} = 2.42$, $P < 0.05$). There was no such decline in freezing in either HDACi-treated group (NaB WT: $t_{(9)} = 0.23$, $P > 0.05$; NaB APP/PS1: $t_{(9)} = 0.35$, $P > 0.05$), indicating that the memory was better preserved in the WT and APP/PS1 animals receiving the HDACi. It is possible that extinction was affected by HDACi treatment, perhaps explaining the preservation of freezing between test days 1 and 15 in both WT and APP/PS1 mice. To address this possibility, we analyzed freezing at 1 min intervals during the 5 min expression test 24 h after training (Figure 5c). There was no difference in freezing among WT groups and the HDACi-treated APP/PS1 group at any point during this test (P 's > 0.05), indicating that extinction learning was not altered by these compounds and was not a likely contributor to the preservation of freezing over the 2-week period.

We next isolated histone proteins from the hippocampus of WT and APP/PS1 mice and samples were subsequently immunoblotted for relative changes in histone acetylation. Interestingly, we did not detect significant differences in global H3 or H4 acetylation in the hippocampus when comparing genotypes in chronically injected mice (H3: $F_{(1,14)} = 0.67$, $P > 0.05$, $n = 7$ (WT), $n = 9$ (APP/PS1); H4: $F_{(1,16)} = 0.02$, $P > 0.05$, $n = 9$ (WT), $n = 9$ (APP/PS1)). These data suggest that memory deficits in these mice are not caused by global abnormalities in histone acetylation. However, HDACi injections do cause transient elevations in histone acetylation in mice (Figure 6), as we detected significant H4 acetylation within 2 h of a single vorinostat injection ($F_{(1,18)} = 4.59$, $P < 0.05$). Therefore, when given daily for several weeks, these inhibitors cause repeated cycles of histone acetylation/deacetylation globally throughout the genome, providing a reasonable mechanism underlying the restoration of memory formation in this AD model.

Understanding the specific HDAC isoforms that are targeted by HDACi to enhance memory formation is important. This would provide not only insight into the underlying molecular mechanisms involved in memory processes, but it may lead to the eventual design of more

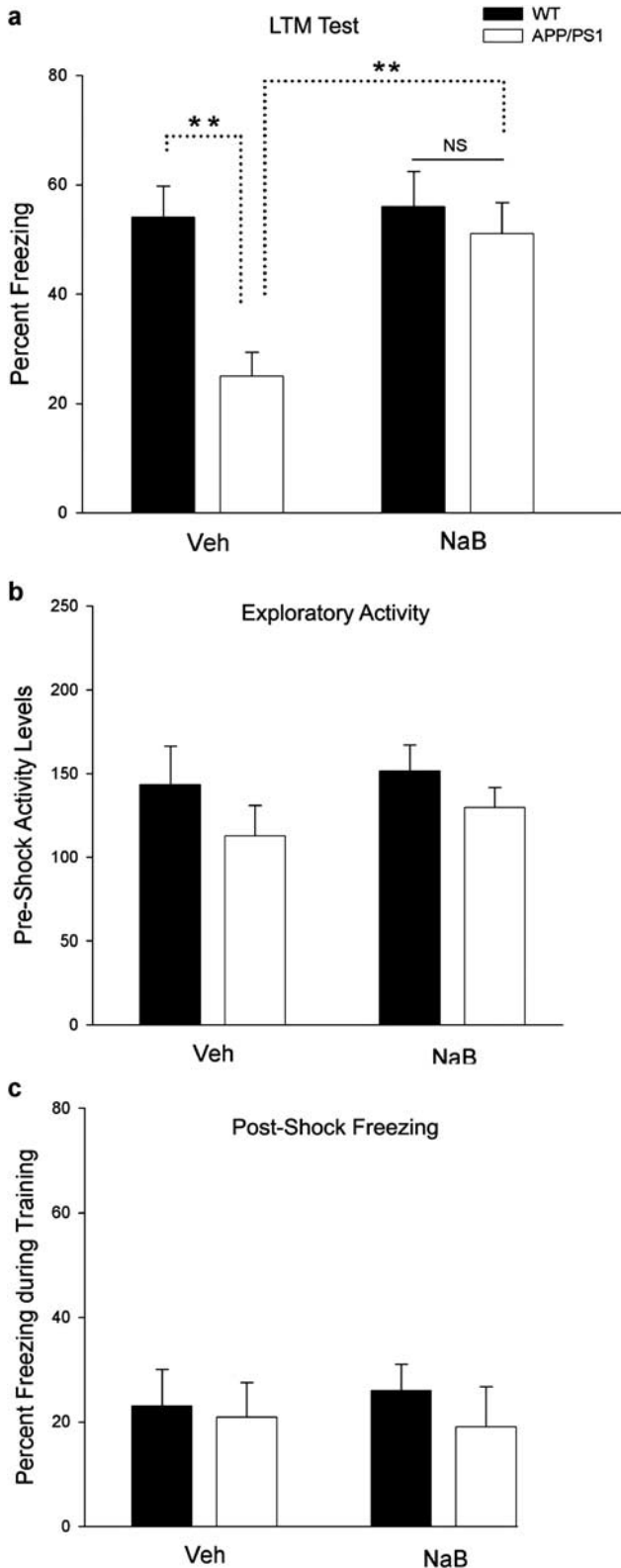


Figure 4 Sodium butyrate (NaB) rescues memory deficits in APPsw/PS1dE9 mice. (a) Wild-type (WT) and APP/PS1 (6-month-old) mice were injected with either vehicle (VEH) or 1.2 g/kg NaB for 21 days (VEH: WT $n = 9$, APP/PS1 $n = 9$; NaB: WT $n = 10$, APP/PS1 $n = 10$). All mice were then trained in a contextual fear conditioning paradigm and tested for long-term memory 24 h later. Freezing was measured for each animal. Two-way univariate analyses with Scheffe's *post hoc* tests were performed (Drug by Genotype: $F_{(3,34)} = 4.81$, $P < 0.05$; Veh WT vs Veh APP/PS1: $t_{(16)} = 4.14$, $P < 0.005$; Veh APP/PS1 vs NaB APP/PS1: $t_{(17)} = -3.57$, $P < 0.005$). ** $P < 0.01$. (b) Exploratory activity was measured for each animal during 150 s period before the footshock. (Drug: $F_{(3,34)} = 0.52$, Genotype: $F_{(3,34)} = 0.47$, Drug by Genotype: $F_{(3,34)} = 0.14$; $P > 0.05$ for all). (c) Percent freezing was calculated for each animal during the 28 s period immediately following the footshock. (Drug: $F_{(3,34)} = 0.01$, Genotype: $F_{(3,34)} = 2.28$, Drug by Genotype: $F_{(3,34)} = 0.07$; $P > 0.05$ for all).

selective and efficacious agents for the treatment of disorders involving cognitive deficits. However, to date there are conflicting reports on the isoform selectivity profile and potency of HDACi's. Previous studies have

reported that VPA, butyrate, and SAHA have a pan-isoform profile with half-inhibitory concentration (IC_{50}) values ranging from low micromolar to millimolar ranges. However, it has become clear recently that immunoprecipitated and even recombinantly expressed and purified class IIa HDACs (eg, HDAC4) associate with class I HDACs and that these co-purifying enzymatic activities can occlude the true selectivity and potency of inhibitor treatments (Lahm *et al*, 2007; Jones *et al*, 2008).

To overcome these limitations, we have implemented biochemical assays of the deacetylase activity of recombinant human HDAC1-9 expressed and purified from Sf9 insect cells. These assays rely on fluorogenic class I and class II HDAC substrates that show an improved ability in the latter case to specifically detect deacetylase activity from class II HDAC isoforms free of contribution of any co-purifying class I HDAC activity that otherwise confounds the data interpretation (Bradner *et al*, 2009). As summarized in Table 1, using these assays in a kinetic mode under conditions where appropriate substrates for each isoform are used at their K_m and the rate of the reaction is monitored over time, VPA (Supplementary Figure 1), NaB (Supplementary Figure 2), and phenylbutyrate (data not shown) were found to have IC_{50} 's in the low micromolar range against HDAC1-3 and 8 with effectively no inhibition against HDAC4-7 and HDAC9. In contrast, and similar to

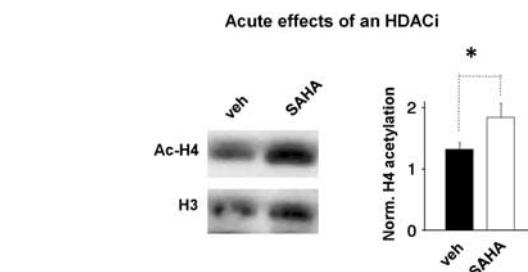
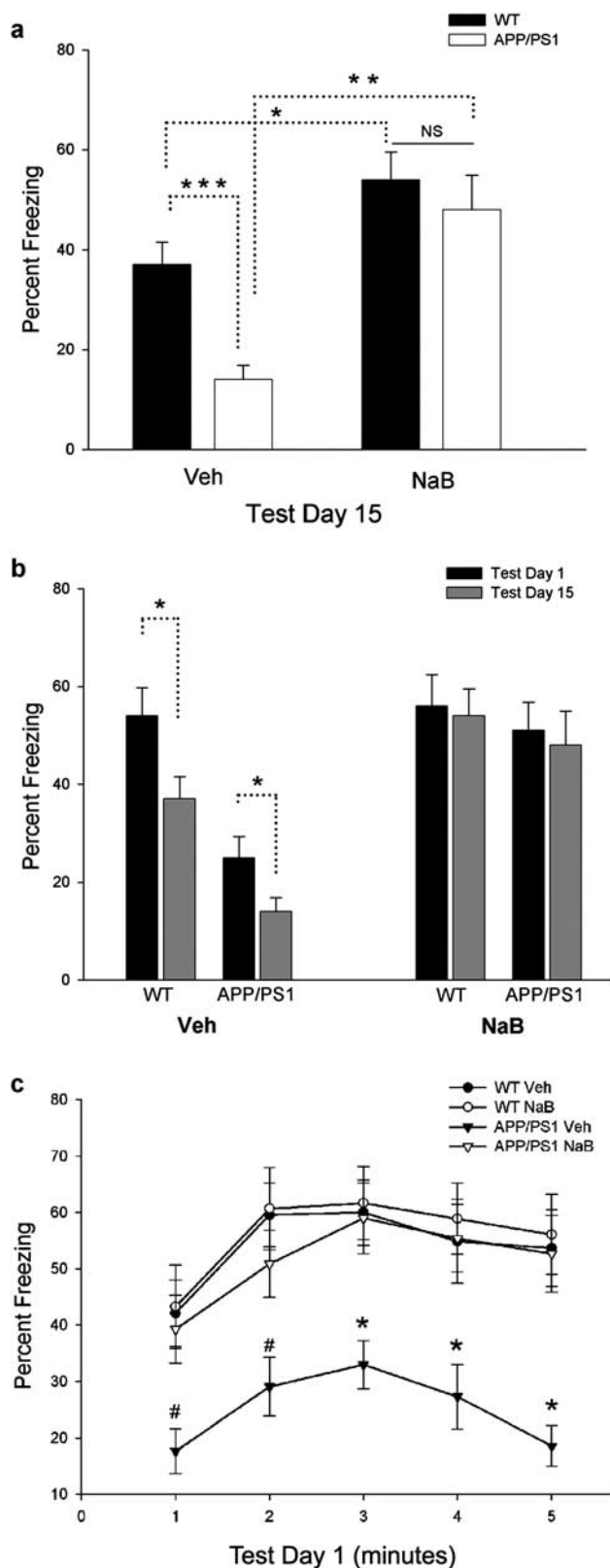


Figure 6 Systemic injections of inhibitors of histone deacetylases (HDACi's) cause elevations in global histone acetylation. Wild-type C57/Bl6 mice were injected once with vehicle ($n=5$) or 50 mg/kg suberoylanilide hydroxamic acid (SAHA, $n=5$) and then killed 2 h later. Purified histone proteins from the hippocampus were immunoblotted for acetylated H4 (Ac-H4) and total H3 (H3; loading control). Normalized H4 acetylation was calculated by dividing the integrated density (ID) of Ac-H4 by the ID of H3 for each sample. One-way analysis of variance (ANOVA, $*P<0.05$).

Figure 5 Sodium butyrate (NaB) treatment stabilizes newly consolidated memories in APPswe/PS1dE9 mutants. (a) Mice trained and tested (Figure 4) continued to receive daily vehicle or NaB for an additional 14 days. All mice were then reexposed to the conditioning context to reactivate the memory. Two-way univariate analyses with Scheffé's *post hoc* tests were performed. $*P<0.05$, $**P<0.01$, $***P<0.001$. (b) Freezing performance from the first (test day 1, 24 h after training) and second (test day 15, 2 weeks after training) retrieval tests are directly compared for each drug treatment and genotype. Paired samples *t*-tests were performed. $*P<0.05$. (c) Freezing behavior during the 5 min retrieval test given 24 h after training (Figure 4a) was analyzed in 1 min intervals to visualize the rate, if any, of extinction in each group. As reported, there was no difference at any time point between WT and APP/PS1 mice treated with NaB. APP/PS1 mice treated with Veh were, however, different from WT mice at the first two time points ($^{\#}P<0.05$) and different from all groups during minutes 3-5 ($*P<0.05$).

Table 1 IC₅₀ Values of HDAC Inhibitors that Increase Memory Formation in APPswe/PS1dE9 Mutant Mice

Compound	Class I				
	HDAC1	HDAC2	HDAC3	HDAC8	
Butyric acid	8.3 ± 0.5	7.0 ± 0.2	4.8 ± 0.2	10.4 ± 0.7	
Valproate	35.5 ± 3.1	59.3 ± 3.6	218.5 ± 22.5	97.1 ± 5.8	
SAHA	0.002 ± 0.0001	0.003 ± 0.0001	0.006 ± 0.0003	0.7 ± 0.07	
Compound	Class IIa				Class IIb
	HDAC4	HDAC5	HDAC7	HDAC9	HDAC6
Butyric acid	5725 ± 98.3	6403 ± 370	4380 ± 441	5614 ± 359	5881 ± 1303
Valproate	> 15 000	> 15 000	> 15 000	> 15 000	> 20 000
SAHA	> 25	> 15	> 25	> 25	0.004 ± 0.0004

IC₅₀ values (μM) of HDAC inhibitors that increase memory formation in APPswe/PS1dE9 mutant mice using recombinant human HDAC1–9 and an *in vitro* deacetylase assay with synthetic substrates for both class I and class IIa/IIb HDACs. Data shown are average values ± s.d. from *n* = 2 replicates performed on different 384-well plates.

what has been reported previously in the literature, SAHA (Supplementary Figure 3) showed nanomolar potency toward HDAC1–3 and 6 and a mild inhibition of HDAC8 while having IC₅₀ values >10 μM for HDAC4–7 and HDAC9. Thus, VPA, NaB, phenylbutyrate, and SAHA share in common the inhibition of the class I HDACs1–3, suggesting that *in vivo* the activity of all three, or a subset, of these HDAC family isoforms may be responsible for modulating memory formation. These data also explain the shared efficacy of these three distinct compounds regarding rescue of cognitive deficits in this AD model.

DISCUSSION

The key finding in our study is that three distinct inhibitors of class I HDACs completely rescue memory deficits in a mouse model of AD. Importantly, SAHA, which is FDA approved for the treatment of cancer under the trade name Zolinza®, was effective in restoring memory performance in this AD model. We believe that HDACi's rescue freezing deficits in APP/PS1 mice by improving memory formation. After chronic HDACi treatments, the fear memory of mutants is indistinguishable from WT mice. Importantly, HDACi treatments do not affect other measures of behavior in these tasks, including exploratory activity and immediate freezing. Thus, the observed rescue of freezing in response to exposure to the associative stimulus cannot be explained by state-dependent effects, such as reduced locomotion. Therefore, our data suggest that HDACi's rescue freezing deficits in the APP/PS1 mice by promoting memory formation through enhancing consolidation or by rescuing a retrieval deficit. This exciting finding provides important preclinical evidence for the hypothesis that HDACi's may improve cognitive function in AD patients.

We hypothesize that HDACi's promote memory formation in this AD model by enhancing histone acetylation. The mammalian genome encodes for 18 HDAC isoforms, which can be divided into four distinct classes (I–IV) based on a variety of factors, including catalytic mechanism, cofactors,

patterns of expression, and sensitivity to various classes of inhibitors (Haberland *et al*, 2009). Although the specific isoforms involved in memory and other neurological processes are poorly understood at this point, very recent work has begun the task of defining their functional roles in the CNS. For example, HDAC1 and 2, which are class I HDACs and nearly identical, are required for normal neuronal differentiation during development (Montgomery *et al*, 2009). Interestingly, Tsai and colleagues showed an additional key function for HDAC2 as a negative regulator of synaptic plasticity and memory formation in the adult CNS (Guan *et al*, 2009). In addition, the emotional salience of stimuli such as stress or drugs of abuse is regulated by activity of the class II HDAC5 within the nucleus accumbens, the brain's reward center (Renthal *et al*, 2007). In this study, we used contextual fear conditioning, a hippocampus-dependent task. Several HDAC isoforms are highly expressed in this brain regions (Broide *et al*, 2007). Using synthetic substrates that enable one to distinguish between class I and class II HDAC activities (Jones *et al*, 2008; SJ Haggarty, Personal Communication), we found that the HDACi's used in this study predominantly target the deacetylase activity of class I HDAC isoforms (HDAC1, 2, 3, 8), with HDAC6, a class IIb isoform, also being targeted by SAHA (Table 1). Thus, although we cannot determine which isoform(s) is critical for amelioration of the memory deficit or its subsequent maintenance from our current data, our findings are consistent with those of Guan *et al* (2009) in terms of supporting an important role for class I HDAC isoforms. As isoform-selective inhibitors are a vigorous area of development in cancer therapeutics, identifying the isoform(s) critical to our effects will likely provide potent, druggable targets for therapeutics aimed at the cognitive deficits associated with AD.

Our behavioral results are consistent with a recent study showing improvement of APPswe transgenic mice in a spatial learning task after repeated injections of the HDACi, phenylbutyrate (Ricobaraza *et al*, 2009). Our HDAC biochemical assay indicates that phenylbutyrate shares an HDAC isoform profile similar to butyrate and VPA. In contrast to our findings in 6-month-old APP/PS1 mice, the

authors observed a significant reduction in H4 acetylation in much older mice from this distinct transgenic line. The most parsimonious explanation for these contrasting results is that the studies used distinct mouse models at different ages. We used an aggressive model of familial AD for our studies, where plaque deposits appear between 5 and 6 months of age (Jankowsky *et al*, 2004; Jankowsky *et al*, 2005). In contrast, Ricobaraza *et al* (2009) used the TG2567 line that does not begin to develop plaque deposits or cognitive anomalies until 1 year of age (Gotz *et al*, 2004). Indeed, their study reported a deficit in H4 acetylation in animals that were 18 months of age (Ricobaraza *et al*, 2009). Therefore, it is possible that a global decrease in histone acetylation is promoted by a combination of advanced age and elevated levels of A β . In addition, we did not observe effects of NaB on freezing in WT mice at any age tested. This finding contrasts with a recent study that used a similar dosing schedule of NaB and contextual fear conditioning (Guan *et al*, 2009). There are several possible explanations for these discordant results. Our WT mice were much older and had a different genetic background than the mice used in the study by Guan *et al*. In addition, the fear conditioning protocol used in Guan *et al*. study resulted in very low freezing levels in saline-treated mice. Thus, in addition to genetic background differences that may impact the acquisition, encoding, or expression of conditioned fear, we speculate that NaB enhances contextual freezing in young mice presented with weak context-shock associations, but not in older mice that receive stronger training.

From a memory systems standpoint, we envision several processes that could be affected by chronic HDACi treatment. First, HDACi's may facilitate cognitive function in this model through broad network (ie, system) effects, possibly by promoting dendritic growth and synaptogenesis (Fischer *et al*, 2007; Guan *et al*, 2009). This would allow for alternate strategies to consolidate and/or retrieve information through enhanced neural connectivity (Buckner, 2004; Jankowsky *et al*, 2005). Another possibility may involve the ability of particular HDAC isoforms to deacetylate proteins in addition to histones, such as transcription factors (Yeh *et al*, 2004). Finally, this method could enhance memory consolidation by altering the cellular processes that support information storage. This hypothesis is distinct from the others, as it relates specifically to the cell and molecular processes that are believed to contribute to memory formation in the hippocampus. For instance, transcription of memory-promoting genes could be primed by HDACi treatments, essentially increasing the 'gain' of the molecular machinery that supports memory formation. In support of this idea, we show that a single dose of NaB results in a transient elevation of histone acetylation, though we did not detect a global elevation in histone acetylation after 3 weeks of NaB treatments. Thus, repeated, systematic HDACi dosing may have unique effects on gene activation resulting from the phasic regulation of global histone acetylation. This type of chronic treatment likely results in downstream biochemical effects that are distinct from those of a single dose of an HDACi. Indeed, post-training infusions of the HDACi trichostatin A enhance contextual fear conditioning and histone acetylation in WT mice (Vecsey *et al*, 2007). The absence of a similar memory enhancement in our WT

mice after HDACi treatment may be due to differences in the specific compound used, how it was delivered and how many times. For example, direct infusion of HDACi's into the hippocampus may regulate a subset of genes distinct from those targeted by repeated systemic injections. This distinction should not be underestimated. In contrast to acute delivery of HDACi directly into the hippocampus of AD mice (Francis *et al*, 2009), the chronic treatment used in this study likely causes a repeated cycling of histone acetylation and deacetylation. Importantly, this chronic dosing is likely analogous to the therapeutic treatment regimen that would be used in patients with AD. Future studies will hopefully assess the gene- and brain-region-specific effects of systemic vs acute class I HDAC inhibition in both normal and AD mice.

Our remote memory experiment revealed an additional positive effect of HDACi's on contextual memory. As expected, we observed memory deficits between 1- and 15-day retrieval tests in both genotypes treated with saline. In contrast, HDACi-treated mice, regardless of genotype, showed no sign of reduced memory between retrieval tests. These data suggest that HDACi's protect previously consolidated memories from weakening in response to time and/or reactivation. Several possibilities could account for the contextual freezing differences observed in saline-treated mice. Extinction of fear is one possible explanation. However, because fear memory was preserved in HDACi-treated mice, this would require that HDACi's negatively regulate extinction learning. Such an event is unlikely, given that HDACi's have recently been shown to enhance extinction of contextual memories (Bredy and Barad, 2008; Lattal *et al*, 2007). In addition, we did not observe a difference in extinction learning among groups in our studies. Therefore, we believe that the HDACi's are more likely to be regulating another aspect of memory stability; reconsolidation, maintenance or retrieval. It is possible that the process of reconsolidation is not entirely efficient (Bredy and Barad, 2008). This could result in a slight weakening of the memory following multiple retrieval tests. HDACi's may, therefore, improve the efficiency of reconsolidation. Alternatively, the 'HDACi-protected' memory could relate to the growing involvement of the cortex in system consolidation. The role of cortical association areas in contextual fear memories develops over the course of 2 weeks (Frankland *et al*, 2004; Matynia *et al*, 2008; Maviel *et al*, 2004), and synaptic plasticity in cortical circuits is believed to be an important contributor to this process (Frankland *et al*, 2001). Freezing responses may become less robust as fear memories are distributed across multiple networks. In this scenario, HDACi's could act to facilitate 'uploading' of contextual information to other brain areas, perhaps by enhancing cortical synaptic plasticity. Indeed, HDACi's have been shown to facilitate long-term potentiation at hippocampal synapses (Levenson *et al*, 2004; Guan *et al*, 2009), though it is currently not known if these compounds have similar effects on synaptic plasticity in cortical circuits. Finally, the memory deficits observed in vehicle-treated mice could be explained by the relative difficulty of retrieving a remote memory. If a stabilized memory is more difficult than a recent memory to retrieve, then HDACi's may facilitate this aspect of cognition. Given the obvious therapeutic applications for cognitive

improvement, it will be very interesting to further explore the effects of HDACi's on remote memory processes.

In summary, our study provides evidence that several distinct HDACi's rescue cognitive deficits in a genetically engineered mouse model of AD. At present, a variety of specific and potent HDACi's are under development and clinical testing. Our data strongly support the idea that facilitating histone acetylation through systemic delivery of HDACi's targeting class I HDAC's is a promising new avenue for the treatment of cognitive abnormalities associated with AD.

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DISCLOSURE

JDS is a consultant for EvVivo Pharmaceuticals. Except as noted in the acknowledgment, all other authors declare that, except for income received from primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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