SAHA Enhances Synaptic Function and Plasticity In Vitro but Has Limited Brain Availability In Vivo and Does Not Impact Cognition

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Abstract

Suberoylanilide hydroxamic acid (SAHA) is an inhibitor of histone deacetylases (HDACs) used for the treatment of cutaneous T cell lymphoma (CTCL) and under consideration for other indications. In vivo studies suggest reducing HDAC function can enhance synaptic function and memory, raising the possibility that SAHA treatment could have neurological benefits. We first examined the impacts of SAHA on synaptic function in vitro using rat organotypic hippocampal brain slices. Following several days of SAHA treatment, basal excitatory but not inhibitory synaptic function was enhanced. Presynaptic release probability and intrinsic neuronal excitability were unaffected suggesting SAHA treatment selectively enhanced postsynaptic excitatory function. In addition, long-term potentiation (LTP) of excitatory synapses was augmented, while long-term depression (LTD) was impaired in SAHA treated slices. Despite the in vitro synaptic enhancements, in vivo SAHA treatment did not rescue memory deficits in the Tg2576 mouse model of Alzheimer’s disease (AD). Along with the lack of behavioral impact, pharmacokinetic analysis indicated poor brain availability of SAHA. Broader assessment of in vivo SAHA treatment using high-content phenotypic characterization of C57Bl6 mice failed to demonstrate significant behavioral effects of up to 150 mg/kg SAHA following either acute or chronic injections. Potentially explaining the low brain exposure and lack of behavioral impacts, SAHA was found to be a substrate of the blood brain barrier (BBB) efflux transporters Pgp and Bcrp1. Thus while our in vitro data show that HDAC inhibition can enhance excitatory synaptic strength and potentiation, our in vivo data suggests limited brain availability may contribute to the lack of behavioral impact of SAHA following peripheral delivery. These results do not predict CNS effects of SAHA during clinical use and also emphasize the importance of analyzing brain drug levels when interpreting preclinical behavioral pharmacology.

Introduction

Histone deacetylases (HDACs) mediate epigenetic changes by decreasing histone acetylation, leading to condensed chromatin structure and decreased transcription [1,2]. HDACs can also impact cellular functions at various levels through deacetylation of non-histone proteins [3]. SAHA is a HDAC inhibitor that targets Class I and Class IIb Zn2+–dependent HDACs, causing increased acetylation. The altered gene regulation induced by SAHA treatment can arrest proliferation of cancer cells [4]. Also known as Vorinostat and marketed as Zolinza, SAHA is currently approved for the treatment of CTCL and is under consideration for treatment of other malignancies [5–7]. SAHA is also being considered for non-oncology indications including treatment of malaria infection and depletion of latent HIV reservoirs during antiretroviral therapy [8–10]. In the context of brain diseases, recent studies implicate excess HDAC function in Alzheimer’s disease (AD) and suggest decreasing HDAC function with drugs like SAHA could potentially improve cognitive functions [11]. In particular, HDAC2 has been shown to be upregulated in the brains of both AD patients and mouse AD models, and knocking down HDAC2 rescues impaired synaptic plasticity and neurodegeneration-associated memory deficits in an AD mouse model [12]. Furthermore, while transgenic HDAC2 over-expression impairs cognitive functions, HDAC2 knockout mice exhibit enhanced synaptic plasticity and memory function [13]. In another example, reducing HDAC6 function has been shown to protect against neurodegeneration induced by oxidative stress and promote axon outgrowth [14]. As SAHA can block numerous HDACs including HDAC2 and HDAC6, these observations raise the possibility that patients taking SAHA could experience...
neurological impacts. Such impacts could potentially be beneficial to improve brain function in AD patients. To address this possibility, we explored the impacts of SAHA treatment on neuronal function in vitro and on fear memory in AD model mice and general behavioral activity in wild type mice using the SmartCube® System [15–17]. While SAHA enhanced synaptic transmission and potentiation in vitro, we could not detect any effect of in vivo treatment on the behaviors measured. Consistent with a lack of neurobehavioral activity, SAHA exhibited poor brain penetration and was found to be a substrate of brain efflux transporters.

Materials and Methods

Ethics Statement

All animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Experiments performed at Genentech were approved by the Genentech Institutional Animal Care and Use Committee. Experiments performed at PsychoGenics were approved by the PsychoGenics Institutional Animal Care and Use Committee. Experiments performed at Cerebricon were approved by the National Animal Experiment Board of Finland, State Provincial Office of Southern Finland.

Slice Cultures

Interface cultures of hippocampal slices were made from 7–8 day old Sprague Dawley rats as previously described [18]. Briefly, hippocampi were dissected in minimum essential medium (MEM; Invitrogen, Eugene, OR) with 15 mm HEPES and 10 mm Tris buffer (Invitrogen). Four-hundred micrometer slices were cultured on Millicell CM culture plate inserts (Millipore, Temecula, CA). The culture medium consisted of 50% MEM, 25% HBSS, and 25% horse serum, with 12.5 mM HEPES buffer and penicillin (100 U/ml)streptomycin (100 μg/ml) (all from Invitrogen). Cultures were maintained in 5% CO2, at 37°C. Slices were maintained in vitro for one week prior to transfection.

Electrophysiology

Patch clamp recordings were made in oxygenated Artificial Cerebrospinal Fluid (ACSF) containing (in mM) 127 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1.25 Na2HPO4, 25 NaHCO3, 25 glucose. For voltage-clamp recordings patch pipette internal solution consisted of 120 Cs-methanesulfonate, 20 CsCl, 0.5 EGTA, 10 HEPES, 2.5 MgCl2, 4 Na2ATP, 0.3 Na3GTP and 10 Phosphocreatine. Action potential threshold was measured during the smallest depolarizing current injection steps that triggered spiking. Input resistance was calculated from the steady-state response to the smallest hyperpolarizing current injection steps to avoid contamination from hyperpolarization-induced inward currents (h-current). The h-current “sag” was measured based on the difference between the maximal and steady-state voltage responses to strong hyperpolarizing current injection steps. Significance of differences in all measures between treatment groups were assessed using a student’s t-test.

Tg2576 Fear Conditioning

Contextual fear conditioning was performed at Cerebricon, Ltd., (Finland). Tg2576 mice overexpressing human amyloid precursor protein (APP) with the ‘Swedish’ mutation, K670N/ M671L, under control of the prion promoter, were used. Female Tg2576 mice were treated once a day for 35 days, starting at approximately 5 months of age, with i.p. injections of 25 mg/kg SAHA, 50 mg/kg SAHA or vehicle (DMSO). Injection volume was 10 ml/kg and testing was done a minimum of 2 hours after dosing. Open field testing was performed on dosing day 32. Mice were brought to the experimental room for at least 1 h acclimation prior to testing in the open field. At least two hours after the last dosing, mice were placed in open field chambers equipped with infrared photocells (Med Associates, Inc., St. Albans VT; 27x27x20.5 cm) and their locomotor activity was monitored for 30 minutes. Contextual fear conditioning was performed on dosing days 33–34 using modification of published protocols [20]. The training and testing were conducted on two consecutive days, using a Coulbourn Freezeframe system (Coulbourn, Whitehall PA, USA). Day 33 training consisted of placing a mouse in a chamber, bright house light on, and allowing exploration for 2 min. Afterward an auditory cue (1700 Hz, 80 dB, the conditioned stimulus) was presented for 15 s. A 2 s foot shock (1.5 mA, the unconditioned stimulus) was administered for the final 2 s of the auditory cue. This procedure was repeated, and the mouse was removed from the chamber 30 s later. Freezing behavior was recorded during (2 s) and after the shocks (5 s) by a computerized camera tracking system. The next day, the mouse was returned to the same chamber in which the training occurred and freezing behavior was recorded (memory for context). At the end of the 5 min context test, the mouse was returned to its home cage. One hour later, freezing was recorded in a novel environment (altered context) and in response to the cue (memory for cue). Freezing scores for each subject were expressed as a percentage for each portion of the test (memory for context, altered context, memory...
for cue). Reductions in freezing in Tg2576 mice compared to non-transgenic mice were assessed using a student’s t-test. Effects of treatment (vehicle, 25 mg/kg or 50 mg/kg SAHA) on freezing or open field activity in Tg2576 mice were assessed using a one way ANOVA.

**Bioanalysis of SAHA**

Liquid chromatographic-tandem mass spectrometry (LC-MS/MS) was used for the analysis of SAHA. Each brain was homogenized in water (0.25 g/ml) using an OmniPrep homogenizer (OmiPrep, Las Vegas, NV, USA) and each CSF sample was collected into mouse plasma (CSF to plasma 1:1, v/v), 25 µl of plasma, CSF or tissue sample was extracted using acetonitrile and supernatant was diluted with water and then analyzed using reversed-phase chromatography with an XB-C18, 50 µM analytical column (Phenomenex). Peak retention time was 1.0 min using the following gradient (time, %B) at flow rate 0.80 ml/min: (0.00 min, 10%) (0.30 min, 10%) (0.60 min, 90%) (1.10 min, 90%) (1.12 min, 10%) (1.60 min, 10%). Mobile phases were water with 0.1% formic acid and acetonitrile with 0.1% formic acid. Mass analysis was conducted on a TSQ Vantage mass spectrometer (Thermo Scientific, West Palm Beach, FL, USA) with multiple reaction monitoring (MRM) mode. SAHA was ionized using an atmosphere pressure chemical ionization (APCI) source operating in the positive ionization mode. Declustering potential and collision energy were 65 V and 12 V, respectively. The MRM transition was m/z 265.3 to m/z 232.2 and the calibration curve range was between 30.5–20,000 ng/ml for plasma and brain, and 0.66–2500 ng/ml for CSF. Calibration curves were fit to a linear regression with 1/x2 weighting for all plasma, brain homogenate and CSF curves.

**Enzymatic Assays**

A panel of HDAC enzymatic assays (HDAC1 thru HDAC10) were performed by Nanosyn (Santa Clara, CA) using a Caliper microfluidics platform with fluorescently labeled peptide substrates. SAHA was serially diluted from a top concentration of 10 µM in DMSO and tested at 12 concentrations with 3 x dilution intervals. Final DMSO concentration was 1%. Assays used 0.5–5 nM enzyme and 1 µM peptide substrates.

**SmartCube® System**

SmartCube experiments were conducted at Psychogenics, Inc (Tarrytown, NY). 10 week old C57BL/6 mice from Taconic (n = 12/group) were either given a single injection or 14 daily injections of SAHA or valproate prior to behavioral phenotyping. On the day of testing, mice were injected with SAHA or valproate 15 minutes prior to testing with the SmartCube® system [15–17]. The SmartCube® system can measure numerous spontaneous behaviors and responses to challenges in the same testing environment. The hardware includesforce sensors and a number of aversive stimuli used to elicit behavior. Three high-resolution video cameras provide constant 3D view of the mouse in the SmartCube® apparatus throughout the testing period. Digital videos of the subjects were processed with computer vision algorithms to extract over 2,000 independent measures including frequency and duration of behavioral states such as grooming, rearing, etc., and many other features obtained during the test session. These data were compared with a database of therapeutic class signatures. The database comprises 14 classes of drugs with some of the major classes, such as the antidepressant class, comprising several subclasses with representatives of most of the drugs in the market.

In vitro Transporter Assays

Madin-Darby Canine Kidney (MDCK) cells stably transfected with human MDR1 (Pgp) were obtained from the National Institutes of Health, (Bethesda, MD) MDCKII cells transfected with mouse Bcrp1 were obtained from Albrecht Schinkel’s lab (Netherlands Cancer Institute, Amsterdam, Netherlands). Both cell lines were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS and 5 µg/ml Plasmocin and were harvested with trypsin and seeded on Millipore Millicell-24 well plates at initial concentrations of 1.30 x 10^5 cells/ml and 2.50 x 10^6 cells/ml, respectively. Cell monolayers were equilibrated in transport buffer (HBSS with 10 mM Hepes, pH 7.4) for 30 minutes at 37°C with 5% CO2 and 95% relative humidity, prior to the experiment. Dose solutions were prepared in transport buffer and consisted of SAHA (5 µM) and the monolayer integrity marker lucifer yellow (100 µM) in the presence and absence of the MDR1 inhibitor Elacridar (2 µM) or the Bcrp1 inhibitor Ko-143 (1 µM). The dose solutions were added to the donor chambers and transport buffer (with and without inhibitor) was added to receiver chambers. The transport of SAHA was examined in the apical to basolateral (A-B) and basolateral to apical (B-A) directions. The receiver chambers were sampled (50 µL) at 60, 120, and 180 min and were replenished with fresh transport buffer after the 60 and 120 min samplings. Lucifer yellow permeability was used as a marker of monolayer integrity during the experiment. SAHA concentrations in the donor and receiving compartments were determined by LC-MS/MS analysis. The apparent permeability (P_app) in the apical to basal A–B and basal to apical B-A directions, was calculated as follows: P_app = dQ/dt/(1/AC0), where: dQ/dt = rate of compound appearance in the receiver compartment; A = Surface area of the insert; and C0 = Initial substrate concentration at T0. The efflux ratio (ER) was calculated as (P_app B–A/ P_app A-B).

**Results**

In vitro SAHA Treatment Enhances Excitatory Synaptic Function

Previous studies have reported that HDAC inhibitors can alter synaptic function when applied to acutely prepared brain slices for as little as 10–90 minutes [21–23], a time frame that is unlikely to involve effects of transcriptional alterations due to HDAC inhibition. We wanted to explore the consequences of ongoing exposure to SAHA so as to better reflect therapeutic dosing which is expected to include transcriptional changes that could take hours or days to become evident. Therefore we used organotypic hippocampal brain slices that were treated with 0.5 µM SAHA for 3–4 days prior to assessing synaptic function. First we examined spontaneous miniature excitatory and inhibitory post-synaptic currents (mEPSCs and mIPSCs) in CA1 pyramidal neurons following treatment with SAHA or vehicle (DMSO). These experiments revealed an increase in the amplitude but not frequency of mEPSCs, indicating enhanced excitatory synaptic strength following SAHA treatment (Figure 1A). On the other hand, no changes in the amplitude or frequency of mIPSCs were detected (Figure 1B). These results indicate SAHA treatment enhances excitatory synaptic transmission without affecting inhibitory synaptic transmission. Thus, while SAHA could potentially cause multiple impacts on synaptic function via inhibition of various individual HDAC isoforms, the net impact of several days of pan-HDAC inhibition, including any compensatory responses, is manifested as a selective enhancement of basal excitatory synaptic function.
Presynaptic Function and Intrinsic Excitability are Unaffected by in vitro SAHA Treatment

The increased amplitude but not frequency of mEPSCs is consistent with postsynaptic rather than presynaptic changes following SAHA treatment. To test whether presynaptic functions are altered by SAHA, we measured parameters that are sensitive to alterations in presynaptic release probability. First we examined short-term plasticity, including paired-pulse facilitation at short inter-stimulus intervals, and paired-pulse depression at longer inter-stimulus intervals, measures that are dependent on presynaptic release probability [24]. These recordings showed no differences in paired-pulse facilitation or paired-pulse depression between SAHA and vehicle treated slices (Figure 2A). Second we examined the rate of use-dependent blockade of isolated NMDAR-mediated EPSCs by the NMDAR open channel blocker MK-801. The rate of blockade by MK-801 is dependent on presynaptic release probability and hence serves as another read-out of presynaptic function. The rate of blockade also did not show any difference between SAHA and vehicle treated slices (Figure 2B). Second, these results support the conclusion that the enhanced mEPSC amplitudes result from postsynaptic alteration in the absence of any significant changes to presynaptic release probability. To look for non-synaptic alterations following SAHA treatment, we examined the intrinsic excitability of CA1 neurons. No changes were observed in: 1) the number of action potentials evoked by various levels of current injection; 2) action potential threshold; 3) input resistance; or 4) the membrane sag caused by hyperpolarization-activated inward currents (Figure 3). Together these results suggest that SAHA does not alter intrinsic neuronal excitability.

In vitro SAHA Treatment Augments LTP and Blocks LTD

We tested whether treating slice cultures with SAHA for several days could alter synaptic plasticity. Robust LTP was observed in SAHA-treated slices using a subthreshold LTP-induction protocol that did not evoke significant synaptic potentiation in vehicle-treated slices (Figure 4A). This indicates that SAHA treatment can lower the threshold for inducing LTP. On the other hand no LTD was observed in SAHA-treated slices using an induction protocol that caused significant LTD in vehicle-treated slices (Figure 4B), indicating SAHA treatment blocks LTD induction and/or expression. Together these results demonstrate that SAHA treatment alters synaptic plasticity in a manner that enhances potentiation and limits depression of excitatory synapses.

In vivo SAHA Treatment does not Rescue Fear Conditioning Deficits in Tg2576 Mice

LTP is considered to be a key substrate of memory formation [25,26] and the hippocampus plays a critical role in contextual fear memory [27,28]. Tg2576 AD model mice exhibit impaired
contextual fear conditioning that correlates with impaired hippocampal LTP [29]. Therefore the enhanced LTP seen in the hippocampus following in vitro treatment with SAHA could potentially translate into improvement of contextual fear conditioning in the Tg2576 mice. Furthermore, previous studies have suggested that HDAC inhibition using various pan-HDAC inhibitors can rescue deficits in contextual fear conditioning in AD models [30–33], and SAHA injection in particular has been reported to enhance fear memory [13,30]. Therefore we tested if treatment with SAHA could rescue the robust deficits in fear memory seen in the Tg2576 mice (Figure 5A). To reflect ongoing therapeutic dosing, mice were given daily i.p. injections of 25 or 50 mg/kg SAHA or vehicle for 35 days and were tested for conditioned fear memory during the final days of dosing. Surprisingly, these experiments did not reveal any improvement of contextual fear memory, with no effect of SAHA treatment on the time spent freezing in response to either the conditioned stimulus or the conditioned cue (Figure 5B). Freezing in response to the unconditioned stimulus (foot shock) was also unaffected (Figure 5C), indicating that SAHA did not alter the response to the shock. Locomotor activity can be a confound in fear conditioning experiments, so we also examined activity in the open field, but again saw no effect of SAHA treatment (Figure 5D). One potential explanation for the lack of behavioral impact of in vivo SAHA treatment is poor CNS exposure of SAHA following peripheral delivery. Therefore we analyzed the concentration of SAHA in samples taken from the 50 mg/kg treatment group 1 hour post injection on day 35 of treatment. These measurements revealed that while total plasma levels of SAHA were 1.53 ± 0.80 μM, and CSF levels were 0.61 ± 0.38 μM, levels in the brain were below the lower limit of quantification (LLOQ) of 0.17 μM in these samples. This bioanalysis suggests poor pharmacokinetic properties of SAHA could potentially explain the lack of efficacy.

SAHA has Limited Brain Availability Following Peripheral Administration

To better understand the bioavailability of SAHA we performed a pharmacokinetic study of the time course of both total and unbound SAHA concentrations in the plasma, CSF, and brain following 50 mg/kg i.p. injections (Figure 6A). This analysis revealed: 1) a significant amount of SAHA was bound to protein...
For 50 mg/kg vs 0.10 increased from 0.07 to 0.36 hr does not enhance the peak concentration achieved in the brain. Pharmacokinetic analysis demonstrates low brain concentrations of free SAHA, and SAHA treated slices (p < 0.05, n = 9 vehicle, 8 SAHA). Example traces before and after LTD induction are shown in red for SAHA and black for vehicle treated slices (scale bars represent 25 pA and 20 ms). Data are plotted as mean ± SEM. doi:10.1371/journal.pone.0069964.g004

Figure 4. SAHA treated slices exhibit enhanced induction of LTP and impaired LTD. (A) An induction protocol that was subthreshold in vehicle treated slices readily evoked LTP in SAHA treated slices (p < 0.05, n = 5.5). Example traces before and after LTP induction are shown in red for SAHA and black for vehicle treated slices (scale bars represent 20 pA and 20 ms). (B) An induction protocol that readily induced LTD in vehicle treated slices could not produce LTD in SAHA treated slices (p < 0.05, n = 9 vehicle, 8 SAHA). Example traces before and after LTD induction are shown in red for SAHA and black for vehicle treated slices (scale bars represent 25 pA and 20 ms). Data are plotted as mean ± SEM. (40.7% plasma, 87.0% brain); 2) SAHA was rapidly cleared and undetectable in the brain at time points beyond 1 hour (LLOQ = 0.018 μM); and 3) brain levels of SAHA were much lower than plasma levels, with total and free brain-to-plasma ratios of 0.04 and 0.01 respectively (area under the curve up to the last measurable time point; AUClast ratios). To determine if a higher dose could enhance brain exposure, pharmacokinetic analysis was repeated following 150 mg/kg SAHA injections (Figure 6B). While the higher dose did not increase the maximal observed concentration (Cmax) of free SAHA in the brain (0.11 ± 0.02 μM for 50 mg/kg vs 0.10 ± 0.01 μM for 150 mg/kg), the AUClast was increased from 0.07 to 0.36 hr·μM. Overall the pharmacokinetic analysis demonstrates low brain concentrations of free SAHA, and shows that increasing the dose can prolong SAHA exposure but does not enhance the peak concentration achieved in the brain.

SAHA Inhibits Different HDAC Isozymes with variable Potency

To help interpret the potential impact of the free brain SAHA concentrations achieved by in vivo SAHA injection, we examined the potency of SAHA against the different HDAC isozymes as determined by in vitro enzymatic assays (Table 1). Based on this data, the free brain SAHA Cmax of 0.11 μM (which was transiently achieved in the in vivo pharmacokinetic studies) exceeded the IC50 values of the Class I HDACs, HDAC1 (0.061 μM) and HDAC3 (0.019 μM) but did not reach the IC50 values of HDAC2 (0.251 μM) or HDAC3 (0.827 μM). The IC50 values of all of the class IIa HDACs were far greater than the in vivo Cmax with values all >10 μM. On the other hand the class IIb HDACs had very low IC50 values with the Cmax of the in vivo studies above the IC50 for both HDAC6 (0.009 μM) and HDAC10 (0.029 μM). Moreover the free brain concentrations remained above the HDAC6 IC50 for at least 6 hours after the 150 mg/kg dose. Overall these results suggest in vivo injections do not result in significant inhibition of the HDAC2 isozyme that is critically involved in fear memory and in deficits in AD model mice [12,13]. Furthermore, the in vivo injections of SAHA could only transiently impact a subset of the other HDAC isozymes with the exception of HDAC6, which could potentially be inhibited for a more prolonged period following higher SAHA doses. In contrast the continuous exposure of brain slices to 0.5 μM SAHA in our in vitro studies should be sufficient to broadly inhibit all of the class I HDAC isozymes, including HDAC2, as well the Class IIb isozymes.

High-content Phenotyping does not Reveal a Significant Neurobehavioral Impact of in vivo SAHA Treatment

That the free brain concentration of SAHA did not even briefly reach the IC50 of HDAC2 which is thought to be critical in fear memory and memory impairments in AD model mice [12,13] is consistent with the lack of improvement of fear memory observed in the Tg2576 mice. Nonetheless, given the expected effects of HDAC inhibition on gene expression, it is possible that even a transient inhibition of the other class I HDACs or Class IIb HDACs could be sufficient to result in some behavioral impacts, especially at higher doses or after prolonged treatment. Therefore, to broadly assess any behavioral effects of SAHA in C57BL/6 mice we employed high-content behavioral phenotyping using the SmartCube® technology [15–17]. This technology assesses behavioral responses to test compounds and determines the class of CNS activity corresponding to any observed behavioral activity. For this study 50 mg/kg or 150 mg/kg doses of SAHA were given to wild type mice prior to SmartCube® testing. A known behaviorally relevant dose of valproate (225 mg/kg) was used as a positive control for the sensitivity of the assay, although it should be noted that this drug has activity on many different targets, and its impacts on behavior should not be assumed to be due to HDAC inhibition. Groups of mice were tested following a single injection or following 14 days of injections to allow for any chronic effects of treatment to develop. There were no significant behavioral alterations observed following single injections of 50 mg/kg or 150 mg/kg SAHA (Figure 7A). Following chronic treatment, the only significant behavioral activity detected for SAHA was in the lower dose group and did not correspond to the signature of any known pharmacological class (Figure 7B). The lack of any behavioral activity in the higher SAHA dose group argues against the unclassified activity in the lower dose group reflecting a CNS effect of chronic SAHA treatment. In contrast, valproate showed strong anxiolytic and mild psychostimulant class behavioral...
activity after acute treatment (Figure 7A) and also exhibited a strong anxiolytic activity signature in chronically treated mice, confirming sensitivity of this assay (Figure 7B). Overall these results do not support a behavioral effect of up to 150 mg/kg SAHA following acute or chronic i.p. injections.

SAHA is a Substrate of Brain Efflux Transporters

Given the lack of behavioral impacts of SAHA, we wanted to better understand the causes of limited free SAHA in the brain. One possibility is that low levels of SAHA in the brain could be due to active export of this drug across the BBB. To test if SAHA is a substrate of brain efflux transporters we performed in vitro transporter assays using monolayers of MDCK cells expressing human P-glycoprotein (Pgp; Multidrug Resistance Protein 1) or mouse Breast Cancer Resistance Protein (Bcrp1), two important ATP-binding cassette (ABC) gene family members that are present in the BBB and often limit drug exposure in the brain [34,35] (Figure 8A). These experiments showed efflux ratios for SAHA of 12±0.75 for Pgp and 14±0.86 for Bcrp1 compared to ratios of 1.4±0.64 and 1.2±0.13 in the presence of the respective transporter inhibitors (Figure 8B). These results suggest SAHA is a substrate of both Pgp and Bcrp1 and that active efflux likely makes a major contribution to the low brain distribution of SAHA following peripheral delivery.

Discussion

Our in vitro electrophysiological analysis using cultured brain slices demonstrates that directly treating brain tissue with SAHA can impact both basal synaptic function and synaptic plasticity. The enhanced mEPSC amplitude but not frequency, together with unchanged short-term plasticity and use-dependent NMDAR blockade rate, strongly support a postsynaptic enhancement of excitatory synapses. At the same time we did not detect changes to inhibitory synaptic transmission, which suggests SAHA treatment...
leads to an overall shift in the balance between excitation and inhibition in the neural network. These results are consistent with studies of HDAC2 KO mice that show increased spine density in CA1 pyramidal neurons, which has been interpreted as reflecting increased excitation and attributed to HDAC2 regulation of genes involved in synapse formation and plasticity [13]. At the same time it is important to keep in mind that the state of synaptic and neuronal function measured after several days of drug treatment reflects the combined effects of inhibiting multiple HDAC isozymes by SAHA, as well as any potential network-wide compensatory processes. For example we have recently reported that selective HDAC2 knockdown in individual neurons in this preparation not only enhances excitation, but also reduces GABAergic synaptic inhibition in a cell-autonomous manner [19]. The lack of a change in synaptic inhibition observed after several days of SAHA treatment suggests that either blockade of isozymes other than HDAC2 enhance inhibition so as to negate HDAC2-mediated reductions, and/or that network-wide compensatory mechanisms counteract any initial cell-autonomous changes in synaptic inhibition. Thus while we cannot discriminate isozyme-specific contributions to the neurophysiological alterations observed following SAHA treatment, the net impact of SAHA treatment, is an altered functional state of the network featuring enhanced excitation.

Our in vitro brain slice experiments also showed that, similar to observations of enhanced LTP following very acute treatment with SAHA or other pan-HDAC inhibitors [21–23], enhanced LTP occurs during more prolonged HDAC inhibition. Furthermore we

Table 1. SAHA IC₅₀ values for each HDAC isozyme are shown as measured using in vitro enzymatic assays.

| Class I | Class IIa | Class IIb |
|---------|-----------|-----------|
| HDAC1   | HDAC2     | HDAC3     | HDAC8 | HDAC4 | HDAC5 | HDAC7 | HDAC9 | HDAC6 | HDAC10 |
| IC₅₀(µM) | 0.061     | 0.251     | 0.019 | 0.827 | >10   | >10   | >10   | >10   | 0.009 | 0.029 |
| (CI)    | (0.004)   | (0.016)   | (0.001) | (0.078) | | | | | (0.001) | (0.002) |

Values are in µM and confidence intervals are in parentheses.
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Figure 6. Pharmacokinetic analysis of SAHA following i.p. injection. A) Bioanalysis of the time course of total (top) and unbound (bottom) plasma, CSF, and brain levels of SAHA following a single 50 mg/kg ip injection (n = 3 mice/time point). The dotted red lines represent the SAHA concentration imposed on the in vitro slice cultures for the electrophysiological studies. B) Total (top) and unbound (bottom) SAHA levels are shown following a 150 mg/kg ip injection (n = 3/time point). All data is shown as mean ± SD.
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extended the examination of synaptic plasticity to LTD and found that SAHA treatment impaired this form of synaptic plasticity. Therefore in addition to enhancing basal excitatory synaptic transmission, in vitro SAHA treatment also makes it easier to potentiate excitatory synapse and harder to weaken them, thus shifting the balance towards synaptic potentiation/stabilization rather than weakening/elimination. Overall these results predict that HDAC inhibition will promote excitation and network activity, which could be beneficial in cases of cognitive impairment featuring weakened synaptic function, reduced synaptic potentiation, and loss of excitatory synapse, as is the case in mouse models of AD.

Despite expectations from previous studies of contextual fear conditioning using the HDAC inhibitor phenylbutyrate in Tg2576 mice [31], or using various HDAC inhibitors including SAHA in double transgenic AD model mice (over-expressing the same ‘swedish’ mutant APP as the Tg2576 mice as well as mutant presenilin 1) [30], we did not observe a significant rescue of contextual fear conditioning in Tg2576 mice treated with 25 mg/kg or 50 mg/kg SAHA. Bioanalysis of samples taken from these mice 1 hour after dosing on day 35 of daily injections revealed that SAHA levels were low in the CSF and undetectable in the brain. Detailed pharmacokinetic analysis demonstrated high binding, rapid clearance and very low levels of free SAHA in the brain. Consistent with the low levels of free SAHA that were only transiently detectable in the brain, in vivo transporter assays showed that SAHA is a substrate for the brain export transporters Pgp and Bcrp1. While previous studies have proposed that brain SAHA exposure could be increased by using a vehicle that enhances solubility [36], our results suggest peripheral delivery of SAHA will be hampered in achieving brain exposure due to active export across the BBB regardless of peripheral solubility.

Based on our in vitro enzymatic assay results, peak free brain levels would appear insufficient to significantly impact HDAC2, HDAC3 or any of the Class IIa HDACs. However other reports using in vitro assays have sometimes reported higher potency for SAHA (with IC50 or Ki values in the 1–200 nM range compared to our IC50 of 251 nM for HDAC2, for example) [37–39], which emphasizes the dependence of potency measurements on in vitro assay conditions, and the uncertainty over the precise potency of in vivo SAHA for HDACs in the brain. In addition, based on our in vitro assays, the free brain levels did at least transiently exceed the IC50 values for HDAC1, HDAC3, HDAC6, and HDAC10. Therefore, taken together with reports of increased histone acetylation following peripheral delivery of SAHA [36,40], this suggests that despite limited availability of free drug, some degree of target engagement for some HDAC isozymes may occur in the brain. While the roles of HDACs other than HDAC2 in neurobehavior are not well understood, it is possible their inhibition could affect behavior of wildtype mice even if HDAC2 is not significantly inhibited. However, broadly screening for effects of SAHA injections in wildtype mice using the SmartCube® system failed to detect any impact of SAHA: no relevant neurobehavioral activity consistent with any class of drug action was evident following either a single injection or 14 days of dosing with 50 mg/kg or 150 mg/kg SAHA. Overall the lack of efficacy in Tg2576 mice or any behavioral impacts in wt mice is likely contributed to by the very poor pharmacokinetic profile of SAHA in the brain. However, from our data we cannot exclude the possibility that sufficient HDAC inhibition is achieved to result in significant alteration in histone acetylation levels, but there is simply no effect of such altered acetylation on the behavioral assays we conducted.

Our observations of low free SAHA in the mouse brain and no behavioral impact of SAHA following peripheral injection are at odds with previous studies reporting enhanced contextual fear conditioning memory in mice following peripheral SAHA delivery [13,30]. At the same time it is worth noting that our results do not contradict the broader pharmacological evidence for a role of HDACs in memory, as enhanced contextual fear memory has been demonstrated using various other HDAC inhibitors which likely have distinct pharmacokinetic properties from SAHA [22,30–33,41,42]. In addition, studies using direct infusion of SAHA into the brain [21,43], or genetic reduction of HDAC isozymes [12,13] have also demonstrated the importance of HDAC function in memory formation. Therefore our in vitro results are in agreement with previous studies suggesting HDAC inhibition impacts synaptic function, but our in vivo results argue...
against the usefulness of SAHA in particular in causing such impacts in vivo, which is relevant given the clinical use of this compound. Our results also emphasize that the interpretation of behavioral pharmacology data always include careful analysis of brain drug levels in order to reject true negative results and eliminate false positive results.

It is worth noting that compromised BBBs could allow brain effects of SAHA not seen in our in vivo studies. For example SAHA has been reported to be efficacious in models of glioblastoma [44,45] and is currently being tested in clinical trials for patients with brain tumors. One possibility is that the efficacy of SAHA in preclinical glioma models is facilitated by compromised BBB function in the area of the tumor. Supporting this, tumor cells have been demonstrated to induce changes in the composition of the basal lamina and in astrocytic components of the neurovascular unit and increase vascular permeability in mouse brains [46,47]. Thus while our results do not predict a neurological impact of SAHA in CTCL patients, who are unlikely to have compromised BBBs, it is plausible that SAHA would be more available in the brain of glioblastoma patients. At the same time, our results suggest that in addition to issues with toxicity during chronic dosing, SAHA is also unlikely to be efficacious in neurological conditions such as AD which generally feature intact BBB function. The finding of limited access of SAHA to the brain also has implications beyond neurological indications. For example, given recent excitement around using SAHA to disrupt HIV latency in order to eradicate infection [8], our results suggest it will be critical to consider the extent of HIV brain reservoirs [48,49]. Overall our results highlight that while HDAC inhibition can impact neuronal function, efforts to develop HDAC inhibitors for targeting CNS indications need to focus not only on reducing toxicity and achieving selectivity, but also on avoiding compounds that are substrates for efflux transporters in order to allow adequate brain exposure.

Author Contributions
Conceived and designed the experiments: JH QZ PS KSL EP LL TH VA DB EL. Wrote the paper: JH QZ.

References
1. Jenuwein T, Allis CD (2001) Translating the histone code. Science 293: 1074–1080.
2. Swedlow JD, Allis CD (2000) The language of covalent histone modifications. Nature 403: 41–45.
3. Spange S, Wagner T, Heinzel T, Kramer OH (2009) Acetylation of non-histone proteins modulates cellular signalling at multiple levels. Int J Biochem Cell Biol 41: 185–198.
4. Sakajiri S, Kamagai T, Kawamata N, Saitoh T, Said JW, et al. (2005) Histone deacetylase inhibitors profoundly decrease proliferation of human lymphoid cancer cell lines. Exp Hematol 33: 53–61.
5. Durie M, Vu J (2007) Update on the treatment of cutaneous T-cell lymphoma (CTCL): Focus on vorinostat. Biologies 1: 377–392.
6. Durie M, Vu J (2007) Vorinostat: a new oral histone deacetylase inhibitor approved for cutaneous T-cell lymphoma. Expert Opin Investig Drugs 16: 1111–1120.
7. Martinez-Iglesias O, Ruiz-Llorente L, Sanchez-Martinez R, Garcia L, Zambra A, et al. (2008) Histone deacetylase inhibitors: mechanism of action and therapeutic use in cancer. Curr Opin Oncol 10: 395–398.
8. Archin NM, Liberty AL, Kashuba AD, de la Higuera SK, Korne JD, et al. (2012) Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. Nature 487: 482–485.
9. Couteras X, Schwenerker M, Chen CS, McCormack JM, Deeks SG, et al. (2009) Suberoylanilide hydroxamic acid reactivates HIV from latently infected cells. J Biol Chem 284: 6702–6709.
10. Verheyen K, Karagiannis TC (2011) Potential non-oncological applications of histone deacetylase inhibitors. Am J Transl Res 3: 454–467.
11. Graf J, Kim D, Dobbin MM, Tsai LH (2011) Epigenetic regulation of gene expression in physiological and pathological brain processes. Physiol Rev 91: 605–649.
12. Graf J, Rei D, Yuan JS, Wang WY, Seo J, et al. (2012) An epigenetic blockade of cognitive functions in the neurodegenerating brain. Nature 483: 222–226.
13. Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, et al. (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459: 55–60.
14. Karczewicz MA, Brocher C, Willis DE, Walker BA, D’Annamale MA, et al. (2009) HDAC6 is a target for protection and regeneration following injury in the nervous system. Proc Natl Acad Sci U S A 106: 19599–19604.
15. Brunner D, Alexandrov V, Calderone B, Hanania T, Lowe D (2012) Behavior-Based Screening as an Approach to Polypharmacological Ligands. In: Peters J-U, editor. Polypharmacology in Drug Discovery. Hoboken, New Jersey: John Wiley & Sons, Inc. 301–309.
16. Houghton RA, Pirilla C, Giulianotti MA, Appel JR, Dooley CT, et al. (2006) Strategies for the use of mixture-based synthetic combinatorial libraries: scaffold
rank, direct testing in vivo, and enhanced deconvolution by computational methods. J Comb Chem 10: 3–19.

17. Roberds SL, Filipov I, Alexandrov V, Hanania T, Brunner D (2011) Rapid, computer vision-enabled marine screening system identifies neuropharmacological potential of two new mechanisms. Front Neurosci 5: 103.

18. Hanson JE, Orr AL, Fernandez-Illanes S, Valenzuela RA, Madison DV (2010) Hippocampal Slice Cultures. Protocols for Neuronal Cell Culture. Humana Press. 299–311.

19. Hanson JE, Deng L, Hackos DH, Lo SC, Lauffer BE, et al. (2013) Histone Deacetylase 2 Cell Autonomously Suppresses Excitatory and Enhances Inhibitory Synaptic Function in CA1 Pyramidal Neurons. J Neurosci 33: 5924–5929.

20. Comery TA, Martone RL, Aschner M, Atchison KP, Diamantidis G, et al. (2005) Acute gamma-secretase inhibition improves contextual fear conditioning in the Tg2576 mouse model of Alzheimer’s disease. J Neurosci 25: 8898–8902.

21. Alarcon JM, Malleret G, Touzani K, Vronskaya S, Ishii S, et al. (2004) Chromatin acetylation, memory, and LTP are impaired in CBP+/− mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. Neuron 42: 947–957.

22. Levenson JM, O’Riordan KJ, Brown KD, Trinh MA, Molfese DL, et al. (2004) Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem 279: 40545–40559.

23. Vecsey CG, Hawk JD, Lattal KM, Stein JM, Fabian SA, et al. (2007) Histone deacetylase inhibitors enhance memory and synaptic plasticity via cREB-CBP-dependent transcriptional activation. J Neurosci 27: 6129–6140.

24. Branco T, Staras K (2009) The probability of neurotransmitter release: variability and feedback control at single synapses. Nat Rev Neurosci 10: 373–383.

25. Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361: 31–39.

26. Martin SJ, Grimwood PD, Morris RG (2000) Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci 23: 649–711.

27. Kim JJ, Fanselow MS (1992) Modality-specific retrograde amnesia of fear. Science 256: 673–677.

28. Schenberg EE, Oliveira MG (2008) Effects of pre or posttraining dorsal hippocampus D-APS injection on fear conditioning to tone, background, and foreground context. Hippocampus 18: 1089–1093.

29. Balducci C, Mehdawy B, Mare L, Giuliani A, Lorenzini L, et al. (2011) The gamma-secretase modulator CHF5074 restores memory and hippocampal synaptic plasticity in plaque-free Tg2576 mice. J Alzheimers Dis 24: 799–816.

30. Kilcrease M, Miller CA, Fass DM, Hennig KM, Haggarty SJ, et al. (2010) Inhibitors of class I histone deacetylases reverse contextual memory deficits in a mouse model of Alzheimer’s disease. Neuropsychopharmacology 35: 870–880.

31. Ricobaraza A, Caudrado-Trujero M, Marco S, Perez-Osano I, Garcia-Osta A (2012) Phenylbutyrate rescues dendritic spine loss associated with memory deficits in a mouse model of Alzheimer’s disease. J Alzheimers Dis 18: 131–139.

32. Francis Yi, Fa M, Ashraf H, Zhang H, Staniszewski A, et al. (2009) Dyreduction of histone acetylation in the APP/PS1 mouse model of Alzheimer’s disease. J Alzheimers Dis 18: 131–139.

33. Gorio andaringan N, Agis-Balboa RG, Walter J, Sananbenesi F, Fischer A (2011) Sodium butyrate improves memory function in an Alzheimer’s disease mouse model when administered at an advanced stage of disease progression. J Alzheimers Dis 28: 187–197.

34. Begley DJ (2004) ABC transporters and the blood-brain barrier. Curr Pharm Des 10: 1295–1312.

35. Loscher W, Potschka H (2005) Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. NeuroRx 2: 86–98.

36. Hocky E, Richon VM, Woodman B, Smith DL, Zhou X, et al. (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington’s disease. Proc Natl Acad Sci U S A 100: 2041–2046.

37. Brader JE, West N, Grachen ML, Greenberg EF, Haggarty SJ, et al. (2010) Chemical phylogenetics of histone deacetylases. Nat Chem Biol 6: 238–243.

38. Butler KV, Koszikowski AP (2008) Chemical origins of isoform selectivity in histone deacetylase inhibitors. Curr Pharm Des 14: 505–528.

39. Tessier P, Smill DV, Wahhab A, Leit S, Rahul J, et al. (2009) Diphenylmethylene hydroxamic acids as selective class IIa histone deacetylase inhibitors. Bioorg Med Chem Lett 19: 5604–5608.

40. Beconi M, Araz O, Matthews K, Moumne L, O’Connell G, et al. (2012) Oral administration of the pimelic diphenylamide HDAC inhibitor HDACi 4b is unsuitable for chronic inhibition of HDAC activity in the CNS in vivo. PLoS One 7: e44498.

41. Fass DM, Reis SA, Ghosh B, Hennig KM, Joseph NF, et al. (2013) Cerebromatost: A novel cognitive enhancer that inhibits histone deacetylase activity and modulates chromatin-mediated neuroplasticity. Neuropharmacology.

42. Fischer A, Sananbenesi F, Wang X, Dobrin M, Tsai LH (2007) Recovery of learning and memory is associated with chromatin remodelling. Nature 447: 170–182.

43. Pelay S, Sananbenesi F, Zөvөllıs A, Burkhardt S, Bahari-Javan S, et al. (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 320: 753–756.

44. Spiller SE, Ditzler SH, Pullar BJ, Olson JM (2008) Response of preclinical medulloblastoma models to combination therapy with 13-cis retinoic acid and suberoylanilide hydroxamic acid (SAHA). J Neurooncol 87: 133–141.

45. Yin D, Ong JM, Hu J, Desmond JC, Kawamata N, et al. (2007) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor: effects on gene expression and growth of glioma cells in vitro and in vivo. Clin Cancer Res 13: 1045–1052.

46. Lee J, Land-Smith C, Borboa A, Gonzalez AM, Baird A, et al. (2009) Glioma-induced remodeling of the neurovascular unit. Brain Res 1268: 125–134.

47. Lund CV, Nguyen MT, Owens GC, Pakchoian AJ, Shaterian A, et al. (2006) Reduced glioma infiltration in Src-deficient mice. J Neurooncol 78: 19–29.

48. Liner KJ, 2nd, Ro MJ, Robertson KR (2010) HIV, antiretroviral therapies, and latent virus in presymptomatic HIV-infected individuals. Am J Pathol 179: 1295–1312.

49. Thompson KA, Cherry CL, Bell JE, McLean CA (2011) Brain cell reservoirs of latent virus: a model when administered at an advanced stage of disease progression. J Alzheimers Dis 26: 187–197.

50. Roberds SL, Filipov I, Alexandrov V, Hanania T, Brunner D (2011) Rapid, computer vision-enabled marine screening system identifies neuropharmacological potential of two new mechanisms. Front Neurosci 5: 103.

51. Hanson JE, Orr AL, Fernandez-Illanes S, Valenzuela RA, Madison DV (2010) Hippocampal Slice Cultures. Protocols for Neuronal Cell Culture. Humana Press. 299–311.

52. Alarcon JM, Malleret G, Touzani K, Vronskaya S, Ishii S, et al. (2004) Chromatin acetylation, memory, and LTP are impaired in CBP+/− mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. Neuron 42: 947–957.

53. Levenson JM, O’Riordan KJ, Brown KD, Trinh MA, Molfese DL, et al. (2004) Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem 279: 40545–40559.

54. Vecsey CG, Hawk JD, Lattal KM, Stein JM, Fabian SA, et al. (2007) Histone deacetylase inhibitors enhance memory and synaptic plasticity via cREB-CBP-dependent transcriptional activation. J Neurosci 27: 6129–6140.