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HDAC inhibition combined with behavioral therapy enhances learning and memory following traumatic brain injury

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Abstract

Traumatic brain injury (TBI) induces a number of pathological events ranging from neuronal degeneration and tissue loss to impaired neuronal plasticity and neurochemical dysregulation. In rodents, exposure of brain injured animals to environmental enrichment has been shown to be an effective means of enhancing learning and memory post-injury. Recently, it has been discovered that environmental enrichment may enhance neuronal plasticity through epigenetic changes that involve enhanced histone acetylation, a property that can be mimicked by the use of histone deacetylase (HDAC) inhibitors. We therefore evaluated the consequences of the HDAC inhibitor sodium butyrate on the learning and memory of brain injured mice. In contrast to a previous report using a mouse neurodegeneration model, sodium butyrate (1.2g/kg daily for four weeks) did not improve learning and memory when tested after the completion of the drug treatment paradigm. In addition, sodium butyrate administration during the reported period of neurodegeneration (days 0–5) also offered no benefit. However, when administered concurrently with training in the Morris water maze task (beginning on day 14 post-injury), sodium butyrate improved learning and memory in brain injured mice. Interestingly, when these mice were subsequently tested in an associative fear conditioning task, a continued improvement was observed. Taken together, our findings indicate that HDAC inhibition may mimic some of the cognitive improvements seen following enriched environment exposure, and that the improvement is observed when the treatment is carried out current with behavioral testing.

Keywords

traumatic brain injury; HDAC; Morris water maze; hippocampus; delay fear conditioning

It has been well established that the pathophysiology of TBI is complex with multiple cellular and molecular processes contributing to the observed cognitive and behavioral dysfunctions (Kochanek et al., 2000; Ray et al., 2002; Raghupathi, 2004; Bramlett and

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Dietrich, 2007). While a great deal of effort has been focused on the identification of acute neuroprotective interventions (Zhang et al., 2005; Royo et al., 2007; Jennings et al., 2008), strategies aimed at enhancing residual neurocognitive function following injury have been disproportionately underdeveloped. Accumulating evidence indicates that the brain can reorganize extensively after TBI and that reorganization can be enhanced with appropriate rehabilitation. Consistent with this, a number of studies have demonstrated that exposure of TBI rats to enriched environments improves cognitive function (Hamm et al., 1996; Passineau et al., 2001; Hicks et al., 2002; Wagner et al., 2002; Kozlowski et al., 2004). For example, daily exposure (1 hr/day for 3 weeks) of fluid percussion injured rats to a variety of novel objects, climbing ladders, and racks was found to be sufficient to improve the performance of these animals in the Morris water maze and increase neurogenesis within the granule cell layer of the hippocampus (Gaulke et al., 2005). Although it is clear that environmental enrichment has a profound influence on cognition in brain injured animals, the cellular and molecular basis of this improvement has not been determined.

A number of studies have shown that epigenetic changes such as histone acetylation and methylation are important mechanisms for the regulation of gene expression and neuronal plasticity (Guan et al., 2002; Crosio et al., 2003; Levenson et al., 2006; Miller et al., 2008). A recent study employing a mouse model of neurodegeneration (CK-p25 Tg mice) found that the memory improvement associated with environmental enrichment is due to increased histone acetylation (Fischer et al., 2007). Remarkably, these authors found that intraperitoneal administration of the histone deacetylase (HDAC) inhibitor sodium butyrate for 4 weeks to these mice resulted in a memory improvement comparable to that seen following environmental enrichment. Importantly, this improvement could be observed when the behavioral training was initiated following the completion of the inhibitor treatment. However, a recent study has reported that the ability of enriched environment exposure to improve memory function in TBI animals may be contingent on the timing of the behavioral training. In this study, it was found that exposure of cortical impact injured rats to an enriched environment improved motor and cognitive performance, but only when the behavioral testing and environmental enrichment overlapped (Hoffman et al., 2008). For example, early exposure to an enriched environment (initiated after injury for 1 week) improved motor skills but not water maze behavior tested on days 14–18. In contrast, when the exposure was delayed (initiated 1 week following injury) or continuous (initiated after injury but continued throughout testing), environmental enrichment improved water maze behavior.

In the present study, we have examined if the HDAC inhibitor sodium butyrate given to brain injured animals results in improved memory when given either prior to, or concurrent with, behavioral training. Our findings demonstrate that systemic administration of sodium butyrate improves water maze performance, that this improvement can be generalized to a second task, and that similar to enriched environment, occurs only when sodium butyrate is given concurrent with behavioral training.

Experimental Procedures

Animals

All manipulations of animals were carried out in compliance with the National Institutes of Health guidelines outlined in *Guide for the Care and Use of Laboratory Animals*. Male C57 mice (~25g) were purchased from Charles River Laboratories. Mice were group housed and maintained on a 12:12 light dark cycle with *ad libitum* access to food and water.

Controlled cortical impact injury and drug administration

Animals were initially anesthetized with 5% isoflurane and 2:1 mixture of N₂O/O₂. While being maintained under anesthesia (2% isoflurane and 2:1 mixture of N₂O/O₂), mice were placed in a stereotaxic frame and a 5 mm craniotomy (halfway between bregma and lambda, $\pm$ 1 mm lateral to midline) was performed. A heating pad was used to maintain body temperature at 37°C. Using a 3-mm-diameter impact tip and a cortical impact device, a single impact (1.7 mm deformation, 4 m/s) was delivered to the parietal association cortex at an angle of 10° from the vertical plane, such that the impact was orthogonal to the cortex surface. Sodium butyrate was prepared at a concentration of 120mg/ml in sterile saline. Beginning at the indicated time post-injury, mice were i.p. injected with either 1.2 g/kg sodium butyrate, or an equal volume of vehicle (0.25 ml). For the 4 week administration study, 12 vehicle-treated injured, 12 drug-treated injured, and 10 sham-operated animals were used. For the acute administration study, 8 vehicle-treated injured, 8 drug-treated injured, and 10 sham-operated animals were tested. For the study involving concurrent behavioral training and drug administration, 9 vehicle-treated injured, 7 drug-treated injured, and 10 sham-operated animals were used.

Western blotting

Thirty minutes following injection of either 1.2 g/kg sodium butyrate or vehicle, animals were killed and brains were removed and submerged under ice-cold artificial cerebrospinal fluid (10 mM HEPES pH 7.2, 1.3 mM NaH₂PO₄, 3mM KCl, 124 mM NaCl, 10mM dextrose, 26 mM NaHCO₃, and 2mM MgCl₂). The hippocampi were quickly removed and snap-frozen on dry ice. The tissue was homogenized in a lysis-buffer containing 10 mM Tris pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5 μ M DTT, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, 1 mM PMSF, and 0.1 μ M okadaic acid. The protein concentration was measured using a Bradford assay with BSA as the standard. Samples were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA), followed by blocking overnight in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) plus 5% BSA. Membranes were then incubated with either a mixture of anti-acetylated histone H3 and H4 (Upstate Biotechnologies, 0.1 μ g/ml) antibodies or anti-histone H3 and H4 (Upstate Biotechnologies, 0.5 μ g/ml) antibodies for 3 h at room temperature. After incubation with the primary antibodies, membranes were washed five times in TBST, and immunoreactivity was assessed by an alkaline phosphatase-conjugated secondary antibody and a CDP-star chemiluminescent substrate (Cell Signaling Technology, Beverly, MA). The optical density of the immunoreactive bands was measured using *ImageJ* (available from NIH). Linearity of the western blots was determined by examining the optical density of increasing amounts (in

µg) of starting material. Images of western blots have been cropped to eliminate regions of the gels not containing immunoreactive bands.

Morris water maze

The Morris water maze task was performed essentially as described previously (Hamm et al., 1992; Dash et al., 1995). For each daily block of four trials, mice were placed in the pool by hand facing the wall. Animals started a trial once from each of four randomized start locations. Mice were given a maximum of 60 seconds to find the platform. If a mouse failed to find the platform after 60 seconds, it was led there by the experimenter. All mice were allowed to remain on the platform for 30 seconds before being placed in a 37°C warming cage between trials. The intertrial interval (iti) was four minutes. Either 90 minutes or 24hr following the hidden platform testing, a probe trial was given by removing the platform from the tank and allowing the animals to search for 60 seconds. Swimming paths were monitored by use of a tracking device connected to a video camera. Probe trials were analyzed for both latency to first platform crossing as well as the total number of platform crossings. Counter areas, concentric circles of increasing diameter, were centered on the platform and used to record indices of localization including entries, latency, and dwell times.

Fear conditioning

Animals were trained in this task using a foot shocker and training cage obtained from Coulbourn Instruments (Allentown, PA). Conditioning consisted of three consecutive training trials which began with a two minute period in which the animal was allowed to familiarize itself with the context followed by a 30 second tone. During the last two seconds of tone presentation, a 1.0 mA foot shock was used as the unconditioned stimulus. Freezing behavior, defined as refraining from all movement except for that used in respiration, was monitored throughout training and scored in two-second intervals. For testing of cued conditioning, animals were placed in a distinctly different environment for two minutes. The tone was then presented for three minutes during which freezing behavior was measured. For contextual testing, animals were placed in the conditioning chamber for three minutes and freezing behavior was monitored at two-second intervals.

Statistical analyses

Behavioral data were evaluated using either a repeated measures two-way ANOVA, or a Student's *t*-test for unpaired variables. Significant differences were determined at $P < 0.05$. The points at which differences were observed were identified by *post-hoc* analysis. Optical density values from western blot samples were analyzed using a Student's *t*-test for unpaired variables.

Results

Sodium butyrate increases hippocampal histone acetylation

In order to examine if the HDAC inhibitor sodium butyrate can effectively increase hippocampal histone acetylation, mice ($n=4$ /condition) were injected intraperitoneally with (1.2 g/kg) of sodium butyrate or an equal volume of vehicle. This dose has been previously

used to examine the effect of this compound on memory formation (Fischer et al., 2007). Thirty minutes following injection, hippocampal tissues were dissected and processed for western analysis. Figure 1 shows pictures of representative western blots of acetylated and total histone H3 and H4 immunoreactivity from vehicle- and sodium butyrate-treated animals. Sodium butyrate was found to significantly enhance histone H3 (Figure 1A; vehicle: $99.99 \pm 7.04\%$, sodium butyrate: $166.68 \pm 19.51\%$, $P = 0.023$) and histone H4 (Figure 1B; vehicle: 99.99 ± 20.78 , sodium butyrate: 146.70 ± 6.94 , $P = 0.010$) acetylation in hippocampal extracts. These differences were not due to increases in the total levels of either histone H3 (vehicle: $100.00 \pm 10.94\%$, sodium butyrate: $107.01 \pm 10.41\%$, $P = 0.614$) or histone H4 (vehicle: $100.00 \pm 9.59\%$, sodium butyrate: $118.47 \pm 22.30\%$, $P = 0.404$).

Sodium butyrate administration prior to behavioral training does not improve performance of injured animals

To test the influence of sodium butyrate administration on learning and memory in TBI animals, we first utilized the paradigm outlined by Fischer et al who have reported that daily injection of sodium butyrate to CK-p25 Tg mice, a mouse model of neurodegeneration, can mimic the beneficial effects of exposure to an enriched environment. In this study, sodium butyrate was administered for a total of 4 weeks to CK-p25 Tg mice, with the treatment initiated after the neurodegeneration had occurred. To examine if this paradigm can offer improvement in a clinically relevant model of TBI, mice were injured then injected with sodium butyrate ($n=12$) or vehicle ($n=12$) beginning on day 7 post injury. Mice received daily injections for 4 weeks (Figure 2A). A 7 day post-injury time point was selected for initiation of treatment in order to allow TBI-associated neurodegeneration to occur. Although cell death following TBI is progressive, the majority of cell loss occurs within the first week after controlled cortical impact injury (Colicos et al., 1996). After the completion of the 4-week treatment, animals were trained in the water maze task. Animals were given 4 trials/day for 8 days, after which probe trials were given to test short- and long-term memory. A group of sham operated animals served as baseline controls ($n=10$). Figure 2B shows that injury causes a persistent dysfunction in hippocampal learning, as indicated by a significant difference in performance between the injured animals and sham-operated controls (group main effect: $F_{(2,31)}=3.784$, $P=0.034$; vehicle vs sham: $P=0.028$; drug vs sham: $P=0.017$ by Holm-Sidak *post-hoc*). However, when the performance of the vehicle-treated animals was compared to that observed in animals receiving sodium butyrate, no benefit was observed (vehicle vs drug: $P=0.831$ by Holm-Sidak *post-hoc*; Figure 2B). Figure 2C and 2D show that neither short-term (latency to platform vehicle: 31.33 ± 6.14 sec, sodium butyrate: 25.42 ± 5.36 , $P=0.456$; platform crossings vehicle: 1.75 ± 0.39 crossings, sodium butyrate: 1.75 ± 0.45 crossings, $P=0.904$) nor long-term (latency to platform vehicle: 25.75 ± 5.75 sec, sodium butyrate: 24.67 ± 6.01 sec, $P=0.893$; platform crossings vehicle: 2.17 ± 0.38 crossings, sodium butyrate: 1.83 ± 0.46 crossings, $P=0.567$) probe trials revealed any differences between the two injured groups, respectively. There was no difference in swimming speed detected between the vehicle- and sodium butyrate-treated injured groups (vehicle: 22.63 ± 0.79 cm/sec; sodium butyrate: 22.59 ± 0.79 cm/sec; $P=0.997$).

Recently, Lyeth and colleagues have demonstrated that acute, systemic application of the HDAC inhibitor 4-dimethylamino-N-[5-(2-mercaptoacetylaminopentyl)]benzamide (DMA-

PB) significantly reduces the density of phagocytic microglia. In addition, a trend towards reducing the number of degenerating neurons in the ipsilateral CA2/CA3 subfields of the hippocampus was also observed. Based on these observations, we questioned if treatment of injured mice during the period of ongoing hippocampal neuronal death (days 0–5 post-injury), would improve behavioral performance tested one week later (Figure 3A). Figure 3B shows that by comparison to sham-operated controls (n=10), the injured animals (n=8/group) had significantly poorer performance in the Morris water maze task (group main effect: $F_{(2,23)}=10.45$, $P<0.001$; vehicle vs sham: $P<0.001$; drug vs sham: $P=0.002$ by Holm-Sidak *post-hoc*) when tested on days 14–21 post-injury. However, mice receiving sodium butyrate treatment 30min post-injury and for 4 days thereafter, did not perform significantly better than their vehicle-treated counterparts in either hippocampal-dependent learning (vehicle vs drug: $P=0.550$ by Holm-Sidak *post-hoc*), short-term memory (latency to platform vehicle: 30.35 ± 6.85 sec, sodium butyrate: 32.12 ± 8.15 , $P=0.870$; platform crossings vehicle: 1.62 ± 0.50 crossings, sodium butyrate: 1.71 ± 0.47 crossings, $P=0.899$) or long-term memory (latency to platform vehicle: 31.35 ± 6.82 sec, sodium butyrate: 26.32 ± 6.48 , $P=0.601$; platform crossings vehicle: 1.88 ± 0.40 crossings, sodium butyrate: 2.38 ± 0.46 crossings, $P=0.425$, Figure 3C and D). Swimming speed was also not influenced by sodium butyrate administration (vehicle: 18.03 ± 0.80 cm/sec; sodium butyrate: 19.24 ± 1.57 cm/sec; $P=0.772$).

Concurrent sodium butyrate administration and behavioral training improves spatial learning and memory

In a recent study examining the dependency of neurobehavioral experience on the benefit offered by environmental enrichment, Kline and colleagues report that the improvement in performance seen in brain injured animals is restricted to when the behavioral training overlaps with the cognitive testing (Hoffman et al., 2008). To determine if HDAC inhibition can enhance the memory function of injured animals when given to animals performing a memory task, sodium butyrate (1.2 g/kg) or vehicle was injected beginning 2 weeks post-injury immediately following each day of training in the Morris water maze task (Figure 4A). Training and injection was continued for eight days. Figure 4B shows the daily performance of vehicle- (n=9) and drug-injected (n=7) injured animals in the water maze task compared to a group of uninjured sham-operated controls (n=10). When the performance of these groups was compared, a significant interaction of group by trial was detected ($F_{(14,161)}=2.108$, $P=0.014$). *Post-hoc* analysis revealed that this interaction was due to the progressive improvement of the sodium butyrate-treated animals such that the initial difference observed between this group and sham-operated animals diminished, and a significant difference appeared between the sodium butyrate- and vehicle-treated injured animals (indicated by *, Figure 4B). Figure 4C shows that when the injured animals were tested in a short-term memory probe trial, the sodium butyrate injected animals required significantly less time to cross the previous location of the hidden platform (latency to platform vehicle: 36.10 ± 7.14 sec, sodium butyrate: 15.15 ± 3.36 sec, $P=0.032$). This difference was not due to a difference in swimming speed between the two groups (vehicle: 21.62 ± 1.26 cm/sec; sodium butyrate: 22.18 ± 1.32 cm/sec; $P=0.573$). In contrast to that observed in the short-term memory test, no effect on memory retention was observed when the animals were tested by a probe trial given 24 hr following the completion of training

(latency to platform vehicle: 42.46 ± 7.81 sec, sodium butyrate: 34.40 ± 8.81 sec, $P=0.477$; Figure 4D).

To test if the decreased latency to the hidden platform seen in the short-term memory test is associated with increased measures of localization, the probe traces of vehicle- and sodium butyrate-injected animals were further analyzed. Counter areas, concentric rings of increasing diameter (2X, 3X and 4X platform diameters) centered on the platform, were used to assess the number of entries into the target areas, the latency to enter the inner rings, and the time spent searching in the immediate vicinity of the platform. Figure 5A shows traces of the paths taken by representative vehicle- and sodium butyrate-injected mice during the short-term probe trial. The rings used for quantification of localization parameters are indicated by gray dashed lines. Consistent with enhanced short-term memory, the sodium butyrate-treated animals took a more direct path to the platform location ($\neq$, interaction between group and latency $F_{(3,39)} = 2.98$, $P=0.043$; Figure 5B), entered the target area more frequently ($\neq$, group main effect $F_{(1,13)} = 10.63$, $P=0.006$; Figure 5C), and spent more time searching in the immediate area of the platform ($\neq$, interaction between group and dwell time $F_{(3,39)} = 3.14$, $P=0.036$; Figure 5D). When the long-term probe trial was assessed using these parameters, no significant differences in the latency to enter the different counter areas, in the number of platform crossings, nor the time spent in the rings proximal to the platform, were observed. However, a significant difference was detected in the time spent in the outermost ring (4X platform diameter), suggesting that the sodium butyrate-treated animals may have improved long-term memory for the general location of the platform.

Concurrent sodium butyrate administration and behavioral training improves memory in a subsequent cognitive task

To test if the combination of HDAC inhibition and cognitive training results in enhanced performance in a memory task tested subsequent to the termination of treatment, the mice used above were trained and tested in a delay fear conditioning task. Figure 6A shows the training curves of the vehicle- and sodium butyrate-infused mice. A two-way repeated measures ANOVA revealed a significant increase in freezing by trial ($F_{(2,28)}=54.85$, $P<0.001$), with no difference between the vehicle and drug treated groups ($F_{(1,14)}=2.31$, $P=0.150$). Interestingly, although the vehicle-treated animals displayed similar freezing behavior during training, their cue-elicited fear was less robust compared to the sodium butyrate-injected animals when tested in a short-term memory test (vehicle: $64.07 \pm 4.93\%$, sodium butyrate: $83.81 \pm 4.16\%$, $P=0.012$) (Figure 6B). Similar to that seen in the water maze task, this difference was not observed when the animals were tested 24hr post-training for their long-term cue-elicited fear (vehicle: $74.57 \pm 6.29\%$, sodium butyrate: $80.63 \pm 2.96\%$, $P=0.444$) (Figure 6C). No difference in contextual fear was detected at either time point (Figure 6B and 6C).

Discussion

Memory functions, especially hippocampus-dependent learning and memory, are often impaired after TBI. These dysfunctions are thought to be due, in part, to both neuronal death

and impaired plasticity of the surviving neurons (Lyeth et al., 1990; Zhang et al., 2005). Epigenetic changes, such as histone acetylation and methylation regulate chromatin remodeling, are therefore important contributors to the gene regulation involved in neural development and plasticity. When acetylated, histones become binding sites for bromodomain-containing proteins that participate in the chromatin remodeling required for gene expression. Histone deacetylases (HDACs) remove these acetyl groups, thereby returning the chromatin to its condensed and transcriptionally silent state. Inhibition of HDACs changes the expression of approximately 5–10% of transcribed genes (Scott et al., 2006; Ruthenburg et al., 2007), suggesting that neuronal plasticity may be improved by preserving histone acetylation following behavioral training. Consistent with this, recent studies have demonstrated that administration of HDAC inhibitors enhances long-term potentiation and facilitates memory storage (Levenson and Sweatt, 2005). Recently, it has been demonstrated that the memory enhancement caused by exposure of CK-p25 Tg mice, a model of p25-induced neurodegeneration, to environmental enrichment is associated with increased histone acetylation (Fischer et al., 2007), and that increasing histone acetylation in these mice via inhibition of HDACs was sufficient to improve performance in both spatial and fear conditioning tasks. As several investigators have shown that exposure to enriched environments can be used as a therapeutic strategy to alleviate the cognitive dysfunction associated with TBI (Hamm et al., 1996; Passineau et al., 2001; Wagner et al., 2002; Hoffman et al., 2008), we questioned if HDAC inhibition can act as a pharmacological substitute to environmental enrichment and offer behavioral improvement to brain injured animals.

To address the possibility that HDAC inhibitors can be used to mimic environmental enrichment, we followed the protocol used by Fischer et al., who administered sodium butyrate to mice for a period of four weeks following p25-induced neurodegeneration and observed a behavioral improvement comparable to that seen as a result of environmental enrichment (Fischer et al., 2007). However, when we administered sodium butyrate to brain injured mice after the reported period of maximal TBI-induced neurodegeneration, no benefit to hippocampus-dependent learning and memory was observed. While the reason of this failure is not clear at present, a number of differences between the transgenic model of neurodegeneration (CK-p25 Tg mice) used by Fischer and colleagues, and the TBI model used herein, may have contributed to the differential effects observed. First, CaMKII promoter driven, p25-induced cell death is likely to be restricted to neurons. By comparison, the pathology of TBI is complex and involves numerous processes including neuronal loss, impaired neuronal growth, gliosis, inflammation, and impaired neurovascular function. Second, in recent studies, it was reported that cortical impact injury of immature rats decreases histone H3 acetylation at 6 hr and 24 hr post-injury (Gao et al., 2006), and that acute, systemic administration of an HDAC inhibitor is accompanied by a trend towards a reduction in hippocampal neurodegeneration following lateral fluid percussion injury (Zhang et al., 2008). Thus, it is possible that unlike p25-induced neurodegeneration, the benefit of HDAC inhibition following TBI may be dependent on its ability to reduce neurodegeneration rather than increasing the plasticity of surviving neurons. To address this possibility, we administered sodium butyrate during the acute phase of injury (days 0–5) and initiated behavioral testing one week later. Similar to our findings when HDAC inhibition was

performed after the period of maximal neurodegeneration, no benefit was observed from sodium butyrate treatment during this period.

While beneficial effects of environmental enrichment have been reported following various injury magnitudes and injury models (but see (Wagner et al., 2002)), the improvements observed following this treatment appear to be temporally restricted. For example, a recent study by Kline and colleagues determined that the beneficial effects of environmental enrichment was dependent on the time of exposure relative to the period of behavioral testing (Hoffman et al., 2008). Their findings indicate that cognitive improvements in cortically impacted rats were observed only when the exposure to an enriched environment was carried out at the time of testing in the Morris water maze task, with prior exposure offering no benefit. Similar to these findings, we found that sodium butyrate improved performance in the Morris water maze when administered concurrent with, but not prior to, behavioral training. This improvement was associated with enhanced platform localization during a short-term memory probe trial as indicated by decreased latency to first platform crossing, increased dwell time in the immediate vicinity of the platform, and more platform crossings. This finding is similar to that previously reported by Passineau et al., who demonstrated that environmentally enriched, severely injured (by parasagittal fluid percussion injury) rats tended to cross the location of a hidden platform more frequently than rats kept in standard housing when tested immediately following water maze training (Passineau et al., 2001). Taken together, our findings indicate that HDAC inhibition may mimic some of the cognitive improvements seen following enriched environment exposure, and that the improvement is observed when the treatment is carried out at the time of behavioral testing. However, we cannot exclude the possibility that a different dose/dosing regimen, or the use of a different HDAC inhibitor, may yield a benefit to TBI animals independent of behavioral testing.

While the improved ability to perform the water maze task in animals receiving sodium butyrate is intriguing, this approach may have limited clinical utility if the effect is restricted to the time frame of drug administration. Thus, one of our primary interests regarding the cognitive influences of sodium butyrate in injured animals was whether or not the combination of HDAC inhibition and behavioral training would result in a lasting improvement in cognitive function that could be observed in a subsequent task. Our results using delay fear conditioning demonstrated that animals, which had been previously exposed to a HDAC inhibitor and behavioral training, did retain their enhanced cognitive ability and that similar to that seen in the Morris water maze task, this improvement was found to be enhanced short-term, but not long-term, memory. These results suggest that the benefit offered by HDAC inhibition and cognitive therapy may have enduring influences on neuronal properties. These findings, coupled with the previous reports that acute HDAC inhibition (initiated immediately after injury) can reduce microglial activation and inflammation examined 24hr following lateral fluid percussion injury in rats (Zhang et al., 2008), show that increasing histone acetylation following TBI may be an effective strategy to reduce some of the pathologies associated with brain injury. Further research is required to determine the extent of the benefits offered by HDAC inhibition, and to elucidate the genes that are influenced by sodium butyrate that underlie the cognitive improvements we observed.

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List of Abbreviations

CaMKII:	calcium/calmodulin-dependent protein kinase II
DMA-PB:	4-dimethylamino-N-[5-(2-mercaptoacetylamino)pentyl]benzamide
DTT:	dithiothreitol
HDAC:	histone deacetylase
MWM:	Morris water maze
PMSF:	phenylmethylsulphonyl fluoride
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBI:	traumatic brain injury
TBST:	tris buffered saline with triton x-100;

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Figure 1. Systemic sodium butyrate injection increases hippocampal histone acetylation

A) Photographs of representative western blots illustrating acetylated and total histone H3 immunoreactivity in the hippocampus of vehicle- and sodium butyrate-injected animals. **B)** Summary data showing that sodium butyrate significantly increases acetylated H4 immunoreactivity, but not total H4 levels, in the hippocampus. Data are presented as mean $\pm$ SEM. *, $P < 0.05$.

Figure 2. HDAC inhibition in the absence of behavioral training does not improve memory in injured animals

A) Schematic diagram showing the paradigm for injury, HDAC inhibitor injection (SB), and behavioral testing. HDAC inhibitor treatment in the absence of behavioral training is not sufficient to offer behavioral improvement in injured animals for either **B)** the acquisition of the Morris water maze ($\neq$, significant group main effect between sham and injured animals), or during probe trials administered **C)** 90 minutes or **D)** 24hr following the

completion of training. Data are presented as the mean $\pm$ SEM. MWM: Morris water maze.
SB: sodium butyrate

Figure 3. Acute sodium butyrate treatment, prior to behavioral training, does not improve memory in injured animals

A) Schematic diagram showing the paradigm for injury, HDAC inhibitor injection (SB), and behavioral testing. HDAC inhibitor treatment in the absence of behavioral training is not sufficient to offer behavioral improvement to injured animals in either **B)** the acquisition of the Morris water maze (#, significant group main effect between sham and injured animals), or during probe trials administered **C)** 90 minutes or **D)** 24hr following the

completion of training. Data are presented as the mean $\pm$ SEM. MWM: Morris water maze.
SB: sodium butyrate

Figure 4. Concurrent behavioral training and sodium butyrate injection enhances performance of brain-injured animals in a hippocampus-dependent task

A) Schematic diagram showing the paradigm for injury, HDAC inhibitor injection (SB), and behavioral testing. **B)** Sodium butyrate-treated, injured mice have significantly improved performance in the Morris water maze task. ≠, significant interaction of group and trial; *, $P < 0.05$ between vehicle- and drug-treated groups. Latency to the platform during probe trials administered **C)** 90 minutes and **D)** 24hr following the completion of training. *, $P < 0.05$. Data are presented as mean $\pm$ SEM.

Figure 5. Concurrent behavioral training and sodium butyrate injection improves short-term memory

A) Representative short-term probe traces for a vehicle- and a sodium butyrate-treated mouse showing their path to the hidden platform (solid gray circle). “Counter areas”, concentric circles of increasing diameter (shown by dashed lines), for quantification of localization are shown. Sodium butyrate mice have enhanced localization as indicated by **B)** a more direct path to the platform ($\neq$, significant interaction between group and latency), **C)** increased entries into the counter areas ($\neq$, significant interaction between group and

entries), and **D**) increased dwell times in the vicinity of the platform ($\neq$, significant interaction between group and dwell time). Data are presented as mean $\pm$ SEM. *, $P < 0.05$.

Figure 6. Concurrent behavioral training and sodium butyrate injection enhances performance in a subsequent fear conditioning task

A) Training curves for vehicle- and sodium butyrate-treated, injured mice in a fear conditioning task. Mice that received sodium butyrate during training in the water maze task were found to have enhanced cue-elicited **B)** short-term (*, $P < 0.05$), but not **C)** long-term, fear memory. Data are presented as mean $\pm$ SEM.