Scriptaid, a Novel Histone Deacetylase Inhibitor, Protects Against Traumatic Brain Injury via Modulation of PTEN and AKT Pathway

Guohua Wang · Xiaoyan Jiang · Hongjian Pu ·
Wenting Zhang · Chengrui An · Xiaoming Hu ·
Anthony Kian-Fong Liou · Rehana K. Leak ·
Yanqin Gao · Jun Chen

Published online: 7 November 2012

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Abstract Traumatic brain injury (TBI) is a leading cause of motor and cognitive deficits in young adults for which there is no effective therapy. The present study characterizes the protective effect of a new histone deacetylase inhibitor, Scriptaid (Sigma-Aldrich Corporation, St. Louis, MO), against injury from controlled cortical impact (CCI). Scriptaid elicited a dose-dependent

decrease in lesion size at 1.5 to 5.5 mg/kg and a concomitant attenuation in motor and cognitive deficits when delivered 30 minutes postinjury in a model of moderate TBI. Comparable protection was achieved even when treatment was delayed to 12 h postinjury. Furthermore, the protection of motor and cognitive functions was long lasting, as similar improvements were detected 35 days postinjury. The efficacy of Scriptaid (Sigma-Aldrich Corporation) was manifested as an increase in surviving neurons, as well as the number/length of their processes within the CA3 region of the hippocampus and the pericontusional cortex. Consistent with other histone deacetylase inhibitors, Scriptaid treatment prevented the decrease in phospho-AKT (p-AKT) and phosphorylated phosphatase and tensin homolog deleted on chromosome 10 (p-PTEN) induced by TBI in cortical and CA3 hippocampal neurons. Notably, the p-AKT inhibitor LY294002 attenuated the impact of Scriptaid, providing mechanistic evidence that Scriptaid functions partly by modulating the prosurvival AKT signaling pathway. As Scriptaid offers long-lasting neuronal and behavioral protection, even when delivered 12 h after controlled cortical impact, it is an excellent new candidate for the effective clinical treatment of TBI.

G. Wang · X. Jiang · H. Pu · W. Zhang · C. An · Y. Gao · J. Chen
State Key Laboratory of Medical Neurobiology and Institute
of Brain Science, Fudan University,
Shanghai 200032, China

G. Wang · X. Hu · A. K.-F. Liou · Y. Gao · J. Chen
Center of Cerebrovascular Disease Research,
University of Pittsburgh,
Pittsburgh, PA 15213, USA

R. K. Leak
Division of Pharmaceutical Sciences, Mylan School of Pharmacy,
Duquesne University,
Pittsburgh, PA 15282, USA

G. Wang
Department of Neuropharmacology,
Institute of Nautical Medicine, Nantong University,
Nantong, Jiangsu 226001, China

X. Hu · A. K.-F. Liou · J. Chen
Geriatric Research, Education and Clinical Center,
Veterans Affairs Pittsburgh Health Care System,
Pittsburgh, PA 15240, USA

Y. Gao (✉) · J. Chen (✉)
Department of Neurology,
University of Pittsburgh School of Medicine,
Pittsburgh, PA 15213, USA
e-mail: yqgao@shmu.edu.cn
e-mail: ChenJ2@upmc.edu

Keywords TBI · Histone deacetylase inhibitor · CCI ·
Long-term protection · p-AKT · PTEN

Introduction

Traumatic brain injury (TBI) is a neurological disorder with growing prevalence among young adults and no effective therapeutic agent available to date. Both clinical and

experimental evidence accumulated thus far implicates a complex prognosis for TBI. This disorder not only causes primary brain injury at the time of impact, but also induces secondary brain injury through complex cellular and biochemical processes within minutes to days after the initial trauma [1, 2]. Primary TBI brain injuries include skull fracture, cerebral contusions, lacerations, hematoma, and diffuse axonal injury, resulting in the bulk of irreversible damage to the brain [3]. Secondary TBI may result from intracranial or extracranial effects. Intracranial consequences include mass lesions, focal/diffuse brain swelling, intracranial hypertension, seizures, vasospasm, or infection, whereas extracranial consequences include hypotension, hypoxia, hypercapnia/hypocapnia, hyperglycemia/hypoglycemia, anemia, pyrexia, electrolyte abnormalities, coagulopathy, and infection [4]. These secondary injuries from TBI lead to alterations in cell function and propagation of injury through processes such as depolarization, excitotoxicity, disruption of calcium homeostasis, free-radical generation, blood-brain barrier disruption, ischemic injury, edema formation, and intracranial hypertension [5–7]. Apart from prevention, little can be done to mitigate primary injury, whereas the “rolling” pathology of the delayed secondary injury allows therapeutic intervention within a window of limited time. Therefore, TBI management will likely be comprised of preventive strategies coupled with therapies targeted at secondary brain injuries [5, 8–10]. However, the complicated prognosis of this disorder has caused the identification and development of a viable therapeutic agent elusive, despite much research effort.

The acetylation status of chromatin is determined by the homeostatic activities of 2 classes of enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC), competing to modify the lysine residues within nuclear histones. Mechanistically, HAT acetylates lysine groups in nuclear histones, neutralizing the charge, and resulting in a chromatin configuration that is more accessible to transcriptional activity. On the other hand, HDAC deacetylates the lysine groups within nuclear histones, promoting chromatin condensation and consequent transcriptional repression. Recent work has uncovered the importance of chromatin remodeling, especially via histone acetylation, in the epigenetic control of gene expression in acute central nervous system injuries [11].

With epigenetic modulation in mind, a number of studies have examined the use of histone deacetylase inhibitors (HDI) as therapy for restoring histone acetylation and transcriptional activation in TBI models [12, 13]. The epigenetic influence of HDIs on gene expression make them a useful new class of pharmacological agents that could ameliorate various disease conditions. For example, HDIs also promote neuronal survival in ischemic injury, [14–17] multiple sclerosis [18, 19], and Alzheimer’s and Huntington’s disease

models [20–24]. These multiple roles of HDIs can be attributed to alterations in the expression of different gene sets by increasing acetylation of chromatin. Because of the dependence on phenotypic expression of protein markers, the roles of HDIs are likely to be cell-type dependent. In neuronal systems, HDI treatments enhance neuronal plasticity and survival [25–28] via various molecular mechanisms [11]. It is now increasingly clear that the homeostatic equilibrium in chromatin acetylation is greatly disrupted after TBI [29–33]. Indeed, a loss in histone acetylation has been observed after TBI, and it is attributed to the upstream excitotoxic and stress cascades associated with this injury [11]. Moreover, there are studies indicating that TBI specifically reduces histone H3 acetylation [34], which would increase microglial inflammatory responses after TBI [32]. Therefore, we hypothesized that HDI treatment would protect against behavioral deficits induced by TBI. This would be consistent with a recent study showing that ITF2357, a pan-HDI, protected against behavioral deficits and tissue loss from a closed head injury [13]. However, a more specific class of HDI may be able to achieve a comparable or better protective outcome as the pan-HDI, which would avoid negative side effects.

To address these questions, this study was conducted to investigate the potential benefits of Scriptaid (Sigma-Aldrich Corporation, St. Louis, MO), a novel HDI, in a mouse model of TBI. Scriptaid (Sigma-Aldrich Corporation) is recognized as the least toxic HDI thus far [35]. In this study, we show that Scriptaid also protects against injury from controlled cortical impact (CCI). Postinjury administration of Scriptaid resulted in a significant decrease of lesion volume and protected against the motor and cognitive hallmark deficits of TBI. In addition, the underlying mechanism was partially identified, as Scriptaid reversed CCI-induced AKT pathway inactivation and a p-AKT inhibitor attenuated the protection.

Materials and Methods

Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Animals

Young male C57BL/6 J mice (25–30 g body weight) were obtained from the Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The animals were housed in groups (4–5 per cage) with food and water available ad libitum, in a temperature- and humidity-controlled animal facility with a 12-h light/dark cycle. All procedures used in

this study were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Fudan University. All efforts were made to minimize animal suffering and the number of animals used.

TBI

All animals were randomly assigned into experimental groups through the use of a lottery drawing. Mice were anesthetized with 3% isoflurane in a 67% N₂O/30% O₂ mixture (induction), until they were unresponsive to the tail pinch test and then fitted with a nose cone delivering 1.5 % isoflurane for anesthesia maintenance. Animals were mounted in a stereotaxic frame secured by ear and incisor bars, held in a horizontal plane with respect to the interaural line, shaved, and swabbed with betadine. Using aseptic techniques and principles consistent with Institutional Animal Care and Use Committee surgical policy, the wound site was treated with lidocaine (0.5 %), a midline incision was made, the soft tissues were reflected, and approximately a 4-mm craniotomy was performed over the right parietotemporal cortex using a motorized drill. The CCI was centered 2-mm lateral to midline and 1.0-mm anterior to bregma, and was produced with a pneumatically driven CCI device (Precision Systems and Instrumentation, Fairfax, VA) using a 3-mm flat-tip impounder (velocity, 3.5 m/sec; duration, 150 msec; depth, 1.5 mm). Immediately after the injury, the bone flap was replaced and sealed with Koldmount cement (Vernon Benschoff, Albany, NY), and the scalp was sutured shut. Rectal temperature was controlled at 37°±0.5 °C during surgery and for as long as 30 minutes after TBI using a temperature-regulated heating pad. Sham animals were subjected to all aspects of the protocol (i.e., surgery, anesthesia, craniotomy, recovery), except for CCI. Less than 2 % of mice were excluded for symptoms, such as inability to eat and drink without assistance or evidence of pain or wound infection. Time points of assessment in different parameters are illustrated in Fig. 1A.

Drug Administration

Scriptaid was prepared as a 100 mg/ml stock in dimethyl sulfoxide (DMSO). Prior to use, the compound was diluted in sterile saline, heated to boiling for complete dissolution, and cooled to body temperature or less before intraperitoneal administration. Scriptaid solutions were freshly prepared for each experiment. In the vehicle group, a 0.5 % DMSO saline solution was used in lieu of Scriptaid. To examine dose-dependency and determine the optimal dose, mice were injected with 0, 1.5, 3.5, or 5.5 mg/kg of Scriptaid at 0.5 hr after CCI. This dose range was selected based on the results of our pilot study in which these doses of Scriptaid effectively augmented acetyl-H3 and acetyl-H4 levels in the brain and yet did not cause obvious adverse effects or death in mice. In

Fig. 1 The dose-dependent protection conferred by Scriptaid against traumatic brain injury (TBI). (A) Temporal schematic of the experimental protocol and the time points of sample collection and analysis. (B) Changes in the levels of acetylated-H3, acetylated-H4, and total-H3 24 h postinjury, as visualized by Western blot analysis. Nuclear protein extracts were obtained from ipsilateral cortices. β -actin immunoblotting was used as the control to ensure nuclear extracts were not contaminated by cytoplasm. Quantification of the changes in acetylated-H3 and -H4 levels, normalized to total-H3 histone. Data are presented as mean $\pm$ standard error (n=6; * p <0.05, ** p <0.01 vs sham or vehicle). (C) No statistically significant change in weight by Scriptaid. (D) Wire-hanging test: a lower score represents more severe neurological deficits, ranging from a minimum score of 0 for severe impairments, to a maximum score of 5, indicating near-normal function. The scores for the wire-hanging test significantly decreased in the vehicle group after TBI, but performance of Scriptaid-treated (3.5 mg/kg) mice was significantly less affected from 1 to 7 days (dotted boxes; all p <0.01). The high-dose group (5.5 mg/kg) exhibited greatly improved neurological function only at 1 and 3 days after TBI compared with the vehicle group (p <0.05 or 0.01). For the low-dose (1.5 mg/kg) group, the lesion was similar to controls. Data are presented as mean $\pm$ standard error (n=6; * p <0.05, ** p <0.01 vs vehicle). (E) Forelimb foot-fault: was calculated as F/T, where F is the total number of foot faults for left forelimb and T is the total movement number of left forelimb. Controlled cortical impact increased foot-faults compared to sham. Treatment with Scriptaid at 3.5 mg/kg elicited the greatest reduction in forelimb foot-faults on days 3-7 (dotted boxes) postinjury. Data are presented as mean $\pm$ standard error (n=6; * p <0.05, ** p <0.01 vs vehicle). (F) Cylinder test: final score = (nonimpaired forelimb movement - impaired forelimb movement)/(nonimpaired forelimb movement + impaired forelimb movement + both movements). Contralateral forelimb placing for the vehicle group was significantly decreased at 3, 5, and 7 days after TBI, leading to an increase in the cylinder test score. In comparison, mice from the Scriptaid-treated group exhibited a higher placing frequency, resulting in lower scores than the vehicle group. Data are presented as mean $\pm$ standard error (n=6; * p <0.05, ** p <0.01 vs vehicle). (G) Visualization of the lesion volume with Nissl staining of brain sections from mice euthanized at day 7 after TBI and the comparison of various doses of Scriptaid and vehicle. Data were presented as mean $\pm$ standard error (n=10; * p <0.05, ** p <0.01 vs vehicle). WB = Western Blot

subsequent experiments, Scriptaid was introduced at 3.5 mg/kg at 0.5 or 12 h after injury. For determining the therapeutic window of time, Scriptaid was also administered at 3.5 mg/kg, i.p., 12 h after injury, and this treatment was repeated daily for the next 2 days postinjury.

Intracerebroventricular Injection

For PI3K inhibitor delivery, LY294002 (Cell Signaling Technology, Beverly, MA) was dissolved in 25 % DMSO in phosphate-buffered saline (PBS). Mice were anesthetized and placed in stereotaxic frames. LY294002 (61.5 mg/kg in 25 % DMSO/PBS) or the vehicle (25 % DMSO in PBS) were injected intracerebroventricularly (2 μ l; 1.0 mm lateral to bregma, 0.2 mm posterior to bregma, and 2.5 mm ventral to skull). LY294002 or the vehicle was injected 1 h before TBI surgery.

Neurobehavioral Assessments

All motor sensory tests and cognitive assessments were performed by investigators who were blinded to the experimental groups.

Foot Faults Test

The foot fault test was used to assess deficits in forelimb and hindlimb function. Mice were placed on an elevated grid surface (30 L×35 W×31 Hcm) with a grid opening of 2.5 cm². Each fall and slip between the wires with weight-bearing steps was recorded as a hindlimb or forelimb foot fault.

Wire-Hanging Test

The wire-hanging test apparatus was a stainless steel bar (50 cm; 2-mm diameter) resting on 2 vertical supports and elevated 37 cm above a flat surface. Mice were placed on the bar midway between the supports and were observed for 30 seconds in 4 trials. The amount of time spent hanging was recorded and scored according to the following system: 0, fell off; 1, hung onto the bar with 2 forepaws; 2, hung onto the bar with added attempt to climb onto the bar; 3, hung onto the bar with 2 forepaws and 1 or both hind paws; 4, hung onto the bar with all 4 paws and with tail wrapped around the bar; 5, escaped to 1 of the supports.

Cylinder Test

The cylinder test was adapted for use in mice to assess forelimb use and rotation asymmetry [36]. The mouse was placed in a transparent cylinder 9 cm in diameter and 15 cm in height and was videotaped during the test. A mirror was placed behind the cylinder at an angle that enabled the rater to record all forelimb movements. Forelimb use during the first contact with the wall after rearing and during lateral exploration was recorded by the following criteria: 1) The first forelimb to contact the wall during a full rear was recorded as an independent wall placement for that limb; 2) simultaneous use of both the left and right forelimbs during a full rear and for lateral movements along the wall was recorded as “both” movement; 3) after the first forelimb (e.g., right forelimb) contacted the wall and then the other forelimb was placed on the wall, but the right forelimb was not removed from the wall, a “right forelimb independent” movement and a “both” movement were recorded; however, if the other (left forelimb) made several contacting movements on the wall, a “right forelimb independent” movement and only 1 “both” movement were recorded; and 4) when the mouse explored the wall laterally, alternating both forelimbs, it was recorded as a “both” movement.

A total of 20 movements were recorded during the 10 minute test. The final score was determined based on the following formula: the final score equaled (nonimpaired forelimb movement minus impaired forelimb movement) was divided by (nonimpaired forelimb movement plus impaired forelimb movement plus both movement). This test evaluates forelimb use asymmetry for weight shifting during vertical exploration and provides high reliability even with inexperienced raters. Occasionally mice with large deficits did not initially move frequently enough to obtain an adequate number of vertical movements. Typically, these mice would recover in time when the test was performed. To avoid bias, these mice were not scored until they could perform the test.

Morris Water Maze Test

The Morris water maze test was typically performed between 29 and 35 days after injury. For this test [37], an 11-cm diameter platform is submerged in 2 quadrant of a pool that is 109 cm in diameter. The hidden platform test assesses the ability of the mice to find the platform without being able to directly see it, and the mice have to either remember where it is relative to external spatial cues or perform a search. The platform was placed 1 cm under the water surface, and the water was made opaque with white, non-toxic tempera paint. Each mouse was released from 1 of 4 locations and was allotted 90 seconds to search for the hidden platform. At the end of each trial, the mouse was placed on the platform or allowed to remain on the platform for 30 seconds with prominent spatial cues displayed around the room. Because the investigator was also a spatial cue, they always sat in the same location during each trial. Four trials per day for 5 consecutive days were performed with the location of the platform kept constant. Data are expressed as the time (in seconds) or latency to reach the submerged platform on each day.

After the last day of the hidden platform test, a single, 60-second probe trial was performed. The platform was removed and each mouse was placed in the pool once for 60 seconds, at the same starting location as was used first in hidden platform testing. The time spent in the goal quadrant (where the platform had been located) and the swimming speed were both recorded. This is considered the most specific test for spatial memory. All tests were carried out by researchers blinded to the groups.

Nissl Staining

Mice were sacrificed under chloral hydrate (360 mg/kg body weight, i.p.) anesthesia at 7 or 35 days post-TBI by perfusion through the left ventricle with 0.9 % saline followed by 4 % paraformaldehyde. The brains were then

removed and postfixed for 24 h in 4 % paraformaldehyde followed by cryoprotection in 30 % sucrose in PBS. Coronal sections (25- μ m thick) were cut on a cryostat (CM1900; Leica, Bensheim, Germany). Sections were mounted and Nissl stained for histological assessment of damage and the adjacent free-floating sections were processed for immunohistochemical analysis. For Nissl staining, sections were sequentially processed through the following solutions: chloroform, 30 minutes; acetone, 15 minutes; 100 % ethyl alcohol (EtOH), 30 seconds; 95 % EtOH, 30 seconds; 70 % EtOH, 30 seconds; distilled water, 30 seconds, twice; cresyl violet, 20 minutes; distilled water, 30 minutes, three times; 70 % EtOH, 1 minute; 95 % EtOH, 1 minute; 100 % EtOH, 1 minute; chloroform, 5 minutes; differentiator (95 % EtOH, with glacial acetic acid added to reach a pH of 4.1), 6 minutes; 95 % EtOH, 2 minutes, 100 % EtOH, 3 minutes, twice; xylene, 2 minutes; xylene, 3 minutes, twice. Sections were then coverslipped with neutral balata.

The brain slices were photographed (DM5000B, Leica, Bensheim, Germany), and lesion volumes measured with Image-J analysis software (National Institutes of Health, Bethesda, MD). Percentage lesion volume was calculated by an investigator blinded to the study groups with the formula: $[(V_C - V_L)/V_C] \times 100$, where V_C is the volume of contralateral hemisphere (left side) and V_L is the volume of remaining, noninjured tissue in the ipsilateral hemisphere subjected to CCI (right side).

Stereological Cell Counting of CA3 Neurons

After Nissl staining, viable CA3 neurons (35 days after CCI) were quantified using stereology and the Bioquant Image Analysis program (Bioquant, Nashville, TN) as previously described [38]. Image capturing of the entire CA3 subfield from a given section was done with a color CCD camera, with 50 $\times$ 50- μ m grid squares over the region of interest, and with 100 to 150 cells that were counted in a given structure for the optical dissector method to estimate the total number. The number of points to be assessed within the outlined area was empirically determined. Previous studies have shown that a 25 $\times$ 25- μ m counting frame and a focus depth of 25 μ m in a 40- μ m section with 7.5- μ m guard regions above and below should be used [39]. Neurons within the 25 $\times$ 25 $\times$ 25- μ m box were counted and this was defined as a dissector. On average, 9 dissectors per section yielded the desired number of counted neurons throughout the CA3 at the levels of the dorsal hippocampus. From section to section, the number of dissectors was maintained at a constant ratio in the CA3 region. The rostro-caudal length of the CA3 was 1 mm (from bregma -0.94 mm to bregma -1.94 mm), which was obtained by multiplying section thickness by the number of sections (25 sections). Every fifth section was counted. The total number of neurons was calculated using the optical dissector, equal to the quotient

of the total number of neurons counted and the product of the fractions for sampling section frequency (SSF) or fraction of sections counted, the area section frequency (ASF) or sampling area/area between the dissectors, and the thickness section frequency (TSF) or the dissector depth/section thickness, for $n = \Sigma \text{neurons counted} \times 1/\text{SSF} \times 1/\text{ASF} \times 1/\text{TSF}$. For our study, $\text{SSF} = 1/4$ sections, $\text{ASF} = 25 \times 25 \mu\text{m} / 50 \times 50 \mu\text{m}$, and $\text{TSF} = 25 \mu\text{m} / 40 \mu\text{m}$.

Immunohistochemistry

Immunohistochemical stains for light microscopy were performed using rabbit anti-microtubule-associated protein 2 (anti-MAP2) at a 1:500 dilution (Millipore, Billerica, MA). Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 in methanol. This was followed by incubation in blocking buffer (10 % normal goat serum and 0.3 % Triton-X in TBS) for 30 minutes. Primary antibodies were diluted in commercial antibody diluent (Beyotime, Jiangsu, China) and sections were incubated overnight at 4 °C. The following day sections were processed with the ABC kit (Zhongshan Biotechnology Company, Beijing, China). Sections were then treated with 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Zhongshan Biotechnology Company) to visualize changes in the morphology of neurites.

Quantification of MAP-2 Positive Cells

To quantify MAP-2 positive cells in the pericontusion regions of the cortex as indicated in Fig. 2B, stereological counting was performed by the Institute of Brain Sciences Histopathology Core (Fudan University) based on the methods previously described [40]. In brief, MAP-2 positive cells were counted stereologically using the optical fractionator method on a Stereo Investigator system (MicroBrightfield, Williston, VT) attached to a Leica microscope (Leica, Bensheim, Germany). One of 5 40- μ m-thick serial sections with a total of 4 sections from bregma +2.8 to -1.06 mm was collected for counting. The pericontusional cortex was delineated with a 5 $\times$ objective, and the actual counting was performed under a 63 $\times$ objective. The stereological counting was performed by an investigator who was blinded to the experimental group assignment ($n=6$ per group), and all numbers were normalized to the sham controls.

Dendrite Analysis

Pyramidal neurons in the layers III within the pericontusion region of the cortex were analyzed as previously described [41]. Briefly, 12 pyramidal neurons were randomly selected from the cortical layers III of each coronal section, and the lengths in microns of the apical dendrites of completely impregnated pyramidal neurons

were measured using Image-J software (Bethesda, MD). The data presented were based on 4 equally spaced sections located rostrocaudally between bregma +2.8 and -1.06 mm. The analysis was done by an investigator blinded to the experimental group.

Immunofluorescence

For immunofluorescent staining, rabbit anti-p-AKT (ser473) or anti-p-PTEN (Ser380/Thr382/383) polyclonal antibodies were diluted 1:400 (Cell Signaling Technology). Sections of interest were first blocked with 10 % goat serum/PBS solution for 1 h followed by incubation with primary antibodies for 1 h at 37 °C. For double labeling, sections were subsequently incubated with the second primary antibody, mouse anti-NeuN, diluted 1:200 (Millipore, Billerica, MA) overnight at 4 °C. The following day, sections were incubated in a mixture of species specific secondary antibodies, including DyLight 594 goat anti-rabbit antibody diluted 1:1000 and Alexa Fluor 488 goat anti-mouse antibody diluted 1:500 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at 37 °C. Finally, slides were coverslipped with Vectashield mounting medium containing 4'-6-Diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA) prior to confocal fluorescence microscopy (FV1000, Olympus, Japan).

Fluoro-Jade C Staining

Fluoro-Jade (Millipore, Billerica, MA) C staining was performed in accordance with the manufacturer's protocol. Briefly, sections were mounted on slides and immersed in a basic alcohol solution consisting of 1 % sodium hydroxide in 80 % ethanol for 5 minutes. The slides were then rinsed for 2 minutes in 70 % ethanol followed by another 2 minutes in distilled water. They were incubated in 0.06 % potassium permanganate solution for another 10 minutes. The slides were then immersed in 0.0001 % solution of Fluoro-Jade C dissolved in 0.1 % acetic acid vehicle for 10 minutes. Sections were also counterstained for 5 minutes with DAPI. The slides were washed with distilled water and air dried. Finally, the slides were cleared in xylene for at least 1 minute and then coverslipped with nonfluorescent DPX mounting medium (Fluka, Buchs, Switzerland).

Nuclear Protein Extraction

Nuclear protein extracts of ipsilateral cortex were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Fisher Scientific, Pittsburgh, PA), in accordance with the manufacturer's instructions. In brief, cortical tissue was first washed with PBS and homogenized in a glass dounce homogenizer into a uniform suspension. The sample was then subjected to centrifugation for 5 minutes at 500×g and the supernatant was

Fig. 2 Scriptaid conferred protection against long-term motor and cognitive deficits and tissue loss from controlled cortical impact (CCI) injury. (A) Effect of Scriptaid on long-term motor deficits; a) wire-hanging test: Scriptaid treatment attenuated motor deficits leading to an increase in performance scores compared with the vehicle group for as many as 21 days postinjury. Data are presented as mean ± standard error (n=6; * $p<0.05$, ** $p<0.01$ vs vehicle); b) forelimb foot-fault test; c) hindlimb foot-fault test: Scriptaid treatment reduced forelimb and hindlimb foot-faults for as many as 4-weeks post-CCI when compared to vehicle. Data are presented as mean ± standard error (n=6; * $p<0.05$, ** $p<0.01$ vs vehicle); d) cylinder test: the administration of Scriptaid also diminished biased forelimb use for as many as 4-weeks postinjury. (B) Scriptaid treatment significantly reduced tissue loss (cavity size) produced by CCI trauma at 35 days after traumatic brain injury (TBI) compared to the vehicle group. TBI induced a macroscopic area of cortical tissue loss extending rostrocaudally from bregma + 2.8 mm to bregma -1.06 mm in the vehicle group. The region of interest lies between the internal and external circles (1.5-mm gap; pericontusional cortex). (C) Quantitation of the difference of lesion volume at 5-weeks postinjury between Scriptaid-treated and -nontreated mice. Data are presented as mean ± standard error (n=6; * $p<0.05$, ** $p<0.01$ vs vehicle). (D) Effect of Scriptaid on cognitive deficits postinjury: a) cued learning response: a typical swim pattern of mice from sham, vehicle, and Scriptaid-treated groups while locating the platform within the Morris water maze; b) probed trial response: a typical swim pattern of mice from sham, vehicle, and Scriptaid-treated groups while attempting to locate the platform within the Morris water maze based on memory; c) time required for mice from these 3 groups to locate the platform in the cued learning response at 29- to 33-days postinjury. Data are presented as mean ± standard error (n=6; * $p<0.05$, ** $p<0.01$ vs vehicle and $^{##}p<0.01$ vs sham; d) the percentage of time spent in the same quadrant as the platform after training for mice from sham, vehicle, and Scriptaid-treated groups. Data are presented as mean ± standard error (n=10; * $p<0.05$ vs vehicle and $^{##}p<0.01$ vs sham)

removed from the whole cell pellet. Afterward, the pellet was re-suspended in 400 µl of ice-cold cytoplasmic extraction reagent I with protease inhibitors followed by incubation on ice for 10 minutes. Then, 22 µl of ice-cold cytoplasmic extraction reagent II was added to the sample before centrifugation at 14,000×g for 5 minutes. The cytoplasmic extract in the supernatant was carefully removed. The nuclei-enriched pellet was re-suspended in 200 µl of nuclear extraction reagent and incubated on ice for 40 minutes. This was followed by a final centrifugation at 14,000×g for 10 minutes. The supernatant containing the nuclear protein extract was analyzed by Western blot analysis for changes in acetylated-H3, acetylated-H4, and total-H3, outlined as follows.

Western Blot Analysis

At indicated time points after surgery, each mouse was decapitated under 10 % chloral hydrate anesthesia. The cortical tissues surrounding the contusion site (0.5-1.0-mm extension from the cavity) was harvested (Fig. 2B) for immunoblotting with antibodies against p-AKT (Ser 473), AKT, p-PTEN (Ser380/Thr382/383), and PTEN, all diluted 1:1000 (Cell Signaling, Danvers, MA), as well as an antibody against MAP-2 diluted 1:1000 (Millipore, Billerica, MA). Anti-acetylated-H3, acetylated-H4, and total-H3 (Cell Signaling

Fig. 3 Scriptaid conferred comparable protection when administrated 12 h post-controlled cortical impact at day 7 after injury. (A) Wire-hanging test: Scriptaid treatment attenuated motor deficits, leading to an increase in performance scores compared with the vehicle group. Data are presented as mean $\pm$ standard error ($n=6$; $*p<0.05$ vs vehicle). (B) Cylinder test: the administration of Scriptaid also diminished biased forelimb use. Data are presented as mean $\pm$ standard error ($n=6$; $*p<0.05$ vs vehicle). (C) Forelimb foot-fault test: an overall decrease in foot-faults with Scriptaid treatment was not statistically significant. (D) Hindlimb foot-fault test: an overall decrease in foot-faults with Scriptaid treatment was not statistically significant. (E) Quantification of lesion volume by Nissl staining of brain sections. Scriptaid treatment (3.5 mg/kg) at 12-h postinjury significantly reduced lesion volume. Data are presented as mean $\pm$ standard error ($n=8$; $*p<0.05$ vs vehicle)

Technology) primary antibodies were also used at 1:1000 dilutions. Brain tissue was homogenized in an ice-cold buffer (50 mM tris-(hydroxymethyl) aminomethane, pH 7.4; 150 mM NaCl; 0.5 % Triton X-100; 1 mM acetic acid; 1 M phenylmethylsulfonyl fluoride; and 5 mg/L aprotinin), and centrifuged at $14,000\times g$ at 4°C for 30 minutes. The supernatants were then collected and total protein was determined by the bicinchoninic acid assay (Beyotime Biotechnology, Haimen, China). Equal amounts of protein were resolved by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking the membrane in 5 % nonfat milk dissolved in Tris-buffered saline solution for 1 h, it was incubated overnight in primary antibodies at 4°C . Afterward, the membrane was subjected to 3 consecutive washes with Tris-buffered saline for 5 minutes each followed by incubation with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (HRP, at a 1:1000 dilution; Pierce Biotechnology Inc., Rockford, IL) at room temperature for 1 h. Membranes were scanned and quantified by Scion Image (Scion, Frederick, MD), and protein levels were expressed as a fraction of β -actin or total-H3 levels in the same lane.

Statistical Analyses

All values are presented as the mean $\pm$ standard error. Data with 2 groups were analyzed with the Student's *t* test (nondirectional), and data with repeated groups were analyzed with 1-way analysis of variance and the Student-Newman-Keuls test for *post hoc* comparisons. The Pearson product linear regression analysis was used to correlate the number of CA3 neurons with spatial memory. Differences were considered statistically significant at $p<0.05$.

Fig. 4 Scriptaid prevented neurodegeneration in cortex and hippocampus. (A) Viable neurons from the ipsilateral CA3 region of the hippocampus from mice in sham, vehicle, and Scriptaid-treated groups, as visualized by Nissl staining; scale bar, 100 μm . (B) Stereological quantification of viable CA3 neurons in the hippocampus ipsilateral to traumatic brain injury. Data are presented as mean $\pm$ standard error ($n=6$; $**p<0.01$ vs vehicle). (C) Correlation between spatial memory and number of viable neurons in the ipsilateral CA3 region from mice of sham, vehicle, and Scriptaid-treated groups. (D) Visualization of degenerating neurons in the ipsilateral cortex and hippocampus from mice of sham, vehicle, and Scriptaid-treated groups by Fluoro-Jade C (FJC) staining at day 7 postinjury. These sections are co-stained with 4'-6-Diamidino-2-phenylindole (DAPI) to reveal nuclear morphology. Scale bar, 25 μm

Results

Scriptaid is an Effective Inhibitor of Deacetylase Activity

In this study, male adult C57BL/6 J mice were subjected to CCI (velocity, 3.5 m/sec; duration, 150 msec; depth, 1.5 mm) and then randomly and blindly assigned to groups that received either vehicle or Scriptaid. Scriptaid was introduced intraperitoneally at 30 minutes postinjury and motor deficits were determined within the first week after TBI, whereas cognitive deficits were measured within the fifth week after injury. The time points of sample collection, evaluation, and analysis are shown in Fig. 1A.

First, the inhibitory effect of Scriptaid on deacetylase activity in the cortex at doses between 1.5 and 5.5 mg/kg was evaluated after 24 h via Western blot analysis. As expected, CCI significantly enhanced histone deacetylase activity with a marked decrease in acetylated-histone H3 and no change in total histone H3 levels. However, Scriptaid treatment elicited a dose-dependent increase in acetylated-histone H3, indicating the effective inhibition of histone deacetylase activity (Fig. 1B). A similar result was observed for acetylated histone H4, suggesting that the inhibitory effect of Scriptaid extended to acetylases targeting histone H4 (Fig. 1B).

Scriptaid Treatment Ameliorates Motor Function Deficits and Decreases Lesion Volume

In the vehicle group, TBI caused an acute loss in body weight after 1 day, which gradually recovered within 6 days. Treatment with different doses of Scriptaid did not affect body weight (Fig. 1C), suggesting a lack of inherent toxicity. As expected, TBI elicited significant motor deficits in the vehicle group, as reflected by the decrease in wire-hanging test scores. However, Scriptaid treatment attenuated motor deficits in a dose-dependent manner. Maximum protection from Scriptaid was observed at 3.5 mg/kg during the first 7 days postinjury (all $p < 0.01$) (Fig. 1D). In support of these findings, forelimb foot-fault tests and cylinder tests also indicated that the Scriptaid treatment protected against TBI-induced motor deficits for the first 7 days postinjury (Fig. 1E, F). Consistently, the largest degree of protection was observed at 3.5 mg/kg. In addition, Scriptaid treatment decreased cortical lesion volume quantified by Nissl staining (Fig. 1G). At 3.5 mg/kg, a maximal decrease of 45 % in lesion volume was achieved. Hence, the dose of 3.5 mg/kg was used in subsequent experiments.

Scriptaid Treatment Conferred Long-Term Protection Against Motor and Cognitive Deficits, and Cortical Tissue Loss

Thus far, Scriptaid effectively conferred protection against short-term tissue loss and behavioral deficits from TBI. Next,

Fig. 5 Reductions in neuronal microtubule-associated protein-2 (MAP-2) expression and neurites at the ipsilateral cortex were attenuated by Scriptaid treatment. (A) The decrease in MAP-2 levels after traumatic brain injury (TBI) was prevented by Scriptaid treatment at 7 and 35 days postinjury, as shown by Western blot analysis. β -actin was included as the loading control. Quantification of the changes in MAP-2 levels in vehicle and Scriptaid-treated groups normalized to levels in the sham group. Data are presented as mean $\pm$ standard error ($n=6$; $*p < 0.05$, $**p < 0.01$ vs vehicle). (B) Morphological changes in neuronal neurites induced by TBI at days 7 and 35 postinjury with and without Scriptaid treatments. Images were taken from the pericontusional regions in the cortex, as indicated in Fig. 2B. a, d) sham groups; b, e) vehicle treated groups; c, f) Scriptaid treated groups. TBI significantly decreased the number of neurites and average neurites length when compared to sham controls. Scriptaid treatment prevented neurite loss and shortening of dendrites length compared to the vehicle group at both days 7 and 35 postinjury. Scale bar, 100 μ m. (C) Drawings of neuron morphology in sham, vehicle, and Scriptaid-treated groups at 7 and 35 days postinjury. (D) Stereological analysis of MAP-2-positive neuron at 35 days postinjury. Data were normalized to the sham control and are presented as mean $\pm$ standard error ($n=6$; $*p < 0.05$, $**p < 0.01$ vs vehicle). (E) Bar graphs illustrate the mean length of apical dendrites in layer III pyramidal neurons at 35 days postinjury. Degenerative changes were apparent in the length of the apical dendrites in the vehicle group, and were obviously prevented by Scriptaid treatment. Data are presented as mean $\pm$ standard error ($n=6$; $*p < 0.05$, $**p < 0.01$ vs the vehicle group)

we examined its efficacy against long-term injury. In the vehicle group, the motor deficits induced by CCI gradually recovered for the duration of 4-weeks postinjury, as consistently indicated by wire-hanging, foot-fault, and cylinder tests. Nevertheless, Scriptaid treatment facilitated and enhanced this long-term recovery of motor function (Fig. 2A, panels a-d). At 7 to 21 days postinjury, a statistically significant improvement in motor function was consistently shown in all these tests.

Cortex loss induced by CCI remained to be a macroscopically visible cavity, even after 5 weeks in vehicle-treated animals. In comparison, Scriptaid treatment was able to reduce the cavity size (Fig. 2B); consistent with this, the lesion volume was decreased from 30.4 to 14.4 %, as visualized via Nissl staining (Fig. 2C). Unlike motor deficits, changes in cognition are only apparent at least 4-weeks postinjury and can be determined via the Morris water maze test. After CCI, mice in the vehicle group had weaker memory recall and spent significantly more time locating the submerged platform. However, mice with Scriptaid treatment spent more time in the same quadrant as the platform and located the platform faster than the vehicle group (Fig. 2D, panels a, b). The time needed for mice in the vehicle group to locate the platform did not improve much between 29 and 33 days postinjury and remained longer than 80 seconds. However, Scriptaid shortened the time needed to locate the platform from 82 to 58 seconds (Fig. 2D, panel c). On average, there was a 30 % improvement in performance of the spatial memory test with Scriptaid (Fig. 2D, panel d).

Wide Treatment Time Window with Scriptaid Against Injury from TBI

It is clear that Scriptaid can provide protection against short- and long-term injury from CCI when administered 30 minutes after injury. To determine whether Scriptaid protection is still prominent when treatment time is delayed, we introduced it at 12 h postinjury. With this new paradigm, a comparable protection against motor deficits in the wire-hanging and cylinder tests was observed (Fig. 3A, B, C and D). However, there was less prominent protection in the foot-fault test. In addition, the lesion volume was reduced from 23 to 14 % ($p < 0.05$; Fig. 3E). These results indicate a wide temporal window for Scriptaid efficacy in attenuating CCI injury.

Scriptaid Attenuated Neuronal Degeneration and Neurite Degradation

We characterized Scriptaid protection at the cellular level by examining changes in the number of viable neurons with and without treatment. With Scriptaid treatment, there was a 42 % increase in the number of surviving neurons within the CA3 region of the hippocampus compared to the vehicle (Fig. 4A, B). Interestingly, when we plotted the spatial memory performance against viable neurons within the CA3 region for the mice in the sham, vehicle, and treatment groups, there was a direct positive correlation between the 2 variables. This correlation suggests that the protection of spatial memory by Scriptaid may be due to the increase in surviving neurons within the CA3 region (Fig. 4C). These surviving neurons may also contribute to the maintenance of long-term cognitive function. To assess degenerating neurons after TBI, we used Fluoro-Jade C staining on cortex sections collected 7 days post-TBI. As expected, more degenerating neurons in the cortex and hippocampus were visible in the vehicle than the Scriptaid treatment groups (Fig. 4D). Co-staining with DAPI indicated that many of the degenerating neurons also exhibited abnormal nuclear morphologies, such as condensation and fragmentation.

TBI also induced a significant loss of neurites among pyramidal neurons around the impact site within the cortex. This morphological change in vehicle-treated animals was visualized by immunostaining with microtubule-associated protein 2 (MAP2) (Fig. 5B, C). The expected decrease in MAP2 among the neurons in this group was also confirmed by Western blot analysis (Fig. 5A). In contrast, Scriptaid treatment attenuated MAP2 loss by immunoblotting (Fig. 5A), as well as neurite degradation (Fig. 5B, C). Scriptaid-treated animals exhibited higher numbers of surviving cortical neurons (Fig. 5D), and these neurons, on average, had longer apical dendrites at days 35 post-TBI (Fig. 5E).

Scriptaid Prevented the Inhibition of AKT via Modulation of PTEN in Neurons

The AKT pathway is reported to be activated by HDIs to protect against TBI [13]. Thus, the AKT pathway and its regulator PTEN were examined for their involvement in the protection observed with Scriptaid. In the vehicle group, CCI elicited a considerable decrease in p-AKT at day 7 compared to the sham group (Fig. 6A). In addition, a parallel decrease in p-PTEN was observed. Mechanistically, a decrease in p-PTEN implicates an increase in activated PTEN, which in turn inhibits the AKT pathway (Fig. 8). By 5-weeks post-injury, the levels of p-AKT and p-PTEN returned to sham levels. Notably, Scriptaid prevented the transient changes in p-AKT and p-PTEN levels during the first 7 days postinjury.

To substantiate this result, we performed co-immunostaining for NeuN and p-AKT on the cortex from the sham, vehicle, and Scriptaid-treated groups (Fig. 6B). As expected, there were more p-AKT positive cells in the Scriptaid-treated group than in the vehicle group. In addition, all the p-AKT positive cells were co-localized with NeuN, suggesting that p-AKT levels were altered mainly in neurons. Consistent with the Western blot analysis, there is also a higher number of p-PTEN-positive neurons in the Scriptaid-treated group than the vehicle group (Fig. 6C).

The results suggested that Scriptaid protection was mediated by the AKT pathway. To test this hypothesis, we introduced an inhibitor of the AKT pathway, LY294002 (61.5 mg/kg), in conjunction with Scriptaid. As expected, the presence of LY294002 effectively decreased p-AKT levels (Fig. 7A). LY294002 also attenuated Scriptaid-induced protection against motor deficits, as indicated by the wire-hanging and cylinder tests. Specifically, co-treatment with LY294002 and Scriptaid eliminated the significant difference in motor function between the vehicle and Scriptaid-treated mice (Fig. 7B, C). The partial reversal of the neuroprotective effect of Scriptaid by LY294002 was also extended to prevention of tissue loss in the cortex. As shown by Nissl staining, the reduction in lesion volume by Scriptaid was also attenuated by LY294002 (Fig. 7D). Taken together, the results indicate that the AKT pathway is modulated by Scriptaid to exert protection against injury from TBI.

Discussion

HDIs are receiving increased attention for their ability to ameliorate injury in many disease models, partially because they can alter gene expression in a cell type-dependent manner. The devastating condition of TBI is 1 such example. There are no effective therapies to overcome the cognitive and motor deficits of TBI in humans or to reduce the inexorable neuronal degeneration. Thus, the protection against TBI

Fig. 6 Scriptaid prevented the decrease in phosphorylated phosphatase and tensin homolog deleted on chromosome 10 (p-PTEN) and phospho-AKT (p-AKT) levels after injury. (A) p-AKT and p-PTEN levels were decreased by traumatic brain injury at days 1 and 7 postinjury compared to the vehicle group with no significant change in total AKT levels. However, these decreases were prevented with Scriptaid treatment. β -actin was used as the loading control. Quantification of p-PTEN and p-AKT in the vehicle and Scriptaid-treated groups normalized to sham levels at 1, 7, and 35 days postinjury. Data are presented as mean $\pm$ standard error ($n=6$; $*p<0.05$, $**p<0.01$ vs vehicle). (B) The decrease in p-AKT in the ipsilateral cortex in vehicle-treated mice compared to the sham group was prevented by the Scriptaid group, as shown by immunofluorescent microscopy. Changes in p-AKT were localized to neuronal cell bodies in the sham, vehicle, and Scriptaid groups. Sections of cortex stained with p-AKT, neuronal marker (NeuN), and 4'-6-Diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA). Scale bar, 25 μ m, (C) The decrease in p-PTEN in the ipsilateral cortex in the vehicle group compared to the sham group was also prevented by Scriptaid, as shown by immunofluorescent microscopy. Changes in p-PTEN also occurred in neurons and not glia in the sham, vehicle, and Scriptaid groups. Sections of cortex stained with p-PTEN, NeuN, and DAPI. Scale bar, 50 μ m

Fig. 7 Inhibiting AKT pathway with LY294002 significantly attenuated Scriptaid-induced protection against controlled cortical impact. (A) The increase in phospho-AKT (p-AKT) level due to Scriptaid was reversed by LY294002 treatment, as shown by Western blot analysis. β -actin was used as the loading control. Changes in p-AKT levels for vehicle, Scriptaid-treated, and LY294002 + Scriptaid-treated groups were quantified. The statistical increase in p-AKT due to Scriptaid was nullified by LY294002. Data are presented as mean $\pm$ standard error ($n=6$; $**p<0.01$ vs vehicle). (B) Wire-hanging test: Scriptaid treatment attenuated motor deficits leading to an increase in performance scores compared with the vehicle group. This

improvement in motor function was inhibited by LY294002 treatment. Data are presented as mean $\pm$ standard error ($n=6$; $**p<0.01$ vs vehicle). (C) Cylinder test: LY294002 also inhibited the ability of Scriptaid to diminish biased forelimb use. Data are presented as mean $\pm$ standard error ($n=6$; $**p<0.01$ vs vehicle). (D) Visualization of the lesion volume with Nissl staining of brain sections from mice euthanized at day 7 after traumatic brain injury and quantification of lesion volumes from mice receiving vehicle, Scriptaid, or Scriptaid+LY294002. The decrease in lesion volume due to Scriptaid treatment was attenuated by pre-treatment with LY294002. Data are presented as mean $\pm$ standard error ($n=6$; $*p<0.05$ vs vehicle). LY = LY294002

by novel HDIs in experimental models offers new hope. Nevertheless, research on the use of HDIs against TBI is still very limited. One previous study reported protection against closed head injury by a pan-HDI [13]. The current findings complement this study and additionally suggest that even more specific inhibition of targeted deacetylases with the novel HDI Scriptaid is sufficient to achieve both neuronal and behavioral protection. Scriptaid was able to protect against short (7-day postinjury) and long-term (35-day

postinjury, a period that roughly corresponds with 5 to 7 years in humans) motor and cognitive deficits from CCI without incurring significant weight loss and other visible negative side effects. These observations are consistent with the low inherent toxicity of Scriptaid. Notably, Scriptaid was protective even when introduced 12 h postinjury. This finding has direct translational value because victims of TBI are often not treated until several hours after the cortical impact. In summary, the protective characteristics of Scriptaid observed in

the present study make it a suitable candidate as a potentially therapeutic agent for TBI.

In rodent models of TBI, the pyramidal neurons of the hippocampal CA3 region appear to be 1 of the most vulnerable areas [42–44]. Significantly, the extent of neuronal damage in the hippocampus of this model of brain trauma correlates well with the severity of the post-traumatic cognitive deficits and with persistent memory dysfunction [43, 44]. In our study, neuronal loss and degeneration within the CA3 region of the hippocampus were both attenuated by Scriptaid. This preservation of viable neurons immediately after injury may contribute to the improved long-term spatial and cognitive performance as determined by the Morris water maze test. This notion is consistent with the strong positive correlation between spatial memory and number of surviving CA3 neurons. One might speculate that retention of the synaptic networks already established in the brain may ameliorate any delayed memory deficits or psychosis in TBI patients [45–47]. This is significant because much of the injury suffered by patients in the aftermath of TBI develops along a delayed time frame and this secondary injury significantly impedes their long-term recovery.

TBI can induce a number of pathological events, ranging from neuronal degeneration and tissue loss to impairments in neuronal plasticity and neurochemical dysregulation [5, 48, 49]. Our study documented post-traumatic neurodegeneration after TBI in mice using MAP-2 immunolabeling combined with Fluoro-Jade staining. MAP2 is critical for microtubule stability and neural plasticity and appears to be among the most vulnerable of the cytoskeletal proteins under conditions of neuronal injury [50]. Fluoro-Jade staining is exquisitely sensitive to neuronal cell body degeneration [51, 52]. Our findings indicate that experimental TBI can induce long-term cognitive, motor dysfunction derived from neurodegeneration and/or neuritic/axonal degradation, and that these effects are all blunted by Scriptaid. Indeed, accumulating evidence demonstrates that transcriptional dysfunction resulting from imbalances in HAT and HDAC activities may also contribute to neuronal cell death during disease and injury [53–57]. Scriptaid is thought to shift the HAT-HDAC balance in favor of HAT activity, thus enhancing histone acetylation and promoting gene expression. In particular, several other studies showed that neuronal demise was associated with a global decrease in HAT activity

Fig. 8 Proposed mechanism of neuroprotection of histone deacetylase inhibitors (HDI) Scriptaid against traumatic brain injury (TBI). (A) Mechanistic relationships between phosphorylated phosphatase and tensin homolog deleted on chromosome 10 (p-PTEN), PTEN, and phospho-AKT (p-AKT), as well as the impact of Scriptaid on these pathways. A decrease in p-PTEN results in the concomitant increase in active PTEN, which converts PIP3 to PIP2, preventing the phosphorylation of PDK1 and the activation of AKT. Scriptaid treatment increases p-PTEN, leading to a concomitant decrease in active PTEN. This loss of PTEN function by phosphorylation results in the downstream activation of the AKT pathway. As a consequence of enhanced

phospho-AKT (p-AKT) signaling, cell survival is promoted and neurite integrity is preserved. (B) The loss of histone acetyltransferase (HAT) function or increased HDAC activity leads to a decrease in histone/transcription factor acetylation and transcriptional repression. This may partly underlie neurological damage associated with TBI. HDAC inhibition by Scriptaid may be protective by shifting the HAT-HDAC balance in favor of HAT activity, so that the histones remain acetylated and gene expression is activated. We also propose that the accumulation of acetylated histones in nucleosomes leads to expression of specific genes, which, in turn, are responsible for the protection elicited by Scriptaid

and that this can be reversed by HDAC inhibitors [12, 13, 31, 32]. Similar success in other neurodegenerative disease models with HDAC inhibitors, therefore, point to possible translation of these molecules into the clinic [58–60].

Our study also revealed that the pro-survival AKT pathway was modulated by Scriptaid, consistent with previous reports [13]. Furthermore, our results also identified modulation of PTEN activity by Scriptaid. PTEN closely regulates the AKT pathway through changes in its phosphorylation status. This well-known relationship between p-PTEN, PTEN, and AKT [61, 62] is summarized in Fig. 8. We hypothesize the following scenario. PTEN is a potent indirect inhibitor of AKT via dephosphorylation of PIP3 to PIP2, which in turn prevents the tandem activation of PDK1 and AKT. Phosphorylation of PTEN stabilizes the moiety, but virtually nullifies its inherent phosphatase activity [63, 64]. Therefore, the decrease in p-PTEN by CCI signifies an increase in active PTEN, which then indirectly inhibits the AKT signaling pathway. However, in the presence of Scriptaid, p-PTEN levels were increased, nullifying PTEN activity and perhaps thereby removing the indirect inhibition of AKT (Fig. 5). It is unlikely that Scriptaid has a direct effect on PTEN phosphorylation based on our *in vitro* PTEN protein assays (data not shown). We also performed quantitative PCR to determine the levels of PTEN mRNA in TBI brains with and without Scriptaid treatment ($n=3/\text{group}$). The results showed that Scriptaid treatment did not significantly alter the expression of PTEN mRNA (not shown); moreover, Scriptaid did not alter the protein expression of PTEN as determined by Western blot. Alternatively, HDI may affect PTEN phosphorylation through regulation of genes that participate in protein phosphorylation and dephosphorylation. Thus far, 3 proteins have been found to have a direct role in PTEN phosphorylation, including glycogen synthase kinase 3 β , casein kinase 2, and Rho-associated protein kinase [65]. It will be interesting to determine whether HDI by Scriptaid affects the transcriptional regulation of these 3 kinases after TBI in future studies.

In our study, we also examined the levels of total PTEN during the first 7 days postinjury without Scriptaid, and we observed no difference (data not shown), despite a decrease in p-PTEN (Fig. 5A). The expected increase in PTEN in cortical tissue by Western blotting may be masked by an abundant glial pool of PTEN, because there is threefold more glia than neurons in the cortex [66]. Furthermore, the glia to neuron ratio is expected to be even higher in the vehicle group because of neurodegeneration and astrogliosis. In contrast to PTEN, the impact of Scriptaid and CCI on p-PTEN may not be masked by the large number of glia in the Western blotting lysates if PTEN is only phosphorylated in neurons. This differential expression pattern may then explain the lack of an impact on total PTEN, but a significant change in p-PTEN.

Although our results indicate that the AKT pathway underlies the protection elicited by Scriptaid, LY294002 did not completely eliminate protection. Other signaling pathways,

such as extracellular signal-regulated protein kinases 1 and 2 and c-Jun amino-terminal kinase may also help mediate the effects of Scriptaid. Future studies may wish to apply a combination of inhibitors for multiple kinases to see if the protective effect of Scriptaid can be completely abolished.

In conclusion, the novel HDI Scriptaid effectively elicited short- and long-term protection against motor and cognitive deficits, both hallmarks of TBI. Scriptaid was highly protective, even when administered 12 h postinjury, a feature which will be essential for effective clinical translation. In addition, there was significant protection against neuronal degeneration that correlated positively with behavioral protection. Notably, Scriptaid prevented the TBI-induced loss in p-AKT and p-PTEN levels, whereas inhibition of AKT activation attenuated its protective effect. Taken together, these findings make Scriptaid a promising new candidate for long-term clinical protection against TBI.

Acknowledgments This research is supported in part by the Special Research Funds from the Chinese Ministry of Science and Technology to State Key Laboratories (to G.H.W., Y.G., and J.C.); the National Institutes of Health/National Institute of Neurological Disorders and Stroke (grants NS36736, NS43802 and NS45048 to J.C.); the Veterans Administration Merit Review (grant to J.C.); and the Chinese Natural Science Foundation (grants No. 30870794, No. 81020108021, No. 81171149, No. 81150110494 to Y.G., and No. 81000497 to G.W.) The authors declare no conflict of interest. Full conflict of interest disclosures is available in the electronic supplementary material for this article.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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