Valproic Acid and Neural Apoptosis, Inflammation, and Degeneration 30 Days after Traumatic Brain Injury, Hemorrhagic Shock, and Polytrauma in a Swine Model

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BACKGROUND: A single-dose (150 mg/kg) of valproic acid (VPA) has been shown to decrease brain lesion size and improve neurologic recovery in preclinical models of traumatic brain injury (TBI). However, the longer-term (30 days) impact of single-dose VPA treatment after TBI has not been well evaluated.

STUDY DESIGN: Yorkshire swine were subjected to TBI (cortical impact), hemorrhagic shock, and polytrauma. Animals remained in hypovolemic shock for 2 hours before resuscitation with normal saline (NS; volume = 3× hemorrhaged volume) or NS + VPA (150 mg/kg) (n = 5/cohort). Brain samples were harvested 30 days after injuries. The cerebral cortex adjacent to the site of cortical impact was evaluated using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, immunohistochemistry, and Western blot analysis. Neural apoptosis, inflammation, degeneration, plasticity, and signaling pathways were evaluated.

RESULTS: For apoptosis, VPA treatment significantly decreased ($p < 0.05$) the number of TUNEL (+) cells and expression of cleaved-caspase 3. For inflammation and degeneration, expression of ionized calcium binding adaptor molecule-1, glial fibrillary acid protein, amyloid-β, and phosphorylated-Tau protein were significantly attenuated ($p < 0.05$) in the VPA-treated animals compared with the NS group. For, plasticity, VPA treatment also increased expression of brain-derived neurotrophic factor significantly ($p < 0.05$) compared with the NS group. For signaling pathways, nuclear factor-κB was decreased significantly ($p < 0.05$) and cytosolic IκBα expression was increased significantly ($p < 0.05$) in the VPA-treated animals compared with the NS group.

CONCLUSIONS: Administration of a single dose of VPA (150 mg/kg) can decrease neural apoptosis, inflammation, and degenerative changes, and promote neural plasticity at 30 days after TBI. In addition, VPA acts, in part, via regulation of nuclear factor-κB and IκBα pathways. (J Am Coll Surg 2019;228: 265–275. © 2019 by the American College of Surgeons. Published by Elsevier Inc. All rights reserved.)

Traumatic brain injury (TBI) remains a leading cause of preventable death in injured patients.[1,2] In the US, TBI affects nearly 2 million people each year and contributes to permanent disability and death.[3] Although many healthcare providers consider TBI an "event," TBI is, in fact, a chronic disease process.[4] According to WHO, moderate

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Abbreviations and Acronyms

Aβ	= amyloid-β
BDNF	= brain-derived neurotrophic factor
c-cas-3	= cleaved-caspase 3
GFAP	= glial fibrillary acidic protein
Iba1	= ionized calcium binding activated molecule-1
IHC	= immunohistochemistry
NF-κB	= nuclear factor-κB
NS	= normal saline
p-Tau	= phosphorylated-Tau protein
TBI	= traumatic brain injury
TUNEL	= terminal deoxynucleotidyl transferase dUTP nick end labeling
VPA	= valproic acid

to severe TBI is considered to be a permanent process, resulting in irreversible alterations to the brain; this often requires long-term observation or care.[5] In addition, the long-term impact of TBI has been associated with an increased incidence of neurodegenerative diseases.[1,6-10] Despite improvements in medical care for TBI and its long-term complications, there is a clear need for effective pharmacologic agents that can decrease morbidity and improve long-term neurologic outcomes after TBI.

In recent years, valproic acid (VPA) has emerged as a promising pharmacologic agent for the treatment of TBI. Valproic acid, a histone deacetylase inhibitor, acts through epigenetic modulation to induce post-translational modification of histone and non-histone proteins. In large animal models of TBI and hemorrhagic shock, with and without polytrauma, administration of a single-dose of VPA (150 mg/kg) decreases brain lesion size, improves neurologic outcomes, and promotes faster neurocognitive recovery compared with controls.[11,12] In the short-term (9 hours after injury), these neuroprotective effects have been attributed to increased neurogenesis and decreased neuroinflammation in the injured brain.[13] At 24 hours after TBI, peripheral blood mononuclear cells, which serve as a "window" into the brain alterations after TBI, exhibit an increased expression of genes associated with cell survival and decreased expression of genes associated with apoptosis and inflammation.[14] Despite increasing knowledge of VPA's therapeutic effects in short-term settings, the impact of this treatment at longer follow-up remains unknown.

The aim of this study was to demonstrate the impact of single-dose VPA (150 mg/kg) treatment in a large animal model of TBI during a longer (30-day) period of observation. We hypothesized that VPA treatment would attenuate neural apoptosis, inflammation, and degeneration, and promote neural plasticity in a long-term model of TBI, hemorrhagic shock, and polytrauma in swine.

METHODS

This protocol was reviewed by the Institutional Animal Care and Use Committee at the University of Michigan. Experiments were conducted in compliance with all the regulations regarding research and animal welfare.

Injury model: traumatic brain injury, hemorrhage, and polytrauma

This 30-day survival model of injuries, which was designed to simulate military or severe civilian trauma in an austere setting, has been described in-depth previously.[12] Briefly, female Yorkshire swine (37 to 50 kg; Michigan State University) were used for the study. Animals were anesthetized, mechanically ventilated, and instrumented to monitor hemodynamic and intracranial parameters. A 21-mm burr hole was made anterior and lateral to the bregma on the right side of the skull. A computer-controlled cortical impact device was used to create a standardized TBI with a 20-mm cylindrical impactor (4 m/s velocity, 100-ms dwell time, and 8-mm depth of penetration). Animals were subjected to 40% total blood volume hemorrhage, and concurrent polytrauma (rectus abdominis crush, rib fracture, liver injury, and spleen injury). A sham group underwent instrumentation and monitoring, but no injuries (n = 5).

Shock, resuscitation, and treatment

After the completion of hemorrhage, animals were kept in shock (mean arterial pressure of 30 to 35 mmHg) for 2 hours. This simulated a "worst-case scenario" with prolonged time to treatment in an austere environment. Animals were then randomized to 1 of 2 groups: normal saline (NS) resuscitation or NS resuscitation + VPA treatment (150 mg/kg for 3 hours) (n = 5/cohort). Animals randomized to VPA treatment received a single dose of IV VPA (150 mg/kg) given over 3 hours, starting 1 hour after the initiation of shock (to simulate a realistic medic response time in the battlefield). At the end of the shock period (2 hours after injuries), all of the animals received resuscitation with NS (3× the volume of blood lost) for 1 hour.

Observation, recovery, neurologic evaluation, and imaging

Animals were observed for 2 hours after resuscitation and then given autologous packed RBC transfusion to simulate transfer to a higher echelon of care. After transfusion was complete, invasive monitoring was discontinued, animals were weaned from the mechanical ventilation and fully recovered. They were then carefully monitored for a total of 30 days. They were assessed daily using a validated tool for assessing both the severity of neurologic injury

and the degree of recovery.[11,15] Once animals were able to participate, neurocognitive testing (modified operant conditioning model) was initiated.[16] Animals also underwent T2-weighted MRI of the brains on post-injury day 3.

Euthanasia

After 30 days of observation, animals were euthanized and brains were harvested and sliced into 5-mm coronal sections. Brain sections underwent formalin fixation for 48 to 72 hours, and were then transferred to 70% ethanol. Cortical sections adjacent to the most injured site were used for all subsequent analyses.

Primary end points

To evaluate the impact of a single dose of VPA administration in a 30-day model of TBI, neural apoptosis, inflammation, degeneration, plasticity, and signaling pathways were assessed. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and expression of cleaved-caspase 3 (c-cas 3) were used to detect neural apoptosis. Immunohistochemistry (IHC) and Western blot were used to detect ionized calcium binding activated molecule-1 (Iba1) and glial fibrillary acidic protein (GFAP) to evaluate neuroinflammation. These methods were also used to detect amyloid-β (Aβ) and phosphorylated-Tau protein (p-Tau) to evaluate neural degeneration. Western blot was used to detect brain-derived neurotrophic factor (BDNF), a marker of neural plasticity and to evaluate nuclear factor-κB (NF-κB) and IκBα signaling pathways.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

A TACS2 TdT-Fluor In Situ Apoptosis Detection Kit (4812-30-K; Trevigen) was used according to the manufacturer's guidelines to detect neural apoptosis. Briefly, sections were incubated with Proteinase K for 30 minutes. After washing 3 times, slides were incubated with the labeled reactant for 1 hour. Stop buffer was added, followed by staining with Trep-Fluor solution and 4′,6-diamidino-2-phenylindole. Imaging was obtained using a fluorescence microscope (BX53; Olympus).

Immunochemistry analysis

Immunohistochemistry was used to assess the expression levels of Iba1, GFAP, Aβ, and p-Tau. Cortical sections (40 μm) adjacent to the injury were first incubated in 0.3% hydrogen peroxide (H_2O_2) solution, followed by 30 minutes blocking in Tris-Hcl buffered saline supplemented with 0.05% Triton-100 and 4% normal goat serum. Sections were incubated with antibodies in Tris-Hcl buffered saline supplemented with 0.05% Triton-100 and 4% normal goat serum overnight at 4°C. After washing 3 times, sections were then incubated with biotinylated secondary antibody (1:1,000; Vector Laboratories) in Tris-Hcl buffered saline supplemented with 0.05% Triton-100 and 4% normal goat serum for 1 hour. Sections were then incubated for 1 hour in avidin—biotin substrate (ABC kit; Vector Laboratories). All sections then incubated in 3,3-diaminobenzidine solution (Vector Laboratories) until desired stain intensity develops. Finally, sections were dehydrated in ethanol and a mounting medium was used for cover-slipping. Both positive and negative controls to each antibody were performed to confirm specificity.

Western blot analyses

Western blotting was used to confirm findings from TUNEL assay and IHC. Tissues were lysed in radioimmunoprecipitation assay buffer and mitochondria/cytosol fractions were isolated using Abcam's Mitochondria/Cytosol Fractionation Kit (ab65320) according to manufacturer's instructions. Primary antibodies used and their respective dilutions were as follows: rabbit anti-c-cas 3 (1:1,000) from Cell Signaling Technology; rabbit anti-Iba1(1:1,000), rabbit anti-GFAP (1:1,000); rabbit anti-Aβ (1:1,000); rabbit anti-Tau (1:1,000), rabbit anti-p-Tau (1:1,000), rabbit anti-BDNF (1:1,000), rabbit anti-NF-κB (1:1,000), rabbit anti-IκBα (1:1,000), rabbit anti-histone 3 (H3, 1:1,000), and mouse anti-β-actin (1:3,000) from Abcam.

Statistical analyses

Primary end points involved assessing neural apoptosis, inflammation, degeneration, plasticity, and signaling pathways (TUNEL, c-cas 3, Iba1, GFAP, Aβ, p-Tau, BDNF, NF-κB, and IκBα). Pilot experiments were used to conduct an a priori power analysis. Using pilot data, effect size (d) was determined for each variable for Western blot data and sample sizes were planned with 80% power and 95% confidence. These included c-cas 3 ($d = 3.5$; $n = 3$), Iba1 ($d = 4.5$; $n = 2$), GFAP ($d = 7$; $n = 2$), Aβ ($d = 5$; $n = 2$), p-Tau ($d = 4$; $n = 2$), BDNF ($d = 5$; $n = 2$), NF-κB ($d = 2.7$; $n = 3$), and IκBα ($d = 2.8$; $n = 3$).

GraphPad Prism software, version 6.00, was used for all analyses. One-way analysis of variance with Bonferroni post-hoc testing was used to detect differences among 3 or more groups. Data are presented as mean ± SEM. For all tests, $p < 0.05$ was considered statistically significant.

RESULTS

To achieve the primary end points, $n = 5$ was required for each cohort.

Valproic acid administration decreases brain lesion size and improves neurologic outcomes

As reported previously,[12] VPA-treated animals had significantly lower ($p < 0.05$) neurologic severity scores during the first 5 days of recovery. Time until complete neurologic recovery was also significantly shorter for VPA-treated animals (days to recovery: NS = 9.4 ± 3.4; NS + VPA = 5.2 ± 1.8; $p = 0.04$). The VPA-treated animals were able to begin neurocognitive testing earlier (days to initiation: NS = 6.2 ± 1.6; NS + VPA = 3.6 ± 1.5; $p = 0.002$) and required significantly fewer sessions for task mastery (days to mastery: NS = 7.0 ± 1.0; NS + VPA = 4.8 ± 0.5; $p = 0.03$). Brain lesion size on post-injury day 3 was also significantly decreased for VPA-treated animals compared with the NS group (mean lesion size: NS = 4,956 ± 1,511 mm^3; NS + VPA = 828 ± 279 mm^3; $p = 0.04$).

Valproic acid treatment attenuates neural apoptosis

The TUNEL assay and expression of c-cas-3 were used to evaluate neural apoptosis at the end of the 30-day observation period. The TUNEL-positive cells were not expressed in the sham group (Fig. 1A). In the NS group, there was an increase in TUNEL-positive cells compared with sham. However, VPA-treated animals had a decreased number of TUNEL-positive cells compared with the NS group.

Western blot analysis was used for confirmation of these findings. In the NS group, there was a significant increase ($p < 0.05$) in expression of c-cas-3 compared with sham (Fig. 1B), which was not seen in the VPA-treated animals (relative density, sham: 0.12 ± 0.01; NS: 1.14 ± 0.2; NS + VPA: 0.31 ± 0.05).

Valproic acid treatment attenuates neuroinflammation

Immunohistochemistry and Western blot were used to evaluate Iba1 and GFAP, which are markers of activated microglia and astrocytes, respectively. Immunohistochemistry revealed that Iba1 and GFAP expression were increased in the NS group compared with sham (Fig. 2A). However, VPA-treated animals had decreased expression of Iba1 and GFAP relative to the NS group.

Western blot confirmed the IHC findings. The Iba1 and GFAP expressions were significantly increased ($p < 0.001$) in the NS compared with the sham group (Figs. 2B, 2C) (relative density, Iba1; sham: 0.22 ± 0.03; NS: 1.16 ± 0.1; NS + VPA: 0.4 ± 0.05) (relative density, GFAP; sham: 0.41 ± 0.04; NS: 1.64 ± 0.1; NS + VPA: 0.63 ± 0.04). However, VPA administration significantly attenuated ($p < 0.001$ and $p < 0.01$, respectively) Iba1 and GFAP expression similar to the sham group.

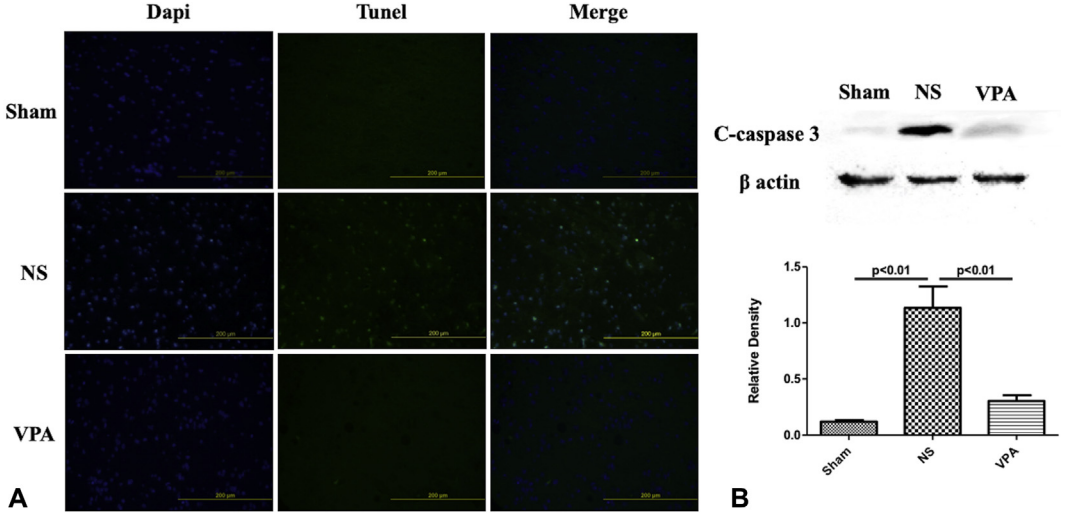

Figure 1. Valproic acid (VPA) attenuates neural apoptosis at 30 days after traumatic brain injury (TBI). Terminal deoxynucleotidyl transferase dUTP nick end labeling (Tunel) assay and expression of cleaved caspase 3 (c-caspase 3) using Western blot were used. (A) In the normal saline (NS) group, there was an increase in Tunel-positive cells compared with sham. However, VPA treatment attenuated the number of Tunel-positive cells. (B) Using Western blot, expression of cleaved-caspase 3 was increased significantly ($p < 0.01$) in the NS group compared with sham. However, treatment with VPA significantly attenuated ($p < 0.01$) these alterations to a level similar to sham. A p value < 0.05 was considered statistically significant. Images shown at 200 μm. β-Actin was used as an internal control. Dapi, 4′,6-diamidino-2-phenylindole.

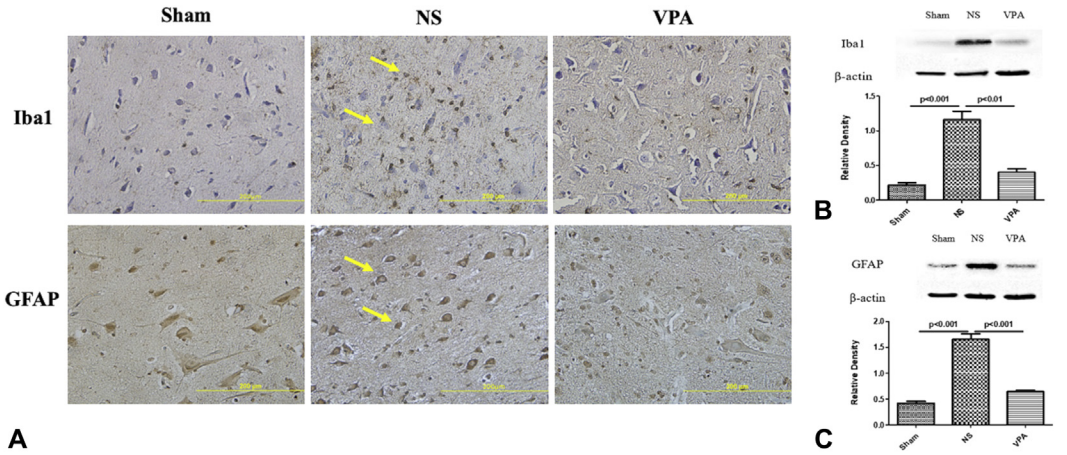

Figure 2. Valproic acid (VPA) attenuates neuroinflammation at 30 days after traumatic brain injury (TBI). Immunohistochemistry and Western blot analyses were used to assess ionized calcium binding adaptor molecule-1 (Iba1) and glial fibrillary acidic protein (GFAP), markers of microglia and astrocytes. (A) Using immunohistochemistry, there was an increase in Iba1 and GFAP-positive cells in the normal saline (NS) group compared with sham. However, VPA treatment decreased the number of Iba1- and GFAP-positive cells. (B) Western blot was used for confirmation. In the NS group, there a significant increase ($p < 0.001$) in Iba1 and GFAP expression compared with sham. However, VPA administration significantly attenuated ($p < 0.01$ and $p < 0.001$, respectively) expression of Iba1 and GFAP. Images shown at 200 μm. Arrows demonstrate Iba1 and GFAP. β-Actin was used as an internal control.

Valproic acid treatment attenuates neurodegeneration

Immunohistochemistry and Western blot were used to detect Aβ and p-Tau, well-known markers of chronic neurodegeneration. Immunohistochemistry revealed that there was a significant increase in Aβ and p-Tau in the NS group compared with sham (Fig. 3A). However, VPA treatment decreased expression of Aβ and p-Tau compared with the NS group.

Western blot analysis confirmed the IHC findings. In the NS group, Aβ and p-Tau were increased significantly ($p < 0.001$ and $p < 0.01$, respectively) compared with

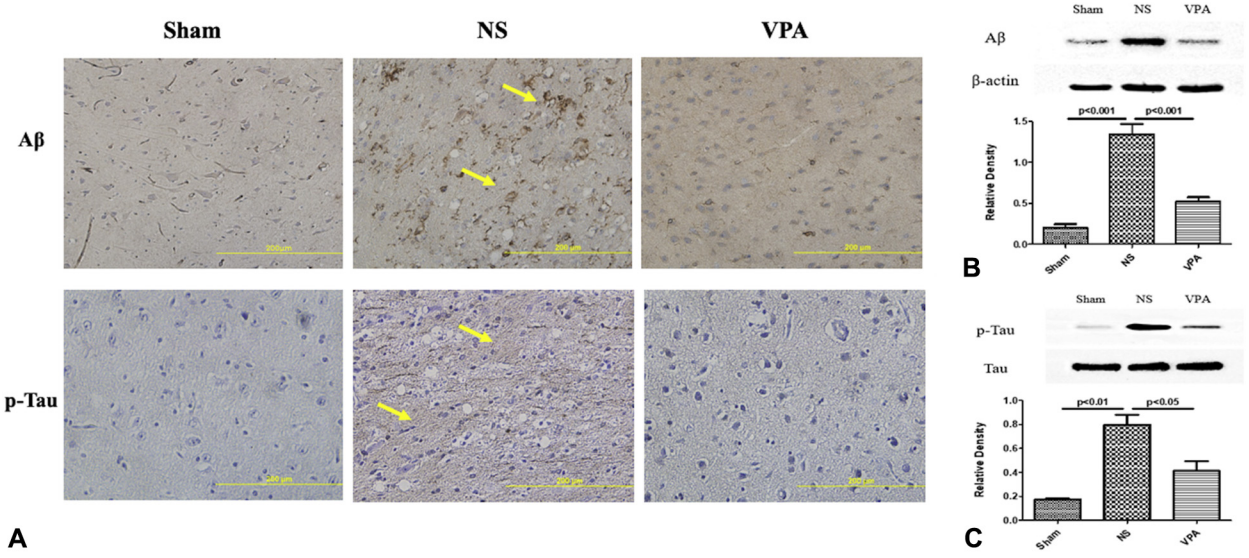

Figure 3. Valproic acid (VPA) attenuates neural degeneration at 30 days after traumatic brain injury (TBI). Immunohistochemistry and Western blot analyses were used to assess amyloid-β (Aβ) and phosphorylated Tau (p-Tau), markers of neurodegenerative diseases. (A) Using immunohistochemistry, there was an increase in Aβ and p-Tau in the normal saline (NS) group compared with sham. However, VPA treatment decreased these alterations. (B, C) Western blot was used for confirmation. In the NS group, there was significant increase ($p < 0.001$ and $p < 0.01$) in Aβ and p-Tau expression compared with sham. However, VPA administration significantly attenuated ($p < 0.001$ and $p < 0.05$, respectively) expression of Aβ and p-Tau. Images shown at 200 μm. Arrows demonstrate Aβ and p-Tau. β-Actin was used as an internal control.

sham (Figs. 3B, 3C) (relative density, Aβ; sham: 0.20 ± 0.03; NS: 1.34 ± 0.12; NS + VPA: 0.52 ± 0.05) (relative density, p-Tau; sham: 0.17 ± 0.01; NS: 0.79 ± 0.08; NS + VPA: 0.41 ± 0.07). However, this increased expression was significantly attenuated ($p < 0.001$ and $p < 0.05$) in the VPA treatment group.

Valproic acid promotes neuroplasticity

Using Western blot analysis, BDNF expression was evaluated in this long-term survival model. Brain-derived neurotrophic factor is a key player in promoting neural plasticity and survival in the CNS. In the NS group, BDNF expression was noted to be decreased significantly ($p < 0.01$) compared with sham (Fig. 4) (relative density, BDNF; sham: 1.0 ± 0.11; NS: 0.2 ± 0.11; NS + VPA: 0.72 ± 0.05). On the other hand, VPA-treated animals showed BDNF expression that was significantly higher ($p < 0.05$) compared with the NS group, and no different than the sham group.

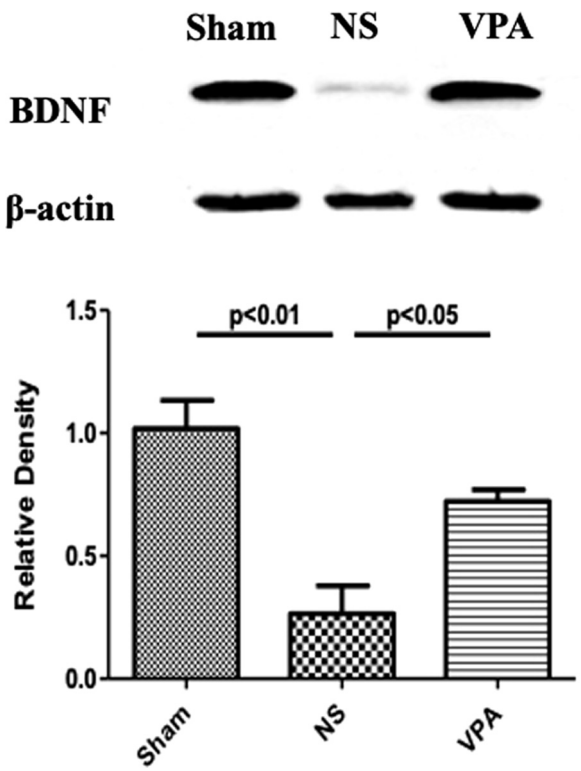

Figure 4. Valproic acid (VPA) promotes neuroplasticity 30 days after traumatic brain injury (TBI). Western blot analysis was used to assess brain-derived neurotrophic factor (BDNF) expression. In the normal saline (NS) group, there was a significant decrease ($p < 0.01$) in BDNF expression compared with sham. However, VPA administration significantly attenuated ($p < 0.05$) the decrease in BDNF. β-Actin was used as an internal control.

Valproic acid regulates nuclear factor-κB and IκBα pathways

Translocation of NF-κB and degradation of cytosolic IκBα were assessed using Western blot analysis. Nuclear factor-κB is a key transcription factor that regulates the neuroinflammation and apoptosis, and IκBα is an inhibitor of NF-κB. In the NS group, there was a significant increase ($p < 0.001$) in NF-κB and decrease ($p < 0.05$) in cytosolic IκBα compared with the sham group (Fig. 5) (relative density, NF-κB; sham: 0.09 ± 0.01; NS: 0.78 ± 0.04; NS + VPA: 0.25 ± 0.08) (relative density, IκBα; sham: 1.25 ± 0.09; NS: 0.48 ± 0.08; NS + VPA: 1.11 ± 0.16). However, VPA treatment significantly decreased ($p < 0.001$) NF-κB expression and increased ($p < 0.05$) cytosolic IκBα expression to levels similar to sham.

DISCUSSION

In this study, we found that administration of a single dose of VPA (15 mg/kg) after TBI confers beneficial effects for up to 30 days. Valproic acid achieves these benefits by promoting a decrease in brain apoptosis, inflammation, and degeneration, and by increasing neural plasticity. Valproic acid acts, in part, by the regulation of NF-κB and IκBα pathways. As such, single-dose VPA administration after TBI confers both neuroprotection and decreases the risk of sequelae and complications of TBI, including neurodegeneration.

Traumatic brain injury remains a leading cause of morbidity and death in trauma.[2,3] Although TBI has direct initial consequences, it can also lead to alterations in the brain that can persistent for months and even years after injury.[6] These long-term consequences, which include apoptosis, inflammation, degeneration, and others, place these patients at increased risk for development of neurologic disorders (eg seizures, sleep disorders, neuroendocrine dysregulation), psychiatric problems, and even neurodegenerative disorders, including Alzheimer's disease and chronic traumatic encephalopathy.[6] These sub-acute and chronic alterations can ultimately increase long-term mortality, reduce life expectations, and drastically affect quality of life.[6] Currently, there are limited pharmacologic strategies that can attenuate the acute and long-term complications after TBI.

Apoptosis, or programmed cell death, is a highly relevant process after TBI. After injury, neural apoptosis can be mediated by the activation of intrinsic and extrinsic apoptotic pathways.[17,18] In contrast to neural necrosis, apoptosis appears to be delayed and chronic, contributing to the progressive nature of TBI.[19-21] Several human studies have demonstrated that pro-apoptotic proteins are upregulated in plasma and cerebrospinal fluid after TBI,[22,23] and

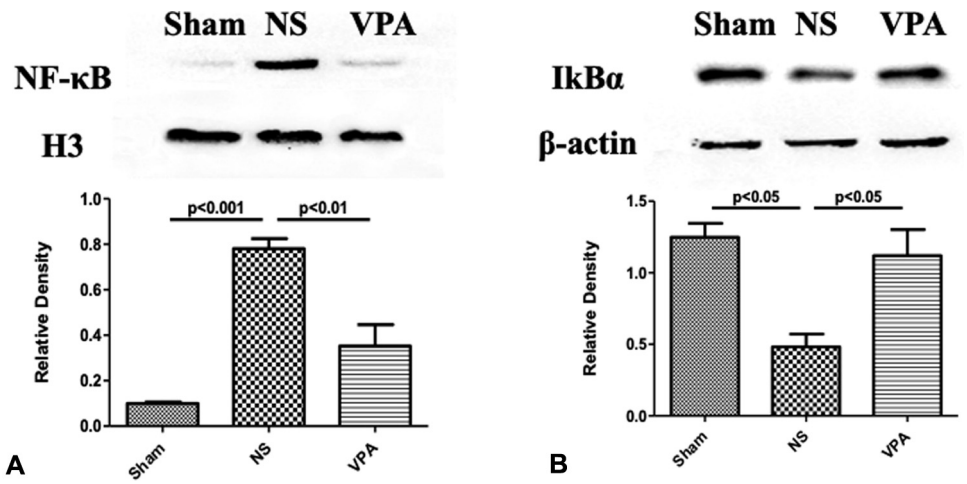

Figure 5. Valproic acid (VPA) promotes activation of nuclear factor-κB (NF-κB) and degradation of cytosolic IκB. Western blot analysis was used. (A) In the normal saline (NS) group, there was a significant increase ($p < 0.001$) in NF-κB compared with sham. However, treatment with VPA significantly decreased ($p < 0.001$) NF-κB expression similar to sham. (B) In contrast, there was a significant decrease ($p < 0.05$) in cytosolic IκBα in the NS group compared with sham. However, VPA treatment significantly increased ($p < 0.05$) cytosolic IκBα expression similar to sham. β-Actin was used as an internal control.

apoptosis in the brain can continue for weeks to months after injury.[20] As such, delayed apoptotic death can determine some of the consequences of TBI, and is therefore an attractive therapeutic target. In this study, we demonstrate that VPA treatment significantly decreased the neural apoptosis at the 30-day time point after TBI.

Neuroinflammatory processes are also an important contributor to secondary injury after TBI.[24] In both experimental models and clinical tissues, TBI promotes activation of microglia and recruitment of circulatory inflammatory cells, including macrophages, to the area of brain injury.[25-27] In the current study, we assessed the Iba-1 expression, a well-known marker of microglial activation that plays a key role in the cytoskeletal reorganization and configuration of the plasmalemma during phagocytosis.[28] We discovered that VPA administration decreased the expression of Iba1 30 days after TBI compared with the controls. As these inflammatory cells are involved in the expression of pro-inflammatory chemokines and cytokines (interleukin-1B, tumor necrosis factor-α, and interleukin-6),[29,30] which can be neurotoxic, VPA can help to ultimately decrease neuroinflammation in the injured brain. In addition, astrocytes, which play a key role in the blood-brain barrier, neural signaling, and scar formation, are often activated after trauma.[31] In recent years, GFAP, a marker of astrocyte activation, has been shown to be a marker of severe TBI.[32] We also noted that VPA treatment significantly attenuated the GFAP expression after TBI, reflecting VPA's ability to decrease the inappropriate astrocyte activation leading to neuroinflammation. These findings correlate with our earlier work demonstrating that VPA administration attenuates plasma GFAP levels after TBI.[33]

In recent years, TBI has been identified as an important risk factor for the development of progressive neurodegenerative diseases, including Alzheimer's disease, chronic traumatic encephalopathy, and Parkinson's disease.[34] There is strong clinical evidence demonstrating that TBI can increase the incidence of neurodegeneration.[35,36] Although this might be multifactorial (eg environment and genetics), it appears that prolonged inflammation and focal protein aggregation in the brain might be responsible. In experimental and clinical models, expression of Aβ and p-Tau proteins, which accumulate in both the gray and white matter, can play a key role in mediating the neurodegeneration.[37-40] In this study, we were able to demonstrate that single-dose VPA treatment decreased expression of both Aβ and p-Tau, reflecting a decrease in Alzheimer's disease-like pathology. We are unsure to what extent these alterations would affect disease onset or clinical manifestations; however, we plan to conduct additional studies for a more comprehensive analysis. There is an increased risk of developing other neurodegenerative diseases (Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease) after TBI; however, this was not assessed in the current study.

Achieving neuroplasticity after TBI has also been a significant area of interest. Neural plasticity is linked to cellular responsiveness reflecting the neuronal capability to adapt and survive after TBI. In recent years, BDNF

has emerged as a critical mediator of neuronal plasticity.[41,42] Numerous studies indicate the beneficial effects of BDNF in promoting neurogenesis.[43-45] After TBI, the significant increase in pro-inflammatory cytokines causes a significant reduction in BDNF gene expression.[46-48] This decrease has even been demonstrated after systemic inflammation (interleukin-1B or lipopolysaccharide administration).[49-51] In this study, we found that concurrent hemorrhagic shock and TBI, promoting systemic and local inflammation, decreased the BDNF expression in the NS group; however, treatment with VPA significantly increased the BDNF expression to a level similar to sham animals. As decreased BDNF expression might ultimately compromise hippocampal learning and spatial memory and increase apoptosis contributing to neuropsychiatric diseases,[52-54] VPA administration might be able to improve these clinical alterations.

We also sought to assess the pathways that might be involved in the attenuation of chronic neural apoptosis, inflammation, and degeneration. In the CNS, NF-κB transcription factors are key players in promoting neurotoxicity after TBI.[55] Although NF-κB is present in latent glia, several studies have demonstrated that glial responses to trauma are mediated through nuclear translocation of NF-κB. After microglial activation of NF-κB, reactive oxygen species and proinflammatory cytokines are released, which contributes to the secondary neurotoxicity.[56,57] In addition, NF-κB can also regulate inflammatory processes that worsen the inflammation-induced neurodegeneration. In this study, VPA administration attenuated the nuclear translocation and activation of NF-κB after TBI. Numerous other pathways (toll-like receptor signaling, cyclooxygenase-2, complement signaling, and cytokines) also mediate neural inflammation and degeneration, which can also be affected after VPA administration[58]; however, we only chose to study NF-κB activation and its inhibitor, *IκBα*.

We would also like to highlight certain aspects of the model used in this study. This model was designed to represent a moderate to severe TBI sustained in a rather austere military or civilian settings. The most common cause of TBI in the current military conflicts is improvised explosive devices. As isolated TBI rarely occurs in such settings, our injury model involved hemorrhagic shock, TBI, and polytrauma. We also created a worst-case scenario by having a delay in medic/first responder time (2 hours after injuries before initial resuscitation), and by introducing additional delay in transfer to higher echelons of care (after initial resuscitation, 2-hour delay before blood transfusion). The findings of this study demonstrate that VPA treatment confers protection after moderate to severe TBI accompanied by the most severe injuries, where concurrent hemorrhage and hypotension can exacerbate TBI progression. Given its marked neuroprotective abilities, we also believe that VPA treatment can demonstrate benefit in isolated TBI models and mild TBI as well, but this was not specifically tested in this study. We also suspect that concurrent administration with popular therapeutic agents, such as tranexamic acid, which has been shown to attenuate the progression of intracranial hemorrhage after TBI,[59] can improve clinical outcomes. Additional work is required to validate VPA's effects in these scenarios.

We would also like to provide the rationale for the early resuscitation strategy used in this model. Currently, there is wide variation in clinical practice resuscitation guidelines for TBI patients. The Tactical Combat Casualty Care Guidelines prioritize fresh whole blood and blood products for patients with TBI and hemorrhage.[60] In their absence, dried plasma can be used, followed by 6% hetastarch and Lactated Ringer's solution. The Joint Trauma System Clinical Practice Guidelines,[61] however, recommend NS resuscitation (1-L bolus as needed) in the absence of fresh whole blood and blood products, and avoiding colloids, hetastarches, and Lactated Ringer's. The Brain Trauma Foundation guidelines recommend isotonic fluids as first-line resuscitation for TBI patients.[62] As the Brain Trauma Foundation recommendations are more relevant to TBI and are widely used in clinical practice, we adhered to these guidelines by administering early NS resuscitation.

We also recognize that these various fluid resuscitation strategies can impact TBI differently. Colloids, including albumin, have demonstrated worse outcomes in TBI patients,[63] and hetastarches might be associated with renal failure and coagulopathy in trauma patients.[64] In addition, hypotonic fluids and even Lactated Ringer's (hypotonic relative to NS) should be avoided whenever possible to prevent brain swelling from worsening.[65] Although isotonic fluids, such as NS, are recommended by the Brain Trauma Foundation and the Joint Trauma System Clinical Practice Guidelines in the absence of fresh whole blood and blood products, aggressive NS resuscitation can also significantly increase brain swelling and lesion size after TBI. In this study, we wanted to exaggerate the insult with early aggressive NS resuscitation, which allowed us to demonstrate that VPA treatment can improve outcomes even in the worst-case scenarios. However, to provide a better consensus of resuscitation strategies in TBI patients, well-designed clinical trials are required.

Overall, these findings contribute to the literature reporting the neuroprotective effects of VPA treatment after TBI. Earlier preclinical studies have only been able to demonstrate benefits in the immediate/early post-TBI periods. For example, 9 hours after TBI, VPA treatment

increased the neurogenesis and inhibited neuroinflammation in the injured brain.[13] Analysis of their peripheral blood mononuclear cells have also demonstrated sustained effects during the first 24 hours after TBI.[14] However, this is the first study to demonstrate that the beneficial effects of single-dose VPA after TBI are sustained during a 30-day period. As VPA continues to move toward human translation,[66] these additional benefits make it even more appealing, especially as there are no other pharmacologic agents that have been proven to attenuate the sequelae and complications of TBI.

There are several limitations to this study. First, although swine are commonly used for human translation, they serve as imperfect surrogates for human subjects. Second, cortical impact was used in this study to produce the TBI. However, this is not representative of all types of TBI and additional work is required to determine whether these beneficial effects can be replicated in other forms of TBI, such as blast injuries. Third, an injured but unresuscitated control group was not used, as survival without resuscitation is extremely poor. In addition, in the rare survivors, the prolonged shock period would have markedly worsened the severity of TBI. Fourth, we used the 3:1 rule of resuscitation to mimic a resource-scare setting where early administration of blood products is not feasible. Current resuscitation regimen, however, favors an end point-based resuscitation, and encourages early resuscitation with blood products. Our resuscitation protocol represents the worst-case scenario, which might not be applicable to urban settings and well-equipped civilian hospitals, where early plasma-based resuscitation can be used. In these settings, however, we have previously demonstrated that the addition of VPA to fresh-frozen plasma can significantly decrease brain swelling and lesion size compared with fresh-frozen plasma alone.[67] Fifth, we evaluated the impact of VPA treatment on brain alterations at 30 days after injuries. However, we realize that longer-term evaluations are required during the course of years to fully assess VPA's protective effects. Sixth, we only assessed the cortex adjacent to the site of injury, which could underestimate VPA's global effects on the brain.

CONCLUSIONS

This study highlights the impact of VPA administration after TBI. In addition to decreasing the brain lesion size and improving neurologic recovery,[11,12] treatment with a single-dose of VPA after TBI appears to have protective effects at longer-term follow-up than previously investigated. Not only does VPA decrease neural apoptosis, inflammation, and degeneration, but it also promotes neural plasticity and neuronal survival. It acts, in part, by modulating the NF-κB-related pathways. Although additional work is required to evaluate its long-term impact, a single-dose of VPA confers early neuroprotection and decreases the risk of sequelae and brain alterations associated with TBI.

Author Contributions

Study conception and design: Chang, Williams, Li, Alam
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