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Examining the role of astrogliosis and JNK signaling in post-traumatic epilepsy

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Abstract

Objective: Post-traumatic epilepsy is a devastating complication of traumatic brain injury that has no targeted pharmacological therapy. Previous literature has explored the role of the c-Jun N-terminal kinase (JNK) pathway in epilepsy and the creation of epileptogenic foci by reactive astrogliosis; however, the relationship between reactive astrogliosis and the c-Jun N-terminal kinase signaling pathway in the development of post-traumatic epilepsy has not been thoroughly examined.

Methods: Four experimental groups, consisting of c57/b16 male mice, were examined: (1) control, (2) traumatic brain injury of graded severity (mild, moderate, severe), (3) sub-convulsive kainic acid alone without traumatic brain injury (15 mg/kg i.p.), and (4) sub-convulsive kainic acid administered 72 h after moderate traumatic brain injury. Modified Racine scale from 1 to 72 h and total beam breaks at 72 h were used to assess seizure activity. Immunohistochemistry and western blot were utilized to examine astrogliosis (GFAP), microglia activation (IBA-1), and phosphorylated JNK in prefrontal cortex samples collected from the contracoup side at 72 h post-injury.

Results: Astrogliosis, measured by GFAP, was increased after traumatic brain injury and increased commensurately based on the degree of injury. Mice with traumatic brain injury demonstrated a four-fold increase in phosphorylated JNK: $p < 0.001$. Sub-convulsive kainic acid administration did not increase seizure activity nor phosphorylation of JNK in mice without traumatic brain injury; however, sub-convulsive kainic acid administration in mice with moderate traumatic brain injury did increase phosphorylated JNK. Seizure activity was worse in mice, with traumatic brain injury, administered kainic acid than mice administered kainic acid.

Conclusions: Reactive astrocytes may have dysfunctional glutamate regulation causing an increase in phosphorylated JNK after kainic acid administration. Future studies exploring the effects of JNK inhibition on post-traumatic epilepsy are recommended.

Keywords: Post-traumatic epilepsy, Traumatic brain injury, JNK signaling, Astrocytes, Animal model

Introduction

Post-traumatic epilepsy (PTE) is one of the most common and devastating complications of traumatic brain injury (TBI) with a variety of consequences for patient care, recovery, and outcomes (TBI) [1]. PTE is defined two or more unprovoked seizures more than a week after injury [1–3]. A unique feature of these unprovoked (occurring 7 < days post TBI), recurrent seizures is the latency; they may initially occur from weeks to years after TBI, typically occurring within 5 years of the head injury [1, 4, 5]. Latency up to 20 years has been described in literature [1]. The development of PTE after TBI varies with type and prevalence but may be as high as 50% [1, 5]. Those at greatest risk are those with penetrating, versus closed head, injury and is directly correlated to injury severity [1, 6]. Many of those studied are veterans as they represent a significant population at risk for TBI, and the impact on quality of life can be significant [3]. Changes in behavior can result from seizures and result

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in incorrectly treatment due to symptom overlap with post-traumatic stress disorder [7]. Seizures also represent a physical danger to health, as accidents, injury and aspiration can happen during seizure; furthermore, in one population, reoccurrence of epileptic seizures within the next 2 years in TBI patients with a single acute post-traumatic seizure (PTS) was as high 86%, suggesting the risk of developing PTE after PTS is extremely high possibly due to alterations in inter-neuronal connections, increasing future susceptibility [1]. Many may also suffer silent, non-convulsive seizures [1, 2]. Sudden death may also occur in uncontrolled seizures possibly due to alterations in blood pressure, heart rate, hyperthermia or hypoxemia [2]. There remains stigma surrounding those with epilepsy, and those with uncontrolled seizures are prevented from driving and may have difficulty attaining and/or maintaining gainful employment, further challenging the re-integration into society that veterans with TBI face [1].

Though its myriad of consequences, there are no current treatments specific to PTE [1, 2, 8]. Development of new therapeutic targets is imperative because modern treatment of PTE is aimed at controlling seizures with antiepileptic drugs; although, up to 30% of those with PTE are medication resistant and their efficacy in prophylaxis is disputed [2, 8]. Of significance, one study showed that none of the drugs used for seizure prophylaxis demonstrated prevented or even suppressed epileptic seizures after TBI [8]. This is possibly due to differing underlying mechanisms between provoked seizures, which antiepileptic drugs have been demonstrated to help, and the unprovoked seizures that characterize PTE. Many studies have proceeded to propose hypotheses that attempt to explain why seizures occur post-TBI, but the lack of robust evidence has continued to make therapeutic target development challenging.

Despite the absence of a definitive mechanism, astrocytes appear to play a role in the development of PTE [9–14]. Astrocytes are a specific type of glial cell in the central nervous system that preserve neural circuit function through the maintenance of neuronal homeostasis [14]. Astrocytes play an integral role in the response to a variety of neuronal insults through reactive astrogliosis—characterized by cellular hypertrophy, astrocyte proliferation, and increased glial fibrillary acidic protein (GFAP) expression—and the resulting formation of a glial scar to protect healthy cells from harmful substances [12, 14, 15]. This glial scar may be directly epileptogenic, or it may be indirectly epileptogenic—through the downstream actions of cytokines on astrocytes [11, 13–17]. Therefore, understanding of the specific cell-signaling pathways involved may allow for the development of future therapeutic regimens. As previous literature has examined the role of reactive gliosis in epileptogenesis [10, 11, 14], the role of the c-Jun N-terminal kinase (JNK) signaling in reactive gliosis [15], and the activation of the JNK signaling pathway by glutamate [18–20], we sought to examine the relationship between reactive gliosis and the JNK cell-signaling pathway in the development of PTE in an animal model.

Methods

Thirty 3-month-old c57/bl6 male mice were used for this study. All procedures were approved by institutional animal care and use committee (IACUC) protocol and in accordance with the National Institute of Health (NIH) guide for the care and use of laboratory animals. TBI consisted of air acceleration injury allowing for coup/contra-coup dynamics. Kainic acid, which is a neuroexcitatory amino acid that acts on glutamate receptors, was used to assess the role of glutamate in PTE. Groups included (1) control, (2) TBI of graded severity (mild, moderate, severe), (3) sub-convulsive kainic acid alone without TBI (15 mg/kg i.p.), and (4) sub-convulsive kainic acid administered 72 h after moderate TBI. Behavioral assays utilized included modified Racine scale from 1 to 72 h after injury to determine seizure and total beam breaks at 72 h. Prefrontal cortex samples were collected from contra-coup side for biochemical analysis. Immunohistochemistry and western blot were utilized to look at hematoxylin and eosin staining (H&E), astrogliosis (GFAP), microglia activation by ionized calcium-binding adapter molecule 1 (IBA-1), and JNK phosphorylation. Standard techniques were utilized as previously reported [21].

Statistical analysis

Graphpad Prism software was utilized for all analysis with $p < 0.05$ considered statistically significant. Results were assessed using a student's t-test or analysis of one-way variance (ANOVA) where appropriate.

Results

Sub-convulsive kainic acid alone did not decrease locomotion or cause seizure activity. When combined with moderate TBI, significant seizure activity was noted as measured by modified Racine score (Fig. 1). H&E showed increased chromatolysis and red/dead neurons with graded TBI severity (Fig. 2).

Significant astrogliosis was noted after moderate TBI (Fig. 3).

The response was graded in a perivascular distribution based on injury severity (Fig. 4). Likewise, microglia activation was graded in similar fashion based on injury severity (Fig. 5).

To tease out mechanism, JNK phosphorylation was examined in correlation with the astrogliosis. Phosphorylated JNK (pJNK) had a four-fold increase in the TBI

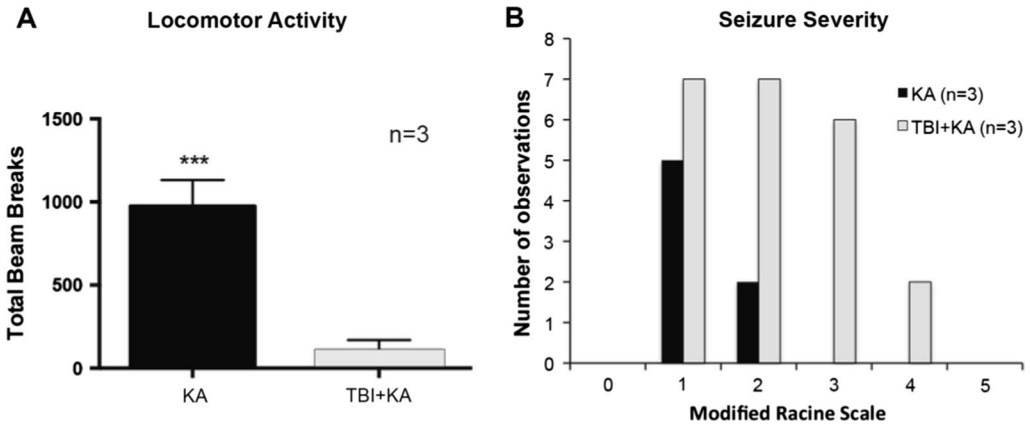

Fig. 1 A sub-convulsvie dose of kainic acid (KA) impedes locomotion and increases seizure severity 72 h after TBI. **a** Beam break number is significantly higher in control mice (black bar) compared to mice exposed to TBI (gray bar). **b** Control mice (black bars) are more resistant to kainic acid-induced seizures than blast-exposed mice (gray bars)

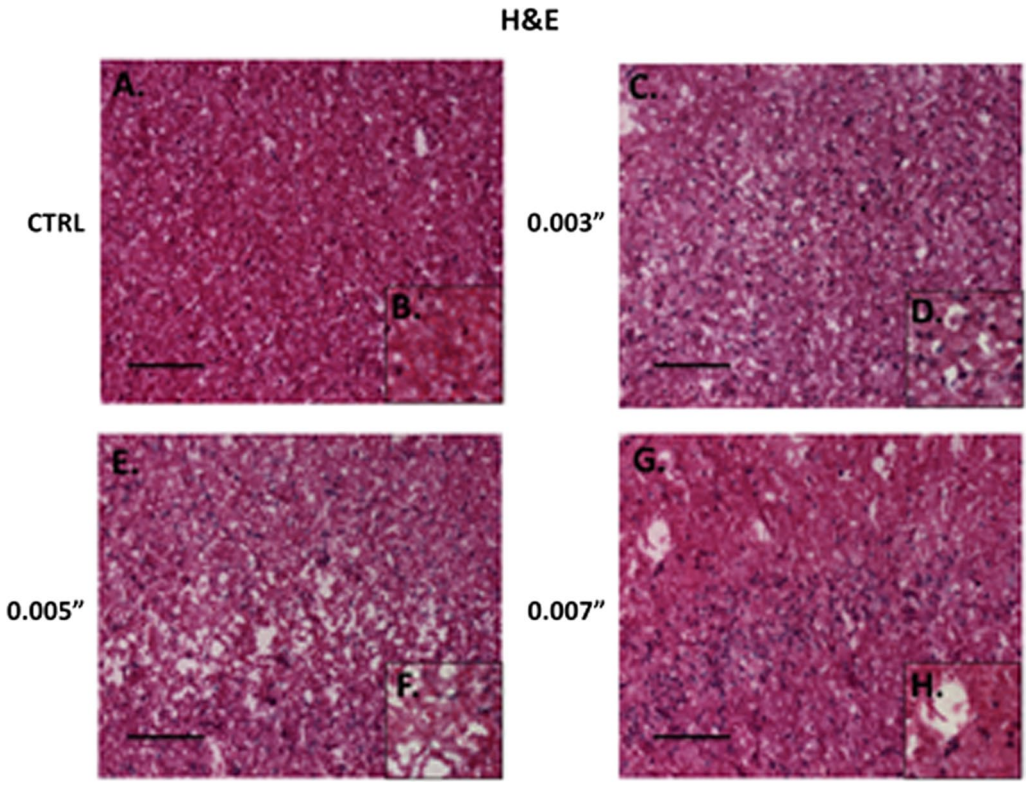

Fig. 2 TBI severity is associated with increased neuronal damage. Increased chromatolysis and red (dead) neurons are seen with graduated increase of severity of TBI from mild to severe. **a** Control group 40x magnification. **b** Control group 63x magnification. **c** Mild TBI 40x magnification. **d** Mild TBI 63x magnification. **e** Moderate TBI 40x magnification. **f** Moderate TBI 63x magnification. **g** Severe TBI 40x magnification. **h** Severe TBI 63x magnification

group (Fig. 6). Kainic acid alone did not increase phosphorylation of JNK but when combined with moderate TBI did increase pJNK indicating possible mechanism for seizure induction (Fig. 7).

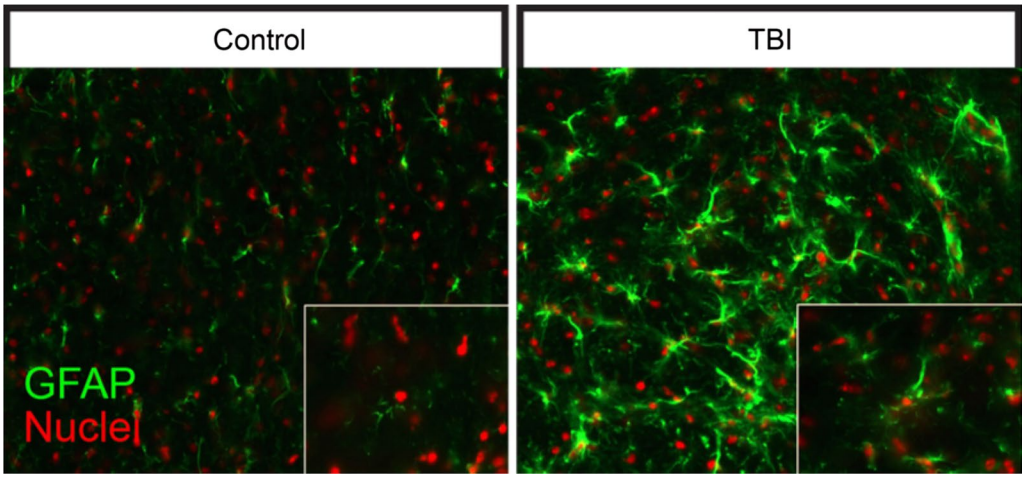

Fig. 3 Reactive astrogliosis after moderate severity TBI. Moderate severity TBI increases astrogliosis at 72 h after injury, as measured by GFAP

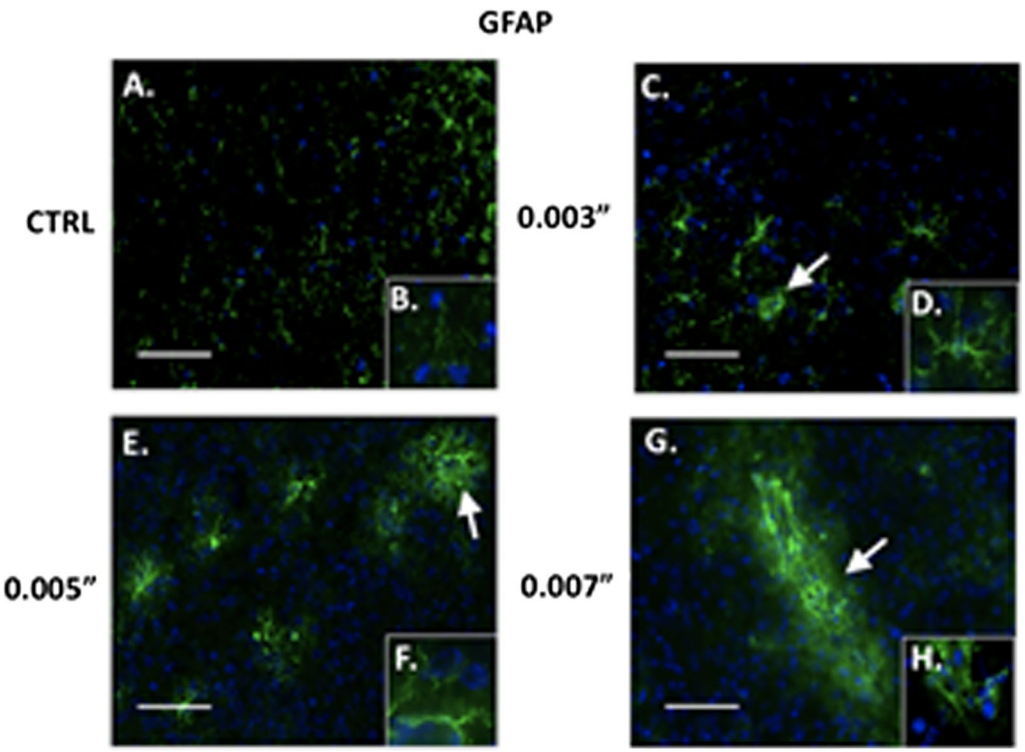

Fig. 4 TBI severity is associated with degree of astrogliosis. Astrogliosis in perivascular distribution is mediated by severity of injury at 72 h after injury, as measured by GFAP. Arrows pointing to reactive astrocytes. **a** Control group 40x magnification. **b** Control group 63x magnification. **c** Mild TBI 40x magnification. **d** Mild TBI 63x magnification. **e** Moderate TBI 40x magnification. **f** Moderate TBI 63x magnification. **g** Severe TBI 40x magnification. **h** Severe TBI 63x magnification

Discussion

Reactive astrogliosis is regulated by cell-signaling mechanisms, which are inducible by a variety of neural insults [13]. In particular, the resulting compressive and sheer strain from mechanical loading deformations associated with TBI present multiple etiologies for astrocyte reactivity [22]. Supra-threshold loading of astrocytes can cause complete structural failure or less overt damage through

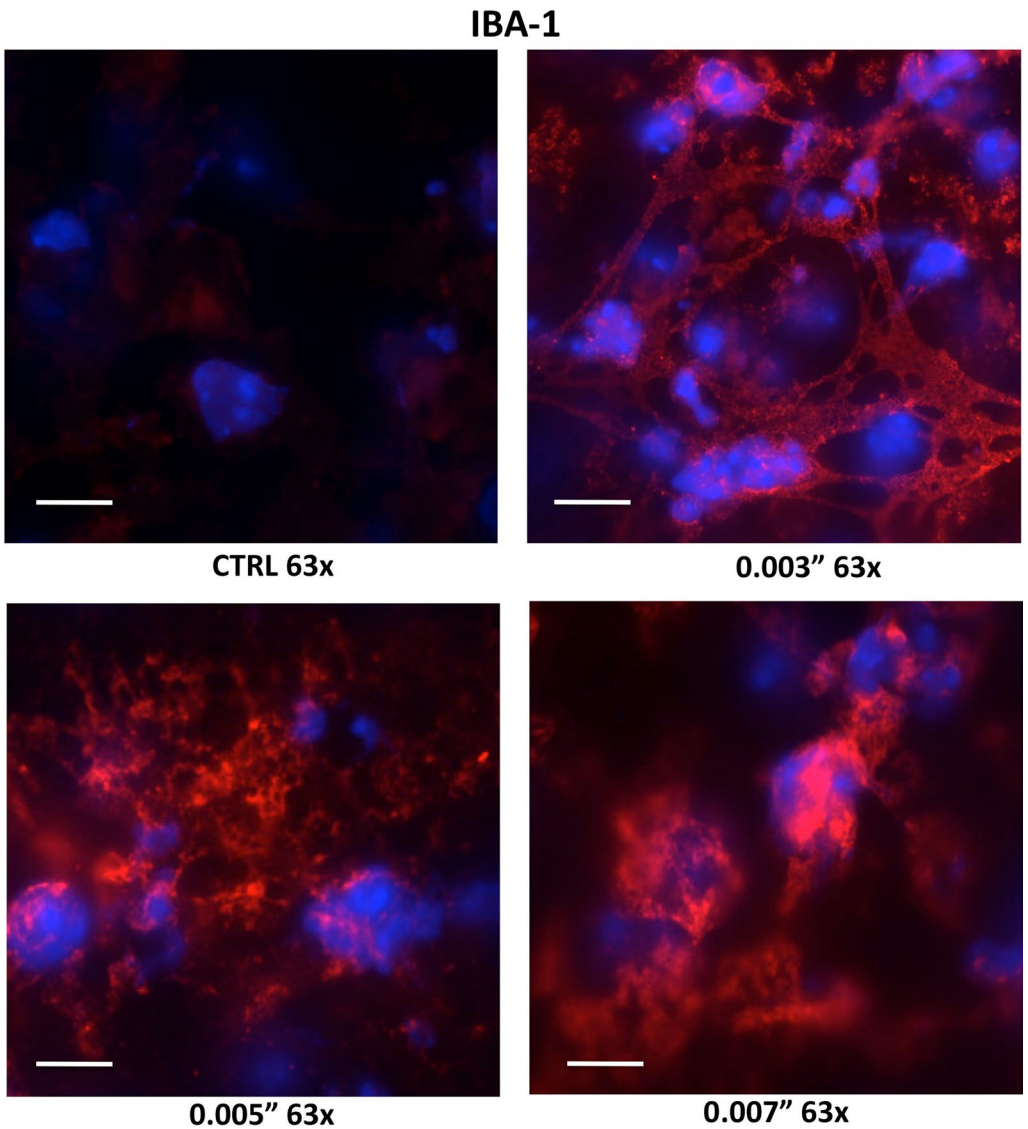

Fig. 5 TBI severity is associated with degree of microglia activation. Microglia activation is also increased in graded fashion with increased severity of injury at 72 h, as measured by IBA-1

intracellular damage to the cytoskeleton, organelles, or plasmalemma [22]. Once these damages occur, intracellular ions are released and an acute inflammatory response triggers an interplay of cell signaling pathways to incite astrocyte migration to the area of injury and subsequent proliferation [10, 14, 23, 24].

In this study, mice that were administered a subconvulsive dose of kainic acid following a blast exposure had a significant increase in gliosis, as measured by GFAP, in the ipsilateral hippocampus and temporal cortex, compared to controls: $p < 0.001$. Additionally, mice exposed to a blast injury and subsequently administered kainic acid had fewer beam breaks and were less resistant to kainic acid-induced seizures than control mice. The implication of these results suggests a possible relationship between reactive astrogliosis and the inability to regulate kainic acid, which is supported by previous literature on reactive astrocytic glutamate regulation. In a similar mouse model, the conditional deletion of the β1-integrin resulted in widespread astrogliosis without causing significant inflammation and continuing to maintain the integrity of the blood–brain barrier [11]. These mice demonstrated neuronal hyperexcitability and defective glutamate uptake by astrocytes; this resulted in increased extracellular levels of the excitatory neurotransmitter glutamate [11]. This

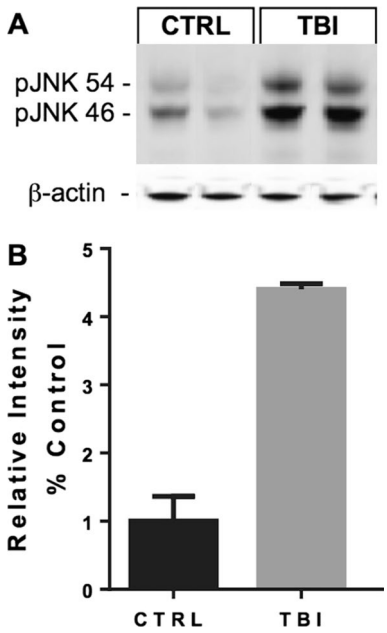

Fig. 6 JNK activity is elevated 72 h after TBI. **a** Western blot data were probed for phosphorylated JNK and beta actin. **b** A fourfold increase in pJNK was seen in TBI samples

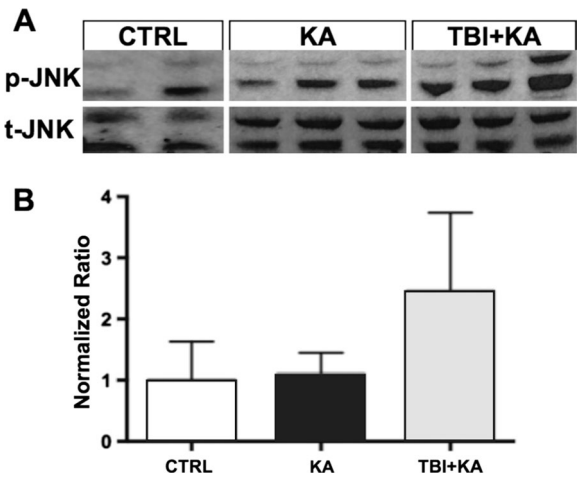

Fig. 7 Elevated JNK activity correlates with enhanced seizure susceptibility. **a** Western blot from control, kainic acid, and TBI + kainic acid. **b** Kainic acid alone did not increase the phosphorylation ratio but combined with TBI the ratio was increased, which can limit seizure threshold

impaired regulation of glutamate homeostasis by reactive astrocytes may be due to decreased expression of the glutamate transporter Glt1, which has been found to undergo a rapid and sustained decrease in expression in the reactive astrocytes of mice after sustaining TBI [12]. Other mice models suggest reactive astrocytes have impaired glutamate metabolism due to downregulation of the enzyme glutamine synthetase [9]. Our results demonstrate that TBI is associated with reactive astrogliosis, decreased resilience to kainic acid-induced seizures, and impedance of locomotion; this suggests that dysfunctional regulation of glutamate in reactive astrocytes may predispose to the development of PTE.

The mice exposed to blast-induced TBI in our study had approximately a fourfold relative increase in hippocampal pJNK, compared to control mice: $p < 0.001$. The increased levels of pJNK in mice administered kanic acid after blast-induced TBI compared to mice administered kainic acid in this study suggest that TBI induces changes in the neuronal homeostasis of glutamate. This is because glutamate activates N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), and the activation of NMDARs and AMPARs result in the downstream activation of JNK [19, 20, 25]. Therefore, impaired glutamate regulation by reactive astrocytes may result in a increased levels of glutamate that are free to act on NMDARs and AMPARs, inducing excitotoxicity and JNK signaling. This glutamate-induced excitotoxicity may an etiology for PTE. This may explain why the total JNK levels were highest in TBI mice subsequently exposed to kainic acid, which also had the lowest threshold for seizures. Previous studies have examined this and found that mice lacking JNK3 gene were resistant to kainic acid-induced seizures, and pharmacological therapies targeting JNK have decreased seizure frequency in epileptic animal models and in glutamate excitotoxity vitro [17, 18]. Additionally, JNK signaling may have a role in the regulation of the inflammatory response of glial cells, and neuroinflammation may also contribute to the genesis of PTE [16, 26]. Therefore, our results further suggest JNK signaling may contribute to the development of epileptogenic foci in PTE, and JNK inhibitors may be a target for pharmacological therapy.

Conclusions

Glutamate may induce JNK signaling through NMDARs and AMPARs. Kainic acid administration was associated with decreased locomotion, increased seizure activity, and increased pJNK in mice with TBI. TBI may cause impaired regulation of glutamate, causing increased levels of the downstream product JNK. Therefore, JNK may present a pharmacological target for PTE, and future animal models examining the efficacy of JNK inhibitors for the treatment of PTE should be explored.

Abbreviations
PTE: Post-traumatic epilepsy; TBI: Traumatic brain injury; PTS: Post-traumatic seizure; GFAP: Glial fibrillary acidic protein; JNK: C-Jun N-terminal kinase; H&E: Hematoxylin and eosin staining; IBA-1: Ionized calcium-binding adapter molecule 1; pJNK: Phosphorylated JNK; NMDARs: *N*-Methyl-D-aspartate receptors; AMPARs: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors.

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Authors' contributions
CS, AD, and BL have given substantial contributions to the conception or the design of the manuscript. All authors have participated in the acquisition, analysis, and interpretation of the data. All authors have participated to drafting the manuscript, and CS and BL revised it critically. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study had full institutional review board approval; all procedures were approved by institutional animal care and use committee (IACUC) protocol and in accordance with the National Institute of Health.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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