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FULL-LENGTH ORIGINAL RESEARCH

Zebrafish model of posttraumatic epilepsy

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Abstract

Objective: Posttraumatic epilepsy (PTE) is defined as recurrent and unprovoked seizures occurring >1 week after traumatic brain injury (TBI). Animal studies of PTE are lengthy and expensive. In this study, we developed a cost-effective PTE animal model using zebrafish to bridge the gap between in vitro studies and low-throughput animal studies.

Methods: We used two different sets of parameters (G1 and G2) to induce closed-head TBI in adult zebrafish using pulsed high-intensity focused ultrasound. Injured fish and naive controls were evaluated for behavioral deficits and spontaneous behavioral seizure activity up to 21 days postinjury (DPI). We also assessed behavioral seizure susceptibility to a subconvulsive dose of pentylenetetrazole (PTZ; 2.5 mmol·L^{-1}) and recorded electrophysiological signals to confirm seizure activity up to 40 DPI. In addition, we investigated injury-related changes in the blood-brain barrier and expression levels of various proteins altered in rodent and human TBI.

Results: The G2 parameters resulted in a more severe TBI, with a mortality rate of 25%, as well as motor dysfunction and heightened anxiety persisting at 21 DPI. One hundred percent of the G2 group showed spontaneous myocloniclike behavior, and 80% demonstrated tonic-clonic–like behavioral seizures by 21 DPI. Such activities were not detected in the naive group. After the application of 2.5 mmol·L^{-1} PTZ, 100% of injured zebrafish had cloniclike seizures at 21 DPI, versus 30% of the naive group. We also demonstrated electrographic seizure activity at 40 DPI, which was not detected in the naive controls. Lastly, we observed acute blood-brain barrier dysfunction and increased levels of HMGB1 and ratios of phosphorylated/total Akt and tau through 21 DPI.

Significance: Together, the results indicate that severe TBI in the adult zebrafish leads to similar behavioral and physiological changes to those of more traditional models, including the development of PTE, and suggest this may be a useful model that can accelerate research in TBI/PTE.

KEYWORDS
posttraumatic epilepsy, seizure, traumatic brain injury, zebrafish

1 | INTRODUCTION

Posttraumatic epilepsy (PTE) can be a debilitating complication after traumatic brain injury (TBI), occurring in up to 30% of TBI patients.[1] There is currently no cure to prevent the development of PTE, although numerous animal models have been developed to study mechanisms of posttraumatic epileptogenesis and potential treatments. Although no single animal model fully recapitulates the heterogeneous spectrum of human TBI, there is no doubt that mammalian models show similar pathophysiological responses and have been critical to understanding the biomechanical and pathophysiological development of secondary injury mechanisms after TBI. Despite the value of these studies, modeling PTE in rodents is expensive and labor-intensive, with low throughput. Given that the incidence of PTE correlates with TBI severity,[1] high mortality rates are unavoidable with animal models. For example, the mortality rate in fluid percussion injury (FPI) modeling severe TBI can be 30%,[2] yet <50% of FPI animals develop epilepsy after 1 year, with a low seizure rate (<1/d).[3] Specific cellular mechanisms of epilepsy can be studied in vitro, which can be useful in developing novel treatments for epilepsy and circumventing these mortality issues. However, in vitro models do not capture the scope of behavioral seizures, limiting the utility of such models in understanding the full complexity of epilepsy.[4] Thus, there is a need for an appropriate animal model that can facilitate the process of studying PTE and overcome the shortcomings of existing methods and models. A robust bridging animal model that can mimic the pathophysiology of PTE with higher throughput and lower costs is needed to facilitate the study of PTE.

Over the past few decades, the zebrafish has emerged as a model organism for the study of human diseases, including epilepsy and TBI.[5–7] Zebrafish have a fully characterized genome, with significant homology to humans, and display robust physiological and behavioral phenotypes, enabling high-throughput disease modeling and drug screening. Zebrafish also develop quickly and produce a vast number of offspring, allowing for lower maintenance costs than rodents and higher productivity.[8] To date, epilepsy studies in zebrafish have focused on chemically kindled epilepsy and genetic mutations. There have been no reports of other models with spontaneous seizures in adult AB-strain zebrafish. With regard to TBI, most brain injury studies in zebrafish rely on a stab wound technique, which is ideal for reproducing human cellular responses to injury, but does not recapitulate closed-head injury, the most common cause of human TBI.[6] To overcome this issue, we developed a closed-head injury model of zebrafish TBI using pulsed high-intensity focused ultrasound (pHIFU) that results in similar pathophysiological responses to rodent and human TBI.

The goal of the current study was to use the pHIFU model to induce TBI of different severities and determine whether zebrafish develop a PTE phenotype with molecular changes similar to those implicated in epileptogenesis in other models. We found zebrafish with severe TBI exhibit motor and neuropsychological deficits, blood-brain barrier (BBB) disruption, and similar molecular changes to those reported in rodent TBI models. Injured zebrafish also demonstrate spontaneous recurrent behavioral seizures (chronic epilepsy), increased susceptibility to chemical convulsants, and spontaneous electrographic seizures. These results support a novel severe TBI zebrafish model for studying PTE, with the potential for higher throughput and cost-efficiency relative to mammalian models.

Key Points

- A severe closed-head model of TBI in zebrafish was modeled for the first time to induce PTE.
- Severe TBI in zebrafish demonstrates increased seizure susceptibility, and spontaneous behavioral and electrographic seizure activity.
- The zebrafish PTE model could be a useful tool in investigating TBI and PTE pathologies.

2 | MATERIALS AND METHODS

2.1 | Animals and reagents

Wild-type (AB strain) zebrafish were raised at the Zebrafish Core Facility of St Michael's Hospital (Toronto, Ontario, Canada) according to standard procedures. Zebrafish were maintained under a 12:12-hour light:dark cycle, and housed in tanks at 25°C and pH 6.8-7.0. Male and female zebrafish aged 6-12 months (young adults) were used. Fish were euthanized using 500 ppm clove oil (Sigma-Aldrich) immediately after each pentylenetetrazole (PTZ) test or electrographic recording. All animal care and experiments were approved by the Animal Care Committee at St Michael's Hospital and conducted in strict accordance with relevant Canadian guidelines and regulations for the amelioration of animal suffering. Zebrafish were randomly assigned to the following groups: naive, G1, and G2. Fish were handled gently to minimize stress throughout the experiments. All behavioral video monitoring was performed in the same room, which was maintained in a uniform condition.

2.2 | Zebrafish model of severe closed-head injury and experimental groups

Closed-head TBI was induced with a pHIFU system (Figure 1), as previously described.[6,7] Briefly, zebrafish were anesthetized with clove oil (50 ppm) and mounted in

a restraint that covered the body and maintained anesthesia during injury induction. The zebrafish and restraint were immediately placed into the pHIFU system, and the target zone (right telencephalon) was aligned to the pHIFU focal spot. Injury parameters were controlled by a function generator (AFG3101, Tektronix) to deliver either two impact forces of 11 MPa (G1 group) or three impact forces of 3 MPa (G2 group) in succession. The fish were then removed from the restraint and returned to anesthetic-free water. The time taken to return to a spontaneous upright position was measured, and the animal was returned to a recovery tank.

2.3 | Behavioral phenotyping

Neurobehavioral and neuropsychological performance after TBI were evaluated in a novel-tank test at 3, 5, 7, 10, 14, 18, and 21 days postinjury (DPI) between 08:00 and 12:00. Zebrafish were placed into a new tank (10 L) and individually video-recorded for 30 minutes (Vixia-HFR800, Canon). Video files were analyzed using an automated video-tracking system (EthovisionXT, Noldus) to calculate mean swimming velocity (cm/s) and mean meandering (°/cm). The percentage of time spent in the upper half of the tank was also calculated as a measure of anxiety levels.

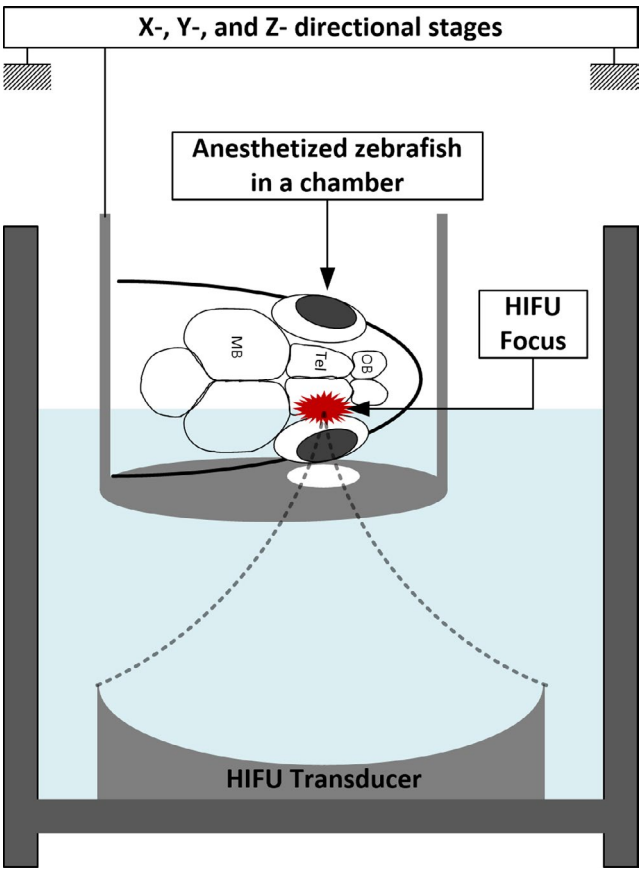


FIGURE 1 Schematic of the noninvasive pulsed high-intensity focused ultrasound (HIFU) injury setup. The device generated the 1-MHz wave, and the wave was emitted with a focal point ~12 cm from the HIFU transducer. A chamber held the anesthetized zebrafish in a right lateral recumbent position. The right telencephalon was positioned over the hole in the chamber. The hole was covered with the ultrasonic membrane, which allows transmitting the wave to the brain. OB, olfactory bulb; Tel, telencephalon; MB, midbrain.

2.4 | Spontaneous recurrent behavioral seizures

The same video recordings were also used to detect spontaneous seizures. Videos were scored manually by a blinded observer based on a zebrafish behavioral seizure scale adopted and modified from the previous studies[9] (Table S1 and Video S1).

2.5 | Behavioral PTZ seizure susceptibility testing

PTZ susceptibility testing was performed at 7, 14, and 21 DPI. A 3-L tank was filled with 2 L of 2.5 mmol·L^{-1} PTZ solution. Ten animals from each injury group at each timepoint were randomly selected, placed in the tank individually, and monitored for 15 minutes. Behavioral seizures were scored as previously described (Table S1). After PTZ exposure, fish were transferred to tanks with clean water to wash out the PTZ and then euthanized.

2.6 | Electrophysiology

Zebrafish from the three injury groups underwent neuroelectrophysiologic recordings at 13, 32, or 40 DPI. Fish were immersed in 200 ppm of clove oil until they show no reflex response,[5] and then intubated with a gravity perfusion system delivering 40 ppm of clove oil with a flow rate of 5-6 mL/min to maintain the surgical anesthesia. Restrained fish were covered with wet gauze to maintain viability and 2-3 mm^2 of the cranium covering the right telencephalon was microdissected. Recording electrodes with a resistance of 2-3 MΩ were pulled from borosilicate glass, backfilled with 150 mmol·L^{-1} NaCl, and placed on the right telencephalon. A chloride-coated silver wire was used as a reference electrode on the supraneural spine.[5] Field potential signals were acquired with Axopatch 200B (Molecular Devices) and bandpass filtered at 0.1-55 Hz. Baseline signals were recorded for 15 minutes while fish maintained constant opercular movements and had good blood circulation, as monitored by visual inspection. Electroencephalographic data were reviewed blindly and manually by two researchers using Clampfit 11.0.3 (Molecular Devices) for the number of interictal epileptiform discharges (IEDs) and the duration of

seizure activity (Figure 2B,C). The rate of having IEDs and seizures indicates the number of animals that had one or more IEDs/seizures during the 15 minutes of electrophysiological recording (Figure 2D).

2.7 | Evans blue staining

In a separate set of experiments, naive or G2 zebrafish (n = 7/group) were anesthetized with 200 ppm clove oil 1 hour after TBI, and 50 μL of 1% Evans blue dye was injected intracardially using a 26-gauge needle. Zebrafish recovered for 30 minutes, then brains were fixed with 4% paraformaldehyde, embedded in 2% agarose gel, sagittally sectioned (300 μm) on a vibratome, and imaged using a fluorescent microscope (ECLIPSE 90i, Nikon). Acquired images were analyzed with ImageJ (National Institutes of Health) to assess fluorescence intensity from the region of interest.

2.8 | Western blotting

Whole brains were collected from all groups at various timepoints and homogenized in radioimmunoprecipitation assay

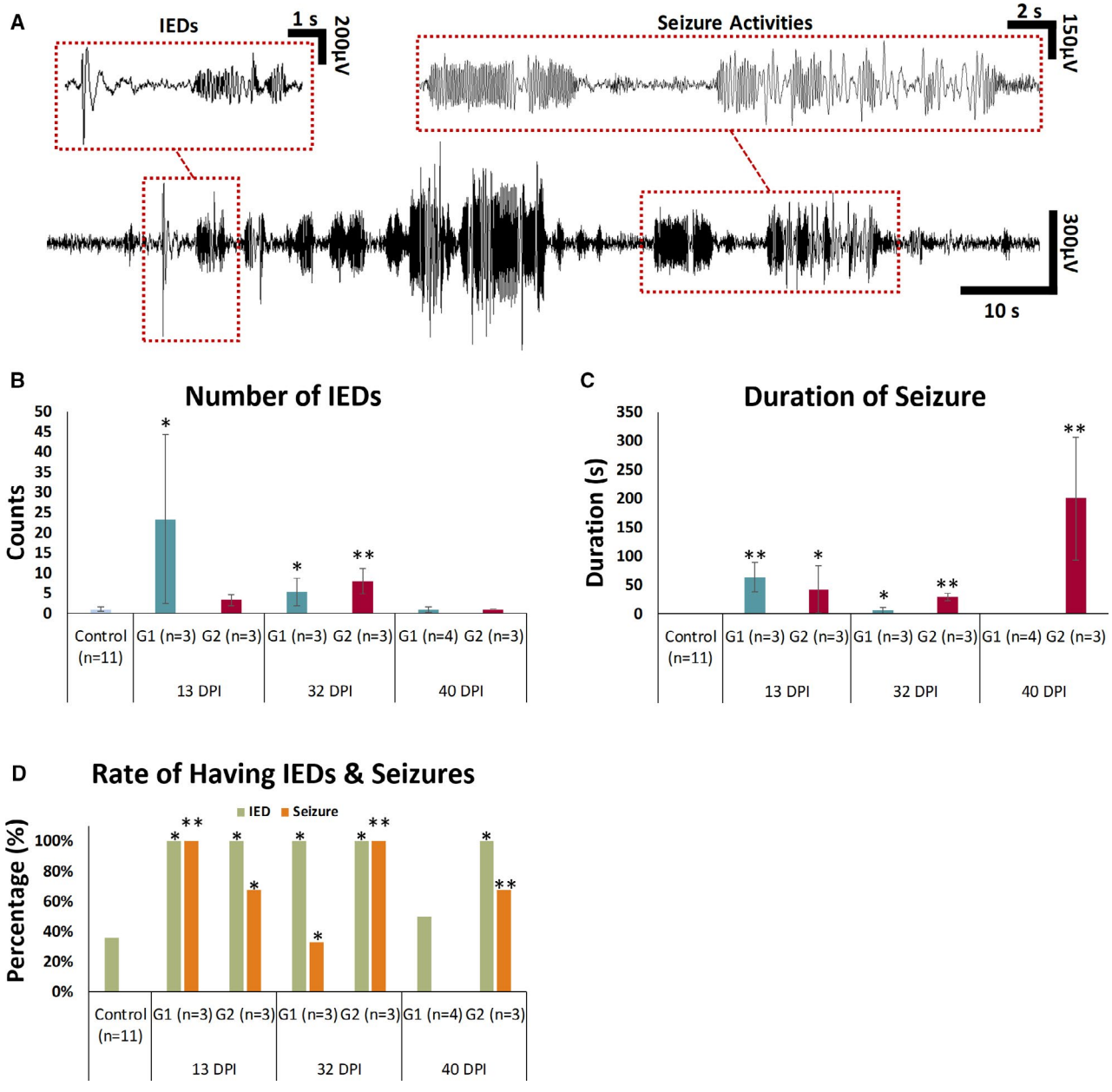
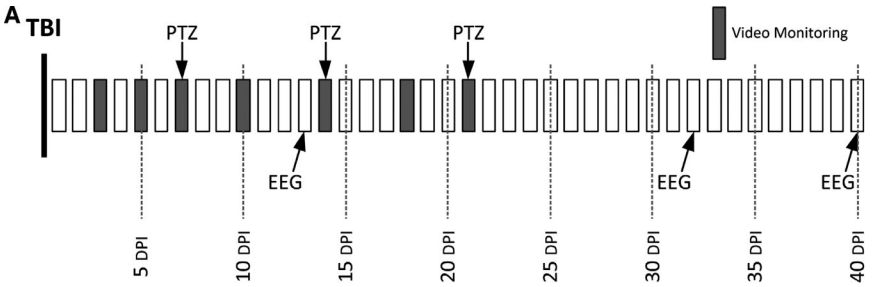


FIGURE 2 Representative electroencephalographic (EEG) trace and quantitative analysis of EEG change after traumatic brain injury. A, In vivo electrophysiology recording (15 minutes) from zebrafish forebrain at 40 days postinjury (DPI) demonstrating seizure activity. B, C, Number of interictal epileptiform discharges (IEDs) and duration of seizure. D, Rate of having IEDs and seizures. Values are expressed as mean ± standard error of the mean in B and C. $*P < .05$, $**P < .01$



B

Video Monitoring

Group	0 DPI	3 DPI	5 DPI	7 DPI	10 DPI	14 DPI	21 DPI
Control	10	X	X	X	X	X	X
G1	X	15	15	15	15	15	15
G2	X	36	34	29	25	23	15

PTZ Test

Group	0 DPI	7 DPI	14 DPI	21 DPI
Control	10	X	X	X
G1	X	10	10	10
G2	X	10	10	10

EEG

Group	0 DPI	13 DPI	32 DPI	40 DPI
Control	11	X	X	X
G1	X	3	3	4
G2	X	3	3	3

FIGURE 3 Representation of experimental design. Video monitoring started 3 days after traumatic brain injury (TBI) and was repeated at 5, 7, 10, 14, 18, and 21 days postinjury (DPI). Behavioral seizure susceptibility tests using a subeffective dose of pentylenetetrazole (PTZ) were performed at 7, 14, and 21 DPI. A, Electrographic recordings were performed at 13, 32, and 40 DPI. B, The number of animals used in each experimental session is shown. All animals were randomized prior to each experiment. EEG, electroencephalogram.

buffer with phenylmethanesulfonyl fluoride (Sigma-Aldrich). Triplicate samples were separated by 7.5% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, and electrotransferred onto 0.2-μm nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked and then incubated with primary antibodies overnight: high mobility group box 1 (HMGB1; 1:400, Abcam), tau (1:5000, Abcam), phospho-tau (1:10 000, Abcam), extracellular signal-regulated kinase (ERK; 1:20 000, Abcam), phospho-ERK (1:10 000, Cell Signaling Technology), protein kinase B (Akt; 1:500, Abcam), phospho-Akt (1:200, Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10 000, Abcam). Immunoblots were washed and incubated for 1 hour with horseradish peroxidase–conjugated goat antirabbit or goat antimouse secondary antibodies at 1:6000 dilutions. Bands were visualized using an enhanced chemiluminescence reagent (GE Healthcare) and imaged with ChemiDoc Imaging System (Bio-Rad Laboratories). Band densities were quantified using Image Lab software (Bio-Rad Laboratories). Results are presented as the relative densities of HMGB1 to GAPDH, or phosphorylated to total proteins.

2.9 | Statistical analysis

Data are reported as the mean ± standard error of the mean. For all experiments, chi-squared or one-way analysis of variance (or the Kruskal-Wallis test with Dunn multiple comparisons test for nonparametric analysis) was used as appropriate, with Tukey test for post hoc comparisons. $P < .05$ was considered significant.

3 | RESULTS

3.1 | Study design

The three experimental groups (naive, G1, and G2) were housed and carefully observed for up to 40 days, with testing performed in different subsets of fish at various timepoints (Figure 3A). The number of animals in each group is shown in Figure 3B.

3.2 | Mortality and righting reflex after TBI

Mortality rate in the first 3 DPI was 15.39% in the G1 group and 25.00% in G2. Mean righting times post-TBI were 3.92 ± 0.42 minutes for G1 and 4.81 ± 0.43 minutes for G2. Both parameters support a more severe injury in the G2 group.

3.3 | Motor and neuropsychological deficits after TBI

The G1 group had motor deficits demonstrated by significantly slower swimming velocity than controls at 3 and 7 DPI

($P = .010$ and $P = .001$, respectively) with recovery by 10 DPI, consistent with our previous findings of reduced velocity up to 48 hours post-TBI.[6] The G2 group had a significantly slower velocity than controls at 3, 18, and 21 DPI ($P = .018$, $P = .006$, and $P = .019$, respectively), indicating the initial motor deficit recovered by 10 DPI, but deteriorated again by 18 and 21 DPI (Figure 4A). Meandering was significantly different from controls for the G1 group at 3 and 5 DPI ($P = .027$ and $P = .023$, respectively) similar to our previous description of a single pHIFU insult, although there were no significant differences between the G2 group and controls.

Exploratory behavior, an established test for anxiety, was significantly reduced in both experimental groups and had not recovered by 21 DPI (Figure 4C). G2 animals exhibited

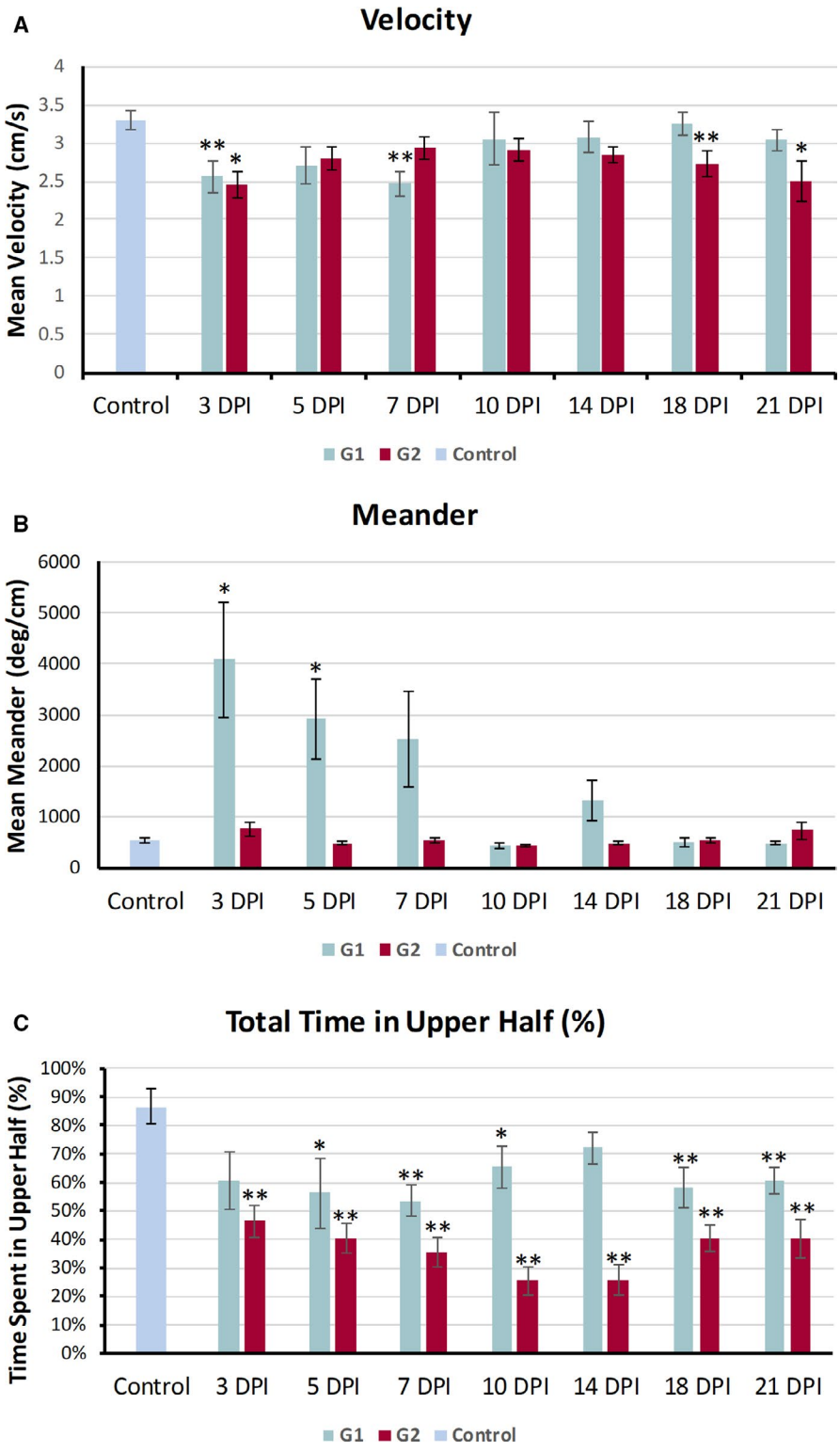


FIGURE 4 Neurobehavioral and neuropsychological deficits after traumatic brain injury. A, Velocities of G1 were significantly decreased at 3 and 7 days postinjury (DPI), and G2 showed a significant decrease at 3, 18, and 21 DPI compared to controls. B, Meandering, which is another measure of locomotor change, measured at 3 and 5 DPI of G1, indicated significant increases, but G2 did not show a significant change in meandering compared to controls. C, The position of zebrafish in the open tank was calculated to measure the anxiety level. Regarding the time spent in the upper half, G1 showed a significant reduction in upper half dwell time at 5, 7, 10, 18, and 21 DPI. Note that G2 showed a significant reduction in upper half dwell time at all timepoints. Values are presented as mean ± standard error of the mean. *$P < .05$, **$P < .01$

significantly less exploratory behavior compared to the control group at all points measured (3, 5, 7, 10, 14, 18, and 21 DPI; P = 3E-04, P = 5E-05, P = 7E-06, P = 4E-08, P = 2E-07, P = 5E-06, and P = 9E-05, respectively), whereas the change in G1 animals was significant only at 5, 7, 10, 18, and 21 DPI compared to controls (P = .041, P = .001, P = .047, P = .008, and P = .002, respectively). The greater effects on swimming velocity and exploratory behavior in the G2 versus G1 group provide further support of a more severe TBI with these injury parameters.

3.4 | Behavioral seizures

Spontaneous myocloniclike seizures were observed in 80%-100% of G2 fish and 93%-100% of G1 fish over the first 21 DPI (Figure 5A). Stage 4 seizures, equivalent to generalized tonic-clonic–like seizures, were consistently observed in ~20% of G1 fish up to 21 DPI. However, ~40% of G2 fish developed stage 4 seizures at 3 DPI, increasing to 80% by 21 DPI (Figure 5B). The higher seizure rate corresponds with the greater behavioral changes in the G2 group.

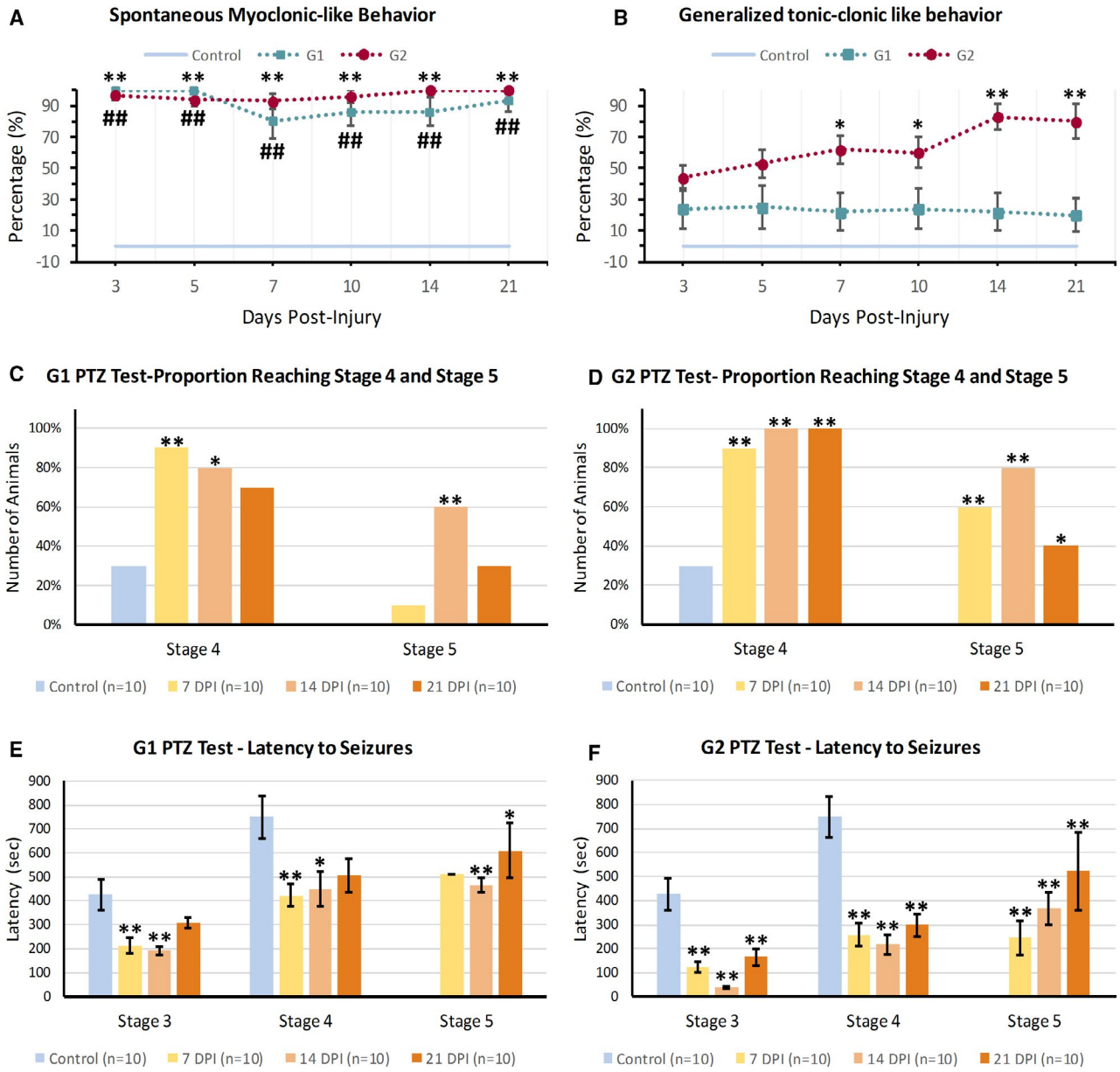


FIGURE 5 Severe traumatic brain injury in zebrafish evoked spontaneous seizures and induced a persistent susceptibility to subconvulsive pentylenetetrazole (PTZ; 2.5 mmol·L^{-1}). A, B, Rate of having a spontaneous myocloniclike behavioral seizure (A), and a generalized tonic-clonic–like behavioral seizure (B). C, D, The percentages of reaching stage 4 and stage 5 after 2.5 mmol·L^{-1} PTZ administration in G1 and G2. E, F, Latencies to the first stage 3, 4, and 5 behavioral symptoms after 2.5 mmol·L^{-1} PTZ administration in G1 and G2. Note that no animals from the control group reached stage 5 during the PTZ test. Values are expressed as mean ± standard error of the mean (A, B, E, F). *P < .05, **P < .01, ##P < .01 (G1 in A and B). DPI, days postinjury.

3.5 | Persistent seizure susceptibility to subconvulsive PTZ after TBI

PTZ-evoked seizure susceptibility was assessed at various timepoints and did not result in mortality in any of the groups. Thirty percent of naive fish exhibited stage 4 seizures; none reached stage 5 (Figure 5C,D). By contrast, 90% of G1 animals reached stage 4 at 7 DPI, decreasing to 80% at 14 DPI and 70% at 21 DPI (Figure 5C). In the G2 group, 90% exhibited stage 4 seizures at 7 DPI, increasing to 100% at 14 DPI and 21 DPI. A greater proportion of fish reached stage 5 seizures at 14 DPI in the G1 group compared with the naive group ($P = .014$), and the proportion of fish with stage 5 seizures was significantly greater in the G2 group versus controls at 7, 14, and 21 DPI ($P = .014$, $P = .004$, and $P = .045$, respectively).

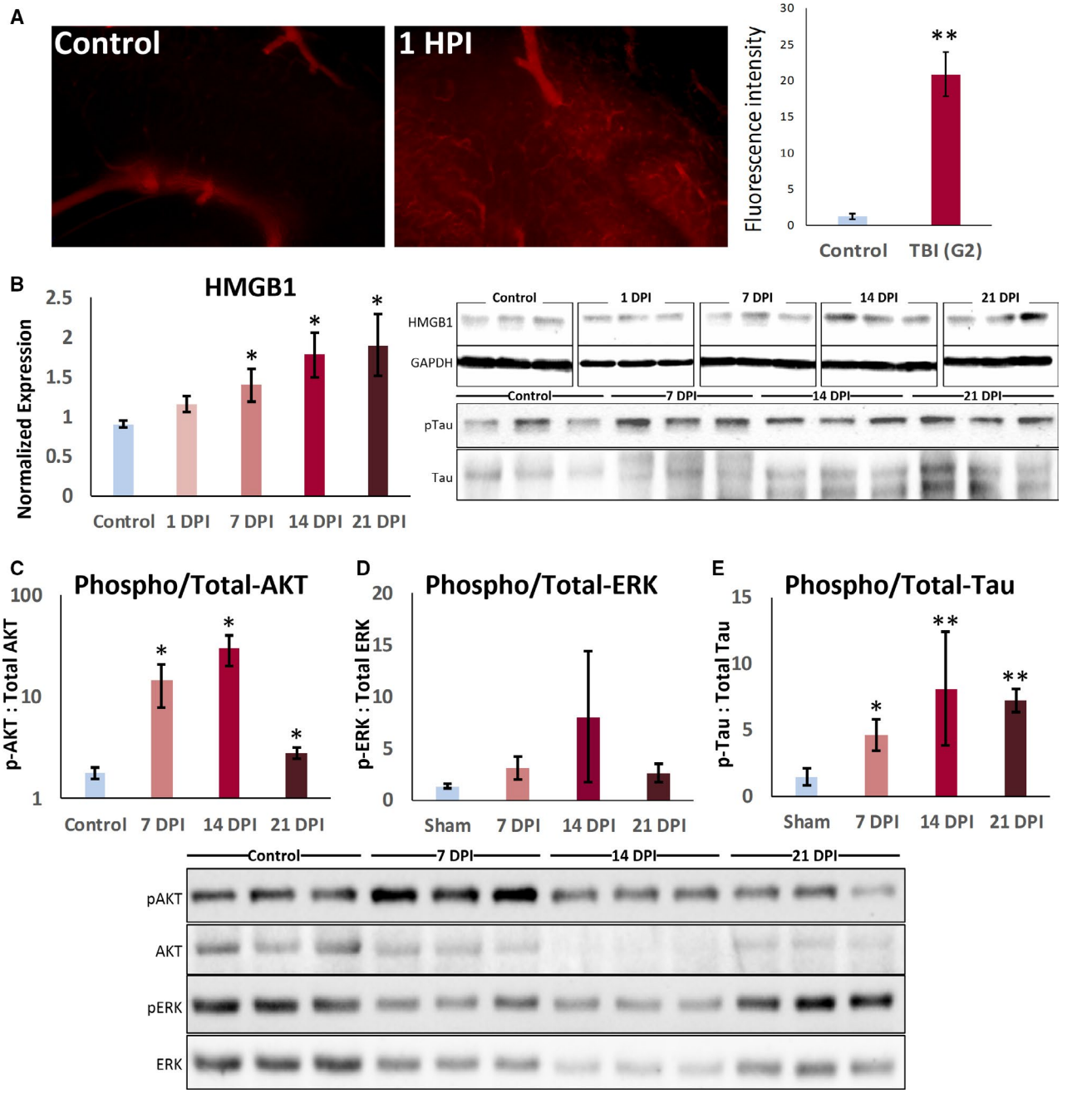


FIGURE 6 Widespread opening of the blood-brain barrier 1 hour after traumatic brain injury (TBI) was monitored by intracardially injected Evans blue dye. A, No extravasation of Evans blue dye in naive and strong extravasation in TBI animal. Anterior midbrains are shown. HPI, hours post injury. B-E, Western blot analysis in TBI animal. high mobility group box 1 (HMGB1) was increased in the TBI-induced epilepsy model until 21 days postinjury (DPI; B). TBI-induced epilepsy model showed upregulation of phospho–protein kinase B (pAKT) at S473 (C), and phosphotau at S396 (E). However, phospho–extracellular signal-regulated kinase (pERK; T202, T204) did not show significant change after TBI (D). *$P < .05$, **$P < .01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

The latencies to reach stage 3 or 4 seizures at 7 DPI were significantly longer in controls (750 ± 86.6 seconds) than in the G1 (423 ± 46.2 seconds) and G2 groups (258 ± 49.0 seconds; Figure 5E,F). The latency to stage 4 seizures was consistently shorter in the G2 versus G1 group: 450 ± 69.9 seconds (G1) versus 217 ± 41.2 seconds (G2) at 14 DPI and 506 ± 69.4 seconds (G1) versus 298 ± 45.2 seconds (G2) at 21 DPI. Seizure latency was no longer significantly different between the G1 group and controls after 21 DPI, but remained lower in the G2 group at this timepoint ($P < .001$). Latencies to reach stage 5 seizures were also lower for the G2 group compared with G1 at all timepoints: 510 ± 0 seconds at 7 DPI, 465 ± 30.8 seconds at 14 DPI, and 610 ± 115.3 seconds at 21 DPI for G1, versus 245 ± 72.4 seconds at 7 DPI, 367 ± 64.8 seconds at 14 DPI, and 522.5 ± 163.4 seconds at 21 DPI for G2. Because no naive animals reached stage 5, the maximum monitoring period of 900 seconds of seizure latency was used for this group for statistical purposes.

3.6 | Electrographic seizures

Electrophysiologic recordings were performed to quantify the number of IEDs and the duration of ictal activity in the various groups (Figure 2). An IED was defined as a sharply contoured discharge with an amplitude at least three times greater than baseline, lasting <3 seconds. This activity was considered ictal if it lasted >3 seconds. Four of 11 naive animals exhibited IEDs; none had ictal activity. The proportion of zebrafish with IEDs or seizures was greater in the G2 versus naive group at all timepoints. A greater proportion of the G1group had electrographic seizures at 13 DPI compared to G2, but this decreased over time, with no seizures in the G1 group at 40 DPI versus ictal activity in 67% of the G2 fish at that timepoint. The G2 groups also showed an enduring increase in the number of spontaneous IEDs and in total seizure duration compared to other groups (Figure 2B,C).

3.7 | BBB breakdown

Early breakdown of the BBB following TBI is an indicator of injury severity and a potential epileptogenic factor.[10] BBB disruption was assessed only in the controls and G2, as this injury paradigm resulted in more severe behavioral and electrophysiological outcomes. After Evans blue injection, fluorescent microscopy showed well-delineated vascular structures in the control group, with low fluorescence in the interstitial areas (Figure 6A). The area of fluorescence in the brain parenchyma of the G2 was noticeably increased, and the contours of cerebral vessels were distinctly less evident. Quantification of fluorescence intensity revealed a 20-fold increase in the TBI group compared with controls.

3.8 | Altered protein expression after TBI

As TBI consequences were more severe in the G2 group, protein levels were only compared between this group and naive controls. The expression of HMGB1, a key initiator of neuroinflammation, was increased 1 day after TBI and was significantly upregulated compared to controls at 7, 14, and 21 DPI ($P = .030$, $P = .011$, and $P = .022$, respectively). Akt, which activates downstream mammalian target of rapamycin (mTOR) and has been linked to PTE, showed an increased ratio of phosphorylated (S473) versus total protein after TBI at 7, 14, and 21 DPI ($P = .050$, $P = .015$, and $P = .031$, respectively). The increased ratio of phosphorylated to total ERK expression was not statistically significant, although there was a trend at 14 DPI ($P = .167$). The microtubule-associated protein tau, which is also upregulated after rodent and human TBI and is linked to epileptogenesis, was hyperphosphorylated as compared to controls at 7, 14, and 21 DPI ($P = .027$, $P = .008$, and $P = 3E\text{-}04$, respectively).

4 | DISCUSSION

In this study, we report the generation and characterization of an adult zebrafish model of severe TBI resulting in PTE. The modification of our previously described pHIFU model of TBI resulted in more severe motor and behavioral deficits than found previously, and a higher mortality rate, demonstrating the pHIFU model can be adjusted to cause TBI of varying severities in adult zebrafish. Moreover, the outcomes in this study demonstrate the development of PTE, with spontaneous recurrent behavioral seizures, increased susceptibility to PTZ, and electrographic seizure activity. Finally, we demonstrated for the first time with this model BBB disruption, increased markers of inflammation, tau hyperphosphorylation, and alterations of the mTOR pathway. Collectively, these outcomes are similar to those described in rodent models and are of relevance to human TBI and PTE.[11,12]

We induced brain injuries using pHIFU with two sets of parameters (G1 and G2). Because the injury procedure is noninvasive, naive zebrafish were used as controls. The mortality rate and righting reflex time were indirect indicators of injury severity.[6] The G2 group had a higher mortality rate than the G1 group, consistent with reports from other animal studies of severe TBI.[13] Fish in the G2 group also had a slower righting reflex than the G1 group. According to our observed mortality rate, physiological alterations, and neurological impairments, the injury parameters in the G2 group produce a model of severe TBI in zebrafish with outcomes comparable to severe TBI in rodents.[13]

TBI results in both primary and secondary injuries to the brain. Primary injury is a result of the mechanical force at the time of impact and is irreversible. Secondary injury occurs as

a result of ionic, molecular, and cellular alterations occurring minutes to weeks after impact, leading to further damage and neurological deficits.[14] The collective trend of greater and sustained behavioral deficits and increased epileptic activity and susceptibility to seizures in the G2 group are highly suggestive of a severe injury. One particular area of focus was the BBB, which functions to partition the brain from circulating blood and confers an "immune privileged" state in the healthy brain. BBB disruption occurs acutely in more severe forms of TBI, and can still be seen 7 DPI in experimental rodent models.[15] BBB impairment leads to the recruitment and extravasation of circulating leukocytes and monocytes, activation of endogenous astrocytes and microglia, macrophage-induced secondary injury, and brain swelling with increased intracranial pressure and ischemia.[16] BBB disruption is also thought to play a role in epilepsy,[17] and has been described both after acute seizures[18,19] and during the latent and chronic epileptic periods of a temporal lobe epilepsy model.[17,20] Focal opening of the BBB can also lead to the generation of an epileptic focus.[21] The zebrafish central nervous system contains a BBB similar to rodents.[22] However, to our knowledge, BBB disruption has not been reported in other zebrafish models of TBI. We found BBB disruption occurs acutely after pHIFU-induced TBI, supporting the use of this zebrafish model to further explore the mechanistic relationship with PTE development.

Neuroinflammation after TBI starts shortly after impact and can last many years after injury.[23] Although neuroinflammation is mounted as a protective response, it can also have detrimental effects. Inhibition of various proinflammatory cytokines after experimental TBI can reduce contusion volume and neurodegeneration, and prevent motor and cognitive deficits.[24–27] In addition, concomitant extracranial trauma in the presence of TBI has been found to worsen neurological outcome,[28] which may be related to a significantly increased neuroinflammatory response.[29] Inflammatory mediators are also implicated in epileptogenesis.[30] One example is HMGB1, which is released from neurons and glia and can both initiate and amplify the neuroinflammatory response. There is evidence HMGB1 promotes seizure activity,[31] and HMGB1 inhibition has neuroprotective effects in chemically induced seizure models.[32] HMGB1 has been investigated in zebrafish in regard to its role in spinal cord injury,[33] but it has not been studied in the context of TBI or epilepsy. The upregulation of HMGB1 after pHIFU injury is further evidence this zebrafish model leads to secondary injuries similar to those occurring in humans and rodents after TBI.

In addition to inflammatory changes, there are a variety of other molecular alterations after TBI that have been implicated in the development of PTE. The mTOR signaling pathway, a regulator of cell metabolism, proliferation, and differentiation, has also been implicated in both TBI recovery[34] and seizures.[35] Surgically resected tissue samples from patients with temporal lobe epilepsy show increased expression of phosphorylated proteins mTOR, s6, and Akt, consistent with mTOR overactivity.[36] mTOR inhibition supports a causative role of this pathway in altered seizure susceptibility.[37] In rodent studies of PTE, the mTOR inhibitor, rapamycin, has demonstrated antiepileptogenic effects.[34] Although mTOR signaling alterations have been shown in various zebrafish models of seizures,[38–40] they have not been investigated in zebrafish TBI or PTE. Similar to rodent studies, we describe increased mTOR activity as evidenced by an increased ratio of phosphorylated/total Akt after pHIFU in zebrafish. We did not find significant changes in ERK phosphorylation after TBI, possibly due to negative regulation of mTOR and mitogen-activated protein kinase pathways on each other, whereby phosphorylation of Akt can inhibit ERK activation.[41]

We also studied tau proteins, which are abundant in the central nervous system and act to stabilize microtubules. In pathological conditions, tau can become hyperphosphorylated, resulting in microtubule destabilization and dysfunction of neurons and glia. Tauopathies contribute to disability after TBI, most notably in chronic traumatic encephalopathy.[42] Animal studies replicate the finding of tau aggregation and hyperphosphorylation after TBI.[43] Elevated tau levels have also been found in various epileptogenic lesions in patients with epilepsy.[44,45] Pharmacological manipulation with sodium selenate to prevent increases in tau hyperphosphorylation leads to reduced seizure frequency and duration in a rodent model of TBI.[43] Tauopathies are being increasingly studied in zebrafish, with novel transgenic lines expressing human tau in neurons.[46] However, there have been no experiments studying the link between tau and seizures in zebrafish. Together, the molecular changes we have found after severe TBI in the zebrafish indicate this model induces secondary damage similar to widely used rodent models and relevant to mechanisms of epileptogenesis.

Zebrafish possess a greater and more rapid capacity for regeneration than mammals.[47] This unique feature of zebrafish may have enabled the rapid onset of epileptogenesis after TBI in our study. Unlike other animal models, where it takes several months for epilepsy to develop after TBI, 80% of our TBI zebrafish exhibited generalized tonic-clonic seizures after 14 DPI.[3,14,48] We confirmed that this rate was maintained until 21 DPI. At 14 DPI, the mean swimming velocity of the injured zebrafish began to decrease after a previous return to normal levels, coinciding with reduced exploratory behavior. Therefore, the development of epilepsy after TBI in the zebrafish appears to occur in parallel with motor recovery. Furthermore, the number of animals that showed stage 4 and stage 5 seizures during the PTZ susceptibility test was also the highest at this timepoint. TBI zebrafish also exhibit a high rate of spontaneous behavioral seizures, as indicated by the spontaneous seizures detected during the 30-minute recording period on

each recording day, and 15 minutes of electrophysiological recording. No naive zebrafish exhibited ictal activities, although four of 11 in the naive group showed IEDs. Previous studies have reported that anesthetized naive zebrafish do not exhibit any abnormal activities,[5] suggesting the IEDs noted in naive zebrafish in this study may have been caused by the craniotomy for electrode insertion. The number of IEDs at 40 DPI was similar to controls. Despite the low number of recorded IEDs at 40 DPI, there were seizures of significantly longer duration at this period. By contrast, rodent models have fewer than one seizure/d.[48] Although this model exhibits a significantly higher rate of seizures than other models, the electrographic results revealed that other characteristics such as the seizure duration corresponded to rodent PTE studies.[48,49] Further analysis of this model, such as long-term multichannel recording, may provide possible electrographic biomarkers for PTE epileptogenesis as previously attempted in other studies.[5,49,50]

In conclusion, we present a novel model of severe closed-head TBI in zebrafish with similar characteristics and consequences to those of rodent TBI models, opening the way for the study of PTE in a nonmammalian species. The rapid onset and high rate of seizures in this pHIFU-induced TBI zebrafish model demonstrate a high potential for higher throughput and cost-efficiency compared to existing PTE models, which could help advance the field at a significantly faster pace.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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