Induction of oxidative stress and apoptosis in the injured brain: potential relevance to brain regeneration in zebrafish

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Abstract
Recent findings suggest a significant role of the brain-derived neurotrophic factor (BDNF) as a mediator of brain regeneration following a stab injury in zebrafish. Since BDNF has been implicated in many physiological processes, we hypothesized that these processes are affected by brain injury in zebrafish. Hence, we examined the impact of stab injury on oxidative stress and apoptosis in the adult zebrafish brain. Stab wound injury (SWI) was induced in the right telencephalic hemisphere of the adult zebrafish brain and examined at different time points. The biochemical variables of oxidative stress insult and transcript levels of antioxidant genes were assessed to reflect upon the oxidative stress levels in the brain. Immunohistochemistry was performed to detect the levels of early apoptotic marker protein cleaved caspase-3, and the transcript levels of pro-apoptotic and anti-apoptotic genes were examined to determine the effect of SWI on apoptosis. The activity of antioxidant enzymes, the level of lipid peroxidation (LPO) and reduced glutathione (GSH) were significantly increased in the injured fish brain. SWI also enhanced the expression of cleaved caspase-3 protein and apoptosis-related gene transcripts. Our results indicate induction of oxidative stress and apoptosis in the telencephalon of adult zebrafish brain by SWI. These findings contribute to the overall understanding of the pathophysiology of traumatic brain injury and adult neurogenesis in the zebrafish model and raise new questions about the compensatory physiological mechanisms in response to traumatic brain injury in the adult zebrafish brain.

Keywords Adult neurogenesis · Brain regeneration · Oxidative stress · Apoptosis · Zebrafish

Introduction
In the past three decades, the teleost zebrafish has emerged as a simple vertebrate model to investigate brain development, normal brain physiology, and the mechanisms of neurological diseases. Both larval and adult zebrafish serve as excellent model organisms to understand normal brain functions, complex brain disorders, and their genetic and pharmacological modulation [1, 2]. At the gene level, the nucleotide sequences of zebrafish genes share at least 70% homology with their counterparts in humans and other mammalian species. Additionally, 84% of the disease-causing genes in humans are known to have their counterparts in zebrafish. Moreover, the zebrafish brain shares a high degree of homology with human brain in terms of genetic architecture, neuroanatomy, neurochemistry and physiology, as a whole [3]. But, one of the most distinguishing features of zebrafish is its extraordinary ability to regenerate its brain after an injury, which is in contrast to the adult mammalian brains with relatively poor regenerative ability [4–8]. The exact reason behind this advanced brain regenerative ability in zebrafish is still unknown. Thus, a comprehensive understanding of the cellular and molecular mechanisms orchestrating adult neurogenesis and brain regeneration in zebrafish can aid in solving this long-standing enigma.

Brain regeneration has been extensively studied in zebrafish in the last decade. As reported in a study, an injury-induced experimentally into the dorsolateral telencephalon of zebrafish was found to be almost completely healed about 35 days after the injury. Besides, accumulation of the Hu positive neurons near the injury site indicated that the damaged neurons were replaced by the new ones [8]. Concomitantly, injury induction in the mediolateral dorsal telencephalon also results in a spectacular increase in cell
proliferation in the ventricular zone and in the area adjacent to the injury sites [8, 9]. Several studies have reported that the peak proliferative response after an injury is observed around 3 to 7 days post-injury [8–10]. Nonetheless, the increased proliferation triggered by an injury or lesion to the brain was found to be a temporary event as it starts to decline after a particular period until it comes back to the baseline level [8, 9]. Interestingly, a recent report suggests that this surge in proliferation is preceded by a rise in the level of brain-derived neurotrophic factor (BDNF) expression [10]. Supporting this finding, our previous studies also observed that antagonizing the TrkB receptor (the concomitant receptor of BDNF protein) reversed the rise in injury induced cell proliferation [9]. BDNF has been reported to affect the proliferation of adult neural progenitors in zebrafish, which clearly indicates the pivotal role played by BDNF during reparative neurogenesis in the injured zebrafish brain. However, the influence of this injury-induced increase in BDNF expression on physiological processes other than neurogenesis are yet to be investigated.

Mounting evidences have suggested that oxidative stress and apoptosis are two crucial biological processes that are affected by the activity of BDNF [11–13]. Thus, it can be hypothesized that increased expression of BDNF might be modulating oxidative stress and apoptosis in the injured zebrafish brain as well. However, this hypothesis has not been tested extensively in the adult zebrafish. In order to test this hypothesis, it is a crucial prerequisite to confirm whether stab wound injury affects oxidative stress and apoptosis in zebrafish brain, the direct evidence for which is lacking. Therefore, the objective of the present study was to examine the potential modulation of oxidative stress and apoptosis induction by stab injury in the adult zebrafish brain. We investigated the injury-induced changes in the oxidative stress parameters, the expression of apoptosis and oxidative stress-related genes in the telencephalon of the adult zebrafish brain. Our findings add to the current understanding of the pathophysiology of brain injury in the adult zebrafish model. Additionally, this study also serves as a pilot study to investigate the cross-talks between BDNF, oxidative stress, and apoptosis in the context of traumatic brain injury.

Materials and methods

Ethics statement

Animal handling and experimental protocols complied with the rules and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Additionally, all the experimental protocols were scrutinized and ratified by the Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru University. All efforts were made to minimize animal suffering, and the number of animals in each experiment was kept at a bare minimum.

Experimental animals

The animal house facility at the Institute of Genomics and Integrated Biology (IGIB), New Delhi, India, provided 200 wild-type ASWT zebrafish. The zebrafish were kept under regulated temperature (26 to 28 °C) and pH (7) conditions with a 12:12 h Light: Dark cycle. The fish were fed twice a day (9:00 am and 7 pm) with brine shrimp plus flakes (Ocean Nutrition). 100 morphologically identical fish of uniform length (3 ± 0.5 cm) were selected for the experiments. The experimental fish were selected at random and comprised of both male and female adult zebrafish.

Stab wound injury

The stab wound injury was induced as previously described [9, 14]. Fish were anesthetized with 0.02% (w/v) tricaine (Ethyl-3-aminobenzoate, Sigma Aldrich) solution, followed by stabilizing them by placing them in a raised cleft on a pile of tricaine soaked tissue paper. Under the visual aid of a dissecting microscope (1X magnification), a 26-gauge needle was inserted vertically in the center of the right telencephalic hemisphere up to a depth of 1–1.5 mm. The injured fish were released into the normal water to allow recovery. Around 90–95% of the injured fish recovered from the injury trauma and survived. These fish were used for further experimentation.

Estimation of oxidative stress

Biochemical assays were performed to evaluate the changes in oxidative stress parameters in the telencephalon of adult zebrafish brain after stab injury, specifically the activity of antioxidant enzymes [Superoxide dismutase (SOD) and Catalase], lipid peroxidation (LPO) and reduced glutathione (GSH) levels. All the biochemical assays were performed in biological triplicates.

Tissue homogenization and PMS preparation

5% tissue homogenate was prepared by homogenizing the telencephalons of 15 zebrafish brains on ice in 0.1 M Phosphate buffer saline (pH 7.4) with the help of a micropestle. The homogenate was subjected to centrifugation at 15,000 rpm for 20 min at 4 °C to obtain 5% post-mitochondrial supernatant (PMS). Protein estimation was done using Bradford’s assay [15].
SOD activity

SOD activity was determined using a method described by Marklund and Marklund in 1974 [16]. 10 µl of 5% PMS was mixed with 2 µl of 12.5% Triton-X-100 to obtain a pre-mixture which was incubated at 4 °C for 30 min. 10 µl of the pre-mixture was added to an assay mixture comprising 500 µl of 0.1 M phosphate buffer, 397 µl of double distilled water (DDW), 33 µl of 3 mM EDTA (pH 8), and 60 µl of pyrogallol (1,2,3-trihydroxy benzene) solution. The absorbance of the resulting mixture was kinetically measured at 420 nm with the help of a UV spectrophotometer (Cary 60 UV–Vis, Agilent Technologies). The SOD activity was calculated using the molar extinction coefficient (ε) 800 × 10³ M⁻¹ cm⁻¹ and expressed as mmol of pyrogallol protected from oxidation per minute per milligram protein.

Catalase activity

Evaluation of catalase activity was based on the method described by Fatima et al. [17, 18], a modified version of the technique given by Claiborne in 1985 [19]. 500 µl of 0.05 M hydrogen peroxide (H₂O₂) was dissolved in a mixture containing 975 µl of 0.1 M phosphate buffer (pH 7.4) and 25 µl of 5% PMS to yield the reaction mixture. Absorbance was measured kinetically at 240 nm. The catalase activity was calculated using the extinction coefficient (ε) 39.6 M⁻¹ cm⁻¹ and expressed as mmol of H₂O₂ consumed per minute per milligram protein in each sample.

Estimation of lipid peroxidation (LPO)

LPO estimation was performed using the procedure of Srivastav et al. in 2020 [20], a modification of the method devised by Mihara and Uchiyama in 1978 [21]. The reaction mixture was composed of 160 µl of 5% PMS, 10 µl of 10 mM butylated hydroxytoluene (BHT), 350 µl of 0.67% thiobarbituric acid (TBA) and 1 ml of 1% orthophosphoric acid (OPA). The reaction mixture was kept at 95 °C for 1 h. This was followed by absorbance measurement at 535 nm. LPO was calculated using the molar extinction coefficient (ε) 1.56 × 10⁴ M⁻¹ cm⁻¹ and expressed as µmoles of thiobarbituric acid reactive substance (TBARS) formed per hour per gram tissue in each sample.

Estimation of reduced glutathione (GSH) levels

Reduced GSH levels in the zebrafish brain tissue were evaluated following the method of Fatima et al. in 2020 [18], a modified procedure developed by Jollow et al., 1974 [22]. 5% PMS and 4% sulphosalicylic acid (SSA) were mixed in equal proportion (1:1) and kept at 4 °C for 1 h. The resulting solution was centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was collected in a separate tube, and 200 µl of the supernatant was added to 1.1 ml of 0.1 M phosphate buffer (pH 7.4). This was followed by the addition of 200 µl of 10 mM DTNB. Absorbance was measured at 412 nm. GSH levels were calculated using the molar extinction coefficient (ε) 1.36 × 10⁴ M⁻¹ cm⁻¹ and expressed as mmol of GSH per gram tissue.

Real time polymerase chain reaction (RT-PCR)

RT-PCR was performed according to a previous protocol [23], with minor modifications. After injury induction at zero time point [0 days post-injury (DPI)], the injured fish were re-anesthetized and euthanized by placing in ice-cold water (0–4 °C) at two different times: 1DPI and 4DPI. Brain telencephalons were removed quickly on ice and transferred to the lysis buffer (GeneJET RNA purification kit, Thermo Fischer Scientific, USA). For each reaction, a total of 10 zebrafish brain telencephalons were pooled, and total RNA was isolated using the GeneJET RNA purification kit (Thermo Fischer Scientific, USA) according to the manufacturer’s protocol. The amount of total RNA in each sample was measured using Nanodrop 2000 in a spectrophotometer (Thermo Scientific, USA). According to the manufacturer’s protocol, cDNA was synthesized using 20 ng of total RNA with the help of Verso cDNA synthesis kit (Thermo Fischer Scientific, USA). RT-PCR was run using a thermocycler coupled to the MyiQ detector (Bio-Rad, USA) with the help of PowerUp™ SYBR® Green master mix (Applied Biosystems, USA). Three biological replicates, each having three technical replicates, were run. The target genes were investigated in this study include bdnf, trkB, pcna, bcl2, bax, caspase 3, caspase 9, sod2, catalase, hsp70 and hsp90. The primer sequences of the target genes were taken from published literature and have been listed in the table S1. β-actin and gapdh were used as standard to normalize the expression levels of the target genes. The relative mRNA expression levels were determined by 2−ΔΔCT analysis.

Immunohistochemistry (IHC)

IHC was performed as previously described [9, 14]. Fish were euthanized at different time points (0DPI, 1DPI and 4DPI), and entire heads were chopped off. The heads were fixed in 10% formaldehyde solution overnight at 4 °C. Zebrafish heads were dissected to isolate the brains. The brains were fixed in 100% methanol overnight at −20°C. Methanol-infused brains were rehydrated by passing through different grades of methanol (75%, 50%, 25%, 0%) that were prepared by diluting 100% methanol with PTW (1X PBS+0.1% Tween20). The brains were embedded in 2% agarose, and coronal sections (30 µm thickness) were cut using a vibratome (Leica VT-1200S). The coronal brain sections were rehydrated in PBS+0.1% Tween20. The brains were embedded in 2% agarose, and coronal sections (30 µm thickness) were cut using a vibratome (Leica VT-1200S). The coronal brain sections were rehydrated in PBS+0.1% Tween20.
were blocked for 1 h at room temperature in blocking buffer [0.1% (v/v) 10% tween 20, 0.2% (w/v) Bovine serum albumin (BSA), 1% (v/v) Dimethyl sulphoxide (DMSO) in 1X PBS]. After this, sections were kept in primary antibody [ab13847, Anti-Caspase3 antibody, Abcam, USA] overnight at 4 °C. This was followed by washing the sections in PTW three times (10 min per wash) and incubation in secondary antibody [ab150077, Goat polyclonal secondary antibody to Rabbit IgG—H&L (1/1000, Alexa Fluor® 488 conjugated, Abcam, USA)] for 2 h at room temperature. The brain sections were washed and counter-stained with DAPI (incubation for 30 min at room temperature). Following this, the sections were mounted on rectangular glass slides and visualized under a fluorescent microscope (Eclipse Ti-E, Nikon) at 10X and 20X magnifications.

Statistical analysis

Statistical analysis was performed with the help of the Sigma Plot 14.0 software. One-way ANOVA, followed by post hoc test Holm-Sidak was applied to evaluate the statistical significance between the different experimental groups. The data is presented here as mean ± standard error of the mean (SEM). The significance value was set at p < 0.05.

Result

The mRNA levels of both the bdnf and trkb genes increase after stab wound injury

In order to validate the animal model and experimental procedures, previously reported study findings pertaining to the expression of bdnf and related genes in stab wound injury condition were replicated. The expression of pcna, bdnf and trkb genes in the telencephalon of injured adult zebrafish brain at the mRNA level was determined by qRT-PCR. In agreement with previous findings [10], the mRNA level of pcna gradually increased after stab wound injury for a temporary period of time and with a peak expression at 4DPI (Fig. 1a). The mRNA level of bdnf and trkb also increased after injury, though in a pattern different from expression of pcna. The expression of both bdnf and trkb was found to be increased 1DPI, but then started declining around 4DPI (Fig. 1b, c). These results indicate a temporal separation between the activation of the BDNF/TrkB signaling pathway and the proliferation response after stab wound injury.

Effect of stab wound injury on the biomarkers of oxidative stress

After stab wound injury in the telencephalon of the adult zebrafish brain, the oxidative stress was found to be increased as indicated by changes in various oxidative stress related parameters. The activity of antioxidant enzymes, SOD (Fig. 2a) and catalase (Fig. 2b) significantly increased after the injury. While the SOD activity was found to be elevated at both 1DPI and 4DPI (Fig. 2a), catalase activity increased only at 1DPI and no significant change in the catalase activity was detected at the stage of 4DPI (Fig. 2b). Stab wound injury also significantly increased lipid peroxidation (Fig. 2c) and reduced glutathione levels (Fig. 2d) at both 1DPI and 4DPI stages, compared to 0DPI. These results indicate enhanced oxidative stress in the injured zebrafish brain.

Heat shock protein 90 (hsp90) responds to brain injury with increased mRNA expression

We analyzed the mRNA levels of four target genes implicated in oxidative stress, namely superoxide dismutase 2 (sod2), catalase (cat), hsp70, and hsp90 in the telencephalon part of the injured zebrafish brain. No change in the mRNA expression of sod2 and cat was detected in the injured fish

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![Fig. 1](image-url)  
Enhancement in proliferation and BDNF/TrkB signaling after stab injury. qRT-PCR results showing the changes in the mRNA levels of pcna (a), bdnf (b) and trkb (c) genes in the telencephalon of injured adult zebrafish brain. ***=P≤0.001, **=0.001 < P < 0.01, *=0.01 < P < 0.05
brain (Fig. 3a, b). In case of hsp70, although the mRNA level increased in the injured fish at both 1DPI and 4DPI stages as compared to the non-injured fish, the difference was found to be statistically non-significant (Fig. 3c). However, a statistically significant increase in the mRNA level of the hsp90 gene was observed in the injured fish brain at both 1DPI and 4DPI stages (Fig. 3d), which suggests an activation of hsp90 in response to stab injury in the brain.

Changes in apoptotic variables induced by stab injury

In this study, we analyzed the expression of apoptotic marker cleaved caspase-3 at protein levels in the injured zebrafish brain through IHC. We also evaluated the expression of an anti-apoptotic gene (bcl2) and two pro-apoptotic genes (bax and caspase9) after injury in the telencephalon of the adult zebrafish brain at the mRNA level by qRT-PCR. Results from IHC studies indicated that cleaved caspase-3 protein levels were enhanced in a subset of cells surrounding the damaged brain tissue at the stage of 1DPI (Fig. 4C-C'). However, no detectable changes were observed in the cleaved caspase-3 protein levels at the stage of 4DPI (Fig. 4D-D'). A significant increase in the mRNA levels of bcl2 and bax was also observed in the injured fish brain at 1DPI and 4DPI stages (Fig. 5a, b), but no change was observed in the mRNA level of caspase9 after the stab injury (Fig. 5c).

Discussion

The present study was undertaken to investigate the effect of stab wound injury on oxidative stress and apoptosis in the adult zebrafish brain. Oxidative stress is known to have fatal impacts on the brain after traumatic brain injury [24]. Several studies have investigated the oxidative stress status in the rodent brain after injury. Oxidative stress has been associated with secondary pathophysiological changes after trauma in the brain [25]. Traumatic brain injury is related to the generation of oxidants, leading to neuronal dysfunction and death [26]. Brain injury is immediately followed by the generation of superoxide that initiates the oxidation cascade [27]. Superoxide dismutase (SOD) rapidly breaks down the superoxide into oxygen and hydrogen peroxide. The resulting oxidant, hydrogen peroxide, can propagate oxidative damage in the brain by interacting with the nearby biological substrates [28]. Lipid peroxidation, induced by hydroxyl free radical, constitutes a primary mechanism of cellular damage in case of traumatic brain injury [28]. The traumatic brain injury-induced oxidative stress has also been correlated to...
blood flow (perfusion) and oxygenation of the neural tissue [29, 30]. Oxidative stress, along with other secondary injury mechanisms such as neuroinflammation and disruption in the blood–brain barrier, may exacerbate brain injury [28, 31]. Cumulatively, increased production of oxidants after traumatic brain injury is associated with further damage to the brain parenchyma with propagation of injury induced inflammation and loss of functional neurons [29, 32].

Unlike the rodent models, the knowledge about the relationship between the brain injury and oxidative stress in the adult zebrafish model is scarce. In this study, we attempted to provide direct evidence for injury-induced oxidative stress in the telencephalon of adult zebrafish brain by assessing the biochemical parameters of oxidative stress. We observed increased activity of antioxidant enzymes (SOD and catalase) and the increased levels of LPO and GSH after brain injury, indicating the disruption of oxidative balance in the injured zebrafish brain. Interestingly, recent studies in rodent brains have also reported injury-induced oxidative stress but with decreased activity of SOD and catalase enzymes [33, 34]. Similarly, the increased GSH levels in the injured zebrafish brain observed in this study disagree with the decreased GSH/GSSG ratio in the injured mouse brain [33]. However, changes in the level of LPO in response to injury in zebrafish brain was found to be in agreement with the changes reported in injured rodent brains [33, 34]. The reasons underlying this disparity in the activity of antioxidant enzymes and GSH levels in response to brain injury in zebrafish and rodents cannot be speculated at this point and thus require further investigation to clarify. In hindsight, it appears that the brain injury-induced oxidative stress is more damaging in rodents (indicated by reduced SOD levels, catalase activity and GSH/GSSG ratio) as compared to zebrafish. It could also be an effect of procedural differences in zebrafish, as compared to rodents. For instance, it is hard to contemplate the severity of injury induction in different species. Alternatively, zebrafish may possess more robust protective mechanisms against brain injury-induced oxidative stress as compared to rodents. For instance, enhanced BDNF expression in the injured zebrafish brain [10] may result in anti-oxidative effects [12, 13]. However, the same cannot be speculated for the rodent models mainly because of the contradicting notions about the expression and role of BDNF in the context of brain injury in rodents and zebrafish [35]. Nevertheless, our results indicate induction of oxidative stress by stab injury in the telencephalon of the adult zebrafish brain.

Although the activity of SOD and catalase enzymes increased after stab injury, the expression of sod2, and catalase genes remained unaffected as no change was observed in the transcript levels of these genes after injury. However, the mRNA level of another well-known antioxidant gene,
hsp90, increased significantly in the injured zebrafish brain. Similar to hsp90, the mRNA levels of hsp70 also seemed to increase after injury, but the change in its expression was not statistically significant. Given the fact that hsp90 is fundamentally a stress protein, implicated in many kinds of physiological and environmental stresses such as heat shock [36], hypoxia [37], infection [38], inflammation [39], etc., it could not be determined precisely whether its upregulation

Fig. 5 Induction of the apoptotic genes after stab injury. qRT-PCR results showing the changes in the mRNA levels of bcl2 (a), bax (b) and caspase9 (c) genes in the telencephalon of injured adult zebrafish brain. *** = P ≤ 0.001, ** = 0.001 < P < 0.01, * = 0.01 < P < 0.05
is indicative of oxidative stress or inflammation or any other physiological response to stab injury. Nonetheless, the increased expression of hsp90 suggests a compensatory mechanism in response to some physiological stress caused due to stab injury in the telencephalon of the adult zebrafish brain.

Apoptosis is an integral part of the pathophysiology of traumatic brain injury [40]. Speculations are that apoptosis after traumatic brain injury may serve a protective role. It helps the brain get rid of the damaged cells without affecting the surrounding brain tissue [41]. Traumatic brain injury in rodents has been observed to cause an imbalance in the pro-apoptotic and anti-apoptotic gene expression in the brain tissue [40, 41]. However, how these genes respond to traumatic brain injury in adult zebrafish brain is not clear.

From this study, it was observed that the expression of cleaved caspase-3 protein, an indicator of apoptosis, increased in the vicinity of the brain tissue in the telencephalon of injured zebrafish brain. Caspase 3 protein is cleaved and activated during the onset of apoptosis. This could be the reason for detecting cleaved caspase-3 positive cells at an earlier stage of post-injury (1DPI) but not at a later stage (4DPI). Similar results were also obtained in a previous study [42]. The detection of cleaved caspase-3 indicates induction of apoptosis by stab wound injury in the adult zebrafish brain. Brain tissue sample taken from the telencephalon region showed enhanced transcript levels of the pro-apoptotic gene bax, which provides additional evidence for the induction of apoptosis in the injured zebrafish brain.

Interestingly, the mRNA levels of the anti-apoptotic gene bcl2 also increased after stab injury. A previous study in rats subjected to “unilateral experimentally controlled cortical contusion” has also documented the upregulation of both bax and bcl2 gene [43]. The enhanced expression of bcl2 gene may partly serve as a compensatory mechanism for preventing the secondary injury damage caused by the induction of apoptosis. Alternatively, the upregulation of both the anti-apoptotic and pro-apoptotic genes may also be perceived as a sign of vigilant and intricate regulation of apoptosis in the injured zebrafish brain in order to maintain a cellular homeostasis by eliminating the damaged cells without any effect on the intact cells. Further investigations should be focused on the precise role and significance of apoptosis in the context of brain injury.

Conclusion

To conclude, this study demonstrated that stab wound injury induces oxidative stress and apoptosis in the adult zebrafish brain. Our findings serve as a preliminary basis for investigating the potential link between the upregulation of BDNF; and oxidative stress and induction of apoptosis in the injured zebrafish brain. However, whether oxidative stress and apoptosis contribute to the secondary brain injury (similar to mammals) or play a protective role during post-traumatic pathophysiology remains a subject for future investigations. Further, we emphasize the need to investigate other cellular and molecular processes influenced by BDNF in order to fully understand and quantify the precise role and significance of BDNF signaling in the regeneration of adult zebrafish brain. This study has the potential to enhance our current understanding of adult neurogenesis, raise new questions about the compensatory physiological mechanisms in response to traumatic brain injury and may even take us one step closer to realizing regeneration-based therapies for neurodegenerative diseases and traumatic brain injury in humans.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06506-7.

Authors' contributions ACM conceptualized the study, designed the experiments. SKA and MRS performed the experiments. The manuscript was written by SKA and edited by ACM.

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Data availability Data and materials supporting this study will be made available on request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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