Induction of Oxidative Stress and Apoptosis in the Injured Brain: Potential Relevance to Brain Regeneration in Zebrafish.

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Research Article

Keywords: Adult neurogenesis, Brain regeneration, Oxidative stress, Apoptosis, Zebrafish

Posted Date: May 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-549302/v1

Abstract

In the recent years, zebrafish, owing to its tremendous adult neurogenic capacity, has emerged as a useful vertebrate model to study brain regeneration. Recent findings suggest a significant role of the BDNF/TrkB signaling as a mediator of brain regeneration following a stab injury in the adult zebrafish brain. Since BDNF has been implicated in a plethora of physiological processes, we hypothesized that these processes are affected in the injured zebrafish brain. In this small study, we examined the indicators of oxidative stress and of apoptosis using biochemical assays, RT-PCR and IHC to reflect upon the impact of stab injury on oxidative stress levels and apoptosis in the injured adult zebafish brain. Our results indicate induction of oxidative stress in the injured adult zebrafish brain. Also, apoptosis was induced in the injured brain as indicated by increased protein levels of cleaved caspase3 as well as enhanced mRNA levels of both pro-apoptotic and anti-apoptotic genes. This knowledge contributes to the overall understanding of adult neurogenesis in the zebrafish model and raises new questions pertaining to the compensatory physiological mechanisms in response to traumatic brain injury in the adult zebrafish brain.

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 for understanding the normal brain functions, complex brain disorders and their genetic and pharmacological modulation. The zebrafish brain has high homology to humans in terms of genetics, physiology, anatomy and neurochemistry (Kalueff et al. 2014). At the gene level, the nucleotide sequences of zebrafish genes share at least 70% homology with their counterparts in humans and other mammalian species (Kalueff et al. 2014). Additionally, 84% of the disease causing genes in humans are known to have their counterparts in zebrafish (Kalueff et al. 2014). One of the most distinguishing features of zebrafish is its extraordinary ability to regenerate its brain after an injury (Kizil et al. 2012; Kroehne et al. 2011; Gemberling et al. 2013; Anand and Mondal 2017; Kishimoto et al. 2012). In contrast to adult mammals, which have a relatively poor regenerative ability, adult zebrafish can repair an injury in almost any part of the brain. The reason for this disparity in the brain regenerative ability is not clear. Also, a comprehensive understanding of the cellular and molecular mechanisms orchestrating adult neurogenesis and brain regeneration in zebrafish is desired.

Brain regeneration has been extensively studied in zebrafish in the last decade. An injury induced experimentally into the dorso-lateral telencephalon of zebrafish is virtually completely healed about 35 days after the injury (Kishimoto et al. 2012). Besides, the Hu positive neurons accumulate near the injury site indicating that the damaged neurons were replaced by the new ones (Kishimoto et al. 2012). The injury induction in the medio-lateral dorsal telencephalon results in a spectacular increase in cell proliferation in the ventricular zone and in the area adjacent to the injury sites (Anand and Mondal 2018; Kishimoto et al. 2012). The proliferation triggered by an injury or lesion to the brain increases only temporarily and starts to decline after a particular time period until it comes back to the baseline level (Anand and Mondal 2018; Kishimoto et al. 2012).

Several studies have reported that the peak proliferative response after injury is observed around 3 to 7 days post injury (Cacialli et al. 2018; Anand and Mondal 2018; Kishimoto et al. 2012). A recent report suggests that this surge in proliferation is preceded by rise in the level of brain derived neurotrophic factor (BDNF) mRNA (Cacialli et al. 2018). Another report suggests that on antagonizing the TrkB receptor (the concomitant receptor of BDNF protein), the rise in proliferation after injury is somewhat reversed (Anand and Mondal 2018). These results emphasize a pivotal role of BDNF during reparative neurogenesis in the injured zebrafish brain. One impact that the increased levels of BDNF seems to have in the injured brain is the enhanced proliferation of neural progenitors. But in the said scenario, what exactly happens to other physiological phenomena that are directly or indirectly influenced by BDNF is not known. Also, the exact mechanism by which BDNF influences adult neurogenesis is unknown.

In this short study, we have assessed the changes in the oxidative stress parameters and the expression of apoptosis and oxidative stress related genes in the injured adult zebrafish brain.

Materials And Methods

Ethics Statement

Animal handling and experimental protocols were in compliance with the rules and guidelines of 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 Institute of Genomics and Integrated Biology (IGIB), New Delhi, India provided wild type ASWT zebrafish. The zebrafish were kept under regulated temperature (26 to 28^{o}C) and pH (7–8) conditions with a 12:12 hour Light: Dark cycle. The fish were fed twice a day (9:00 am and 7 pm) with Brine Shrimp plus Flakes (Ocean Nutrition). Only 3–5 cm long morphologically identical fish were selected for the experiments. The experimental fish were selected at random and comprised of both male and female zebrafish.

Stab Wound Injury

The stab wound injury was induced as previously described (Anand and Mondal 2018; Schmidt et al. 2014). Fish were anaesthetized with 0.02% (w/v) tricaine (Ethyl-3-aminobenzoate, Sigma Aldrich) solution, followed by stabilizing them by placing them in a 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 centre of the right telencephalic hemisphere up to a depth of 1-1.5 mm. The injured fish were released into the normal fish 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 levels (GSH). 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 0.1M Phosphate buffer (pH 7.4) with the help of a micropestle. The homogenate was subjected to centrifugation at 4℃ and 15000 rpm for 20 minutes to obtain 5% Post-mitochondrial supernatant (PMS). Protein estimation was done using Bradford's assay (Bradford 1976).

SOD activity

SOD activity was determined using a method described by Ahmad et al. in 2018 (Ahmad et al. 2018), which is a modified version of the procedure developed by Marklund and Marklund in 1974 (Marklund and Marklund 1974). 10µl of 5% PMS was mixed with 2µl of 12.5% Triton-X-100 to obtain a pre-mixture which was incubated at 4℃ for 30 minutes. 10µl of the pre-mixture was added to an assay mixture comprising 500µl of 0.1M phosphate buffer, 397µl of double distilled water (DDW), 33µl of 3mM EDTA (pH 8) and 60µl of Pyrogallol (1,2,3-trihydroxy benzene) solution. Absorbance of the resulting mixture was kinetically measured at 420nm with the help of a UV spectrophotometer (Cary 60 UV-Vis, Agilent Technologies). The SOD activity was calculated using the molar extinction coefficient (□) 800 × 10^3 M^{-1} cm^{-1}, and expressed as mmoles 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 in 2020 (Fatima et al. 2020), which is a modified version of the method given by Claiborne in 1985 (Claiborne 1985). 500µl of 0.05M hydrogen peroxide (H_2O_2) was dissolved in a mixture containing 975µl of 0.1M phosphate buffer (pH 7.4) and 25µl of 5% PMS to yield the reaction mixture. Absorbance was measured kinetically at 240nm. The catalase activity was calculated using the extinction coefficient (□) 39.6 M^{-1} cm^{-1} and expressed as mmoles of H_2O_2 consumed per minute per milligram protein in each sample.

Estimation of Lipid Peroxidation (LPO)

LPO estimation was done using the procedure of Srivastav et al in 2020 (Srivastav et al. 2020) which is a modification of the method devised by Mihara and Uchiyama in 1978 (Uchiyama and Mihara 1978). The reaction mixture was composed of 160µl of 5% PMS, 10µl of 10mM butylated hydroxytoluene (BHT), 350µl of 0.67% thiobarbituric acid (TBA) and 1 ml of 1% orthophosphoric acid (OPA). The reaction mixture was kept at 95℃ for 1 hour. This was followed by Absorbance measurement at 535nm. LPO was calculated using the molar extinction coefficient (□) 1.56 × 10^5 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

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

Real time Polymerase chain reaction (RT-PCR)

RT-PCR was performed according to a published protocol (Srivastav et al. 2018), with minor modifications. After injury induction at zero time point (0 days post injury (DPI)), the injured fish were euthanized at two different time points: 1DPI and 4DPI. Anaesthetized zebrafish were euthanized by placing in ice cold water (0–4°C). 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 ten 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 Spectrophotometer (Thermo Scientific, USA). cDNA was synthesized using 20ng of total RNA with the help of Verso cDNA synthesis kit (Thermo Fischer Scientific, USA) according to the manufacturer's protocol. RT-PCR was run using a thermocycler coupled to the MyiQ detector (Bio-Rad, USA) with the help of PowerUp™ SYBR^R Green master mix (Applied Biosystems, USA). Three biological replicates, each having three technical replicates, were run. The target genes investigated in this study include *bdnf, trkb, pcna, bcl2, bax, caspase 3, caspase 9, sod2, catalase, hsp70 and hsp90.* The primer sequences of target genes have been listed in table 1. *β-actin and gapdh* were used to normalize the expression levels of target genes. The relative mRNA expression levels were determined by $2^{-\Delta\Delta CT}$ analysis.

Immunohistochemistry (IHC)

IHC was performed as previously described (Anand and Mondal 2018; Schmidt et al. 2014). Fish were euthanized at different time points (0DPI, 1DPI and 4DPI) and entire heads were chopped off. The heads were fixed in 10% formaldehyde overnight at 4°C. Zebrafish heads were dissected to isolate the brains. The brains were fixed in 100% methanol overnight at -20°C. Methanol fixed 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 (30µm thickness) were cut using a vibratome (Leica VT-1200S). The coronal brain sections were blocked for 1 hour 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 minutes 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 hours at room temperature. The brain sections were washed and counter stained with DAPI

(incubation for 30 minutes 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). Significance value was set at $p < 0.05$.

Results

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

In order to validate our animal model and experimental methods, we wanted to replicate previously known results from similar studies. Hence, we determined the mRNA levels of *pcna* and *bdnf* genes in the telencephalon of injured adult zebrafish brain. We also checked the mRNA expression of *trkb* gene, which to the best of our knowledge, hasn't been evaluated before in the context addressed in this study. As previously reported (Cacialli et al. 2018), the mRNA levels of *pcna* increased after stab injury in a temporal manner, i.e. it gradually increased over time (Fig. 1a). Although, the mRNA levels of *bdnf* and *trkb* also increased after injury, but the expression pattern seemed different. The mRNA levels of both *bdnf* and *trkb* increased around 1DPI, but then decreased around 4DPI (Fig. 1b-c). This is in contrast to *pcna*, in which case, the proliferation is maximum around 4DPI. These results indicate that there might be a temporal separation between the activation of the BDNF/TrkB signaling pathway and the proliferation response after stab injury.

Effect of stab injury on biomarkers of oxidative stress

After stab injury in the telencephalon of adult zebrafish brain, the oxidative stress tends to increase as indicated by changes in the various oxidative stress parameters. The activity of anti-oxidant enzymes, SOD (Fig. 2a) and catalase (Fig. 2b) significantly increased after injury. While the SOD activity was seen to be high in both 1DPI and 4DPI experimental groups (Fig. 2a), catalase activity was high only in 1DPI group and no significant change was detected in the catalase activity in 4DPI group (Fig. 2b). Stab injury in the telencephalon also resulted in significantly increased lipid peroxidation (Fig. 2c) and reduced glutathione levels (Fig. 2d) in both 1DPI and 4DPI groups as compared to 0DPI group. These results indicate enhanced oxidative stress in the injured zebrafish brain.

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

We checked the mRNA levels of four genes in the injured zebrafish brain telencephalon that have been implicated in oxidative stress, namely superoxide dismutase 2 (*sod2*), catalase (*cat*), *hsp70* and *hsp90*. We found that the mRNA levels of *sod2* and *cat* did not change in the injured fish brain (Fig. 3a-b). In case of *hsp70*, although the mRNA level increased in the injured fish as compared to non-injured fish at both 1DPI and 4DPI stages, the difference was found to be statistically non-significant (Fig. 3c). However a statistically significant change was observed in the mRNA level of *hsp90* gene in the injured fish brain at both 1DPI and 4DPI stages (Fig. 3d). The mRNA level of *hsp90* increased after stab injury.

Changes in apoptotic variables induced by stab injury

In our study, we checked the protein levels of apoptotic marker cleaved caspase3 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 adult zebrafish brain telencephalon. Our IHC results indicated that cleaved caspase3 protein levels were enhanced in a subset of cells surrounding the damaged brain tissue one day post injury (Fig. 4C-C'). However, no observable changes were seen in the cleaved caspase3 protein levels four days post injury (Fig. 4D-D'). We also observed significant change in the mRNA levels of *bcl2* and *bax* after injury (Fig. 5a-b), but no change was observed in the mRNA level of caspase9 (Fig. 5c). The mRNA levels of both *bcl2* and *bax* were high in the injured fish brain at 1DPI as well as 4DPI stage (Fig. 5a-b).

Discussion

The cellular and molecular mechanisms underlying the restoration of a stab wound injury in the adult zebrafish brain is largely unknown. Commonly known factors that presumably play a role in zebrafish brain regeneration and adult neurogenesis include the Notch (de Oliveira-Carlos et al. 2013), Wnt (Shimizu et al. 2018), Bmp (Zhang et al. 2019), cytokines such as Tumor necrosis factor (Gonzalez-Perez et al. 2010), chemokines such as Cxcll2 (Diotel et al. 2010), estrogen (Diotel et al. 2013) and progesterone (Diotel et al. 2011). Growth factors such as fibroblast growth factor (fgf) and brain derived neurotrophic factor (BDNF) are also believed to mediate these processes (Anand and Mondal 2017; Cacialli et al. 2018). In case of BDNF, recent findings point towards its imperative role in zebrafish brain regeneration after stab wound injury (Anand and Mondal 2018; Cacialli et al. 2018). However, given the multipotent nature of BDNF, it is presumed that change in its expression and activity is liable to affect other physiological parameters as well. These other parameters may or may not influence the brain regeneration process.

Both apoptosis and oxidative stress are major contributors to the pathophysiology of traumatic brain injury (Wong et al. 2005; Cornelius et al. 2013). Given the anti-apoptotic (Chen et al. 2017; Bhave et al. 1999) and anti-oxidation (Numakawa et al. 2011; Chen et al. 2017) effects of BDNF, it is highly probable that increased BDNF expression in the injured zebrafish brain would modulate the expression of apoptotic and oxidative stress related genes. To test this hypotheses, we performed biochemical assays to determine the oxidative stress levels in the injured adult zebrafish brain. We also performed quantitative real time polymerase chain reaction (qRT-PCR) to determine the mRNA levels of two pro-apoptotic genes, one anti-apoptotic gene and four oxidative stress related genes before and after the induction of stab wound injury in the zebrafish brain telencephalon.

In rodent models, oxidative stress has been associated with secondary pathophysiological changes after brain trauma (Petronilho et al. 2010). Traumatic brain injury is associated with generation of oxidants which may lead to neuronal dysfunction and death (Rodríguez-Rodríguez et al. 2014). Brain injury is immediately followed by generation of superoxide that initiates the oxidation cascade (Kontos and Wei 1986). Superoxide dismutase (SOD) rapidly breaks down the superoxide into oxygen and hydrogen peroxide. The resulting oxidant, hydrogen peroxide, can propagate the oxidative damage in the brain by interacting with the nearby biological substrates (Lutton et al. 2019). Lipid peroxidation, induced by hydroxyl free radical constitutes a major mechanism of cellular damage in case of traumatic brain injury (Lutton et al. 2019). The traumatic brain injury induced oxidative stress has also been correlated to blood flow (perfusion) and oxygenation of the neural tissue (Miller et al. 2006; Zagorac et al. 2005). Oxidative stress, along with other secondary injury mechanisms such as neuroinflammation and blood brain barrier disruption, may lead to exacerbated brain injury (Lozano et al. 2015; Lutton et al. 2019). 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 (Miller et al. 2006; Szarka et al. 2018).

Several studies have investigated the oxidative stress status in the rodent brain after injury. But what happens to oxidative stress levels in the injured zebrafish brain is not known. In this study, we observed an increase in the activity of anti-oxidant enzymes, SOD and Catalase after the induction of stab wound injury. We also observed elevation of LPO and GSH levels in the telencephalon of injured fish brain as compared to uninjured fish brain. These results indicate injury induced oxidative stress in the telencephalon of adult zebrafish brain. We also checked the expression of four oxidative stress related genes in the injured zebrafish brain using qRT-PCR. Interestingly, two of the most important anti-oxidant enzyme genes, *sod2* and *catalase* did not show any change in their expression following stab wound injury, although at the protein level, the enzyme activity of both SOD and catalase is increased as is evident from the results of biochemical assays. This might suggest that stab injury in the telencephalon alters anti-oxidant enzyme activity, without affecting their transcription. However, the mRNA levels of another well- known anti-oxidant protein, *hsp90*, increased significantly. 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, hypoxia, infection, inflammation, etc., it could not be determined precisely whether its upregulation 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 kind of physiological stress caused due to stab injury in the telencephalon of adult zebrafish brain.

Apart from acting as an anti-oxidant, BDNF is also a well-known anti-apoptotic factor, acting via the Bcl-2 protein or by modifying the apoptosis related proteins post-translationally (Chen et al. 2017; Wu et al. 2010). It is a well-known fact that apoptosis is an integral part of the pathology of traumatic brain injury (Raghupathi 2004). Speculations are that apoptosis after traumatic brain injury may serve a protective role, since it helps the brain to get rid of the damaged cells without affecting the surrounding brain tissue (Raghupathi et al. 2000). Traumatic brain injury in rodents has been observed to cause an imbalance in the

expression of the pro-apoptotic and anti-apoptotic genes in the brain tissue (Raghupathi 2004; Raghupathi et al. 2000). However, how these genes respond to traumatic brain injury in adult zebrafish brain is not clear.

In our study, we observed that stab injury in the adult zebrafish brain telencephalon enhanced the protein levels of cleaved caspase3, an indicator of apoptosis, in the vicinity of the injured brain tissue 1 day post injury, not 4 day post injury. Caspase 3 protein is cleaved and activated during the onset of apoptosis. May be this is why cleaved caspase3 positive cells were detected at an earlier stage of post-injury trauma, but not at a later stage. Nevertheless, detection of cleaved caspase 3 in the injured zebrafish brain may indicate a potential relevance of apoptosis process in brain regeneration in zebrafish. Additionally, brain tissue sample taken from the telencephalon region showed enhanced transcript levels of the anti-apoptotic gene *bcl2* after stab injury. Interestingly, the mRNA levels of pro-apoptotic gene *bax* also increased. The induction of both pro-apoptotic and anti-apoptotic genes after traumatic brain injury in zebrafish may suggest compensatory mechanisms for regulating apoptosis in the injured brain tissue.

Conclusion

The present study was aimed at communicating the potential alterations in the BDNF influenced physiological processes after traumatic brain injury in zebrafish, particularly oxidative stress and apoptosis. We have tried to touch upon the alterations in the activity and expression of key players in oxidative stress and apoptosis in the context of a stab injury in the telencephalon of adult zebrafish brain. Since the anti-oxidant enzyme activity and LPO and GSH levels increase after telencephalic brain injury, it is highly probable that oxidative stress has a major contribution to the stab injury induced brain pathology and cell death. Interestingly, despite the increased enzyme activity, the mRNA levels of anti-oxidant enzymes do not change in the injured brain as compared to uninjured brain, suggesting alteration at the protein level and not at the transcriptional level. Although *hsp90* has been implicated in oxidative stress (Profumo et al. 2018), it is also associated with many other kind of physiological and environmental stresses. Hence, whether its mRNA level is increased due to oxidative stress or any other kind of physiological stress induced by stab injury could not be determined. Further experiments are required to decipher the exact cause of increased *hsp90* expression after stab injury in the adult zebrafish brain. The increased protein levels of cleaved caspase3 and enhanced transcript levels of both pro-apoptotic gene *bax* and anti-apoptotic gene *bcl2* after stab injury in the zebrafish telencephalon indicate the induction of compensatory mechanisms to regulate apoptosis after stab injury. This may also suggest that apoptosis is involved in the brain regeneration process in zebrafish. Further investigation should be targeted on its precise role in this context. Our results contributes to the understanding of the underlying mechanisms of reparative adult neurogenesis in vertebrates. Further, we emphasize that there is immediate need to investigate the signaling elements downstream to BDNF and other cellular and molecular factors that are influenced by BDNF to fully understand and quantitate the precise role and significance of BDNF signaling in brain regeneration in zebrafish. This may take us one step closer to realizing regeneration based therapies for neurodegenerative diseases and traumatic brain injury in humans.

Declarations

Funding: ACM highly acknowledges the financial supports from DBT NER (BT/PR32907/MED/122/227/2019), DST PURSE- (Phase-II) (PAC-JNU-DST-PURSE-462), UGC RNW, UGC DRS-II, and UPE-II, JNU (Project Id No. 247) and School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. SKA acknowledges the financial support from CSIR-HRDG, New Delhi.

Authors' Contributions: ACM and SKA designed the experiments. SKA and MRS performed the experiments. The manuscript was written by SKA and edited by ACM.

Conflicts of interest: The authors declare no conflict of interest.

Availability of data and materials: Data and materials supporting this study will be made available on request.

Code Availability: Not applicable

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Tables

Table 1: Primers used in the qRT-PCR analysis.

Gene symbol	Gene name	Forward Primer (5’-3’)	Reverse Primer(5’-3’)
β-actin	Beta actin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
gapdh	Glyceraldehyde 3-Phosphate dehydrogenase	CGCTGGCATCTCCCTCAA	TCAGCAACACGATGGCTGTAG
bdnf	Brain derived neurotrophic factor	CTCGAAGGACGTTGACCTGT	CGGCATCCAGGTAGTTTTTG
trkb	Tropomyosin receptor kinase B	GGAAAAGCAAAAACCCTGTCTAGA	TGTAGCATCACTTCCTGCCATT
pcna	Proliferating cell nuclear antigen	CTCACAGACCAGCAACGTCG	GGACAGAGGAGTGGCTTTGG
bcl2	B-cell lymphoma 2	GTCACTCGTTCAGACCCTCAT	GACGCTTTCCACGCACAT
bax	Bax	GGCTATTTCAACCAGGGTTCC	TGCGAATCACCAATGCTGT
cas3	Caspase 3	CCGCTGCCCATCACTA	ATCCTTTCACGACCATCT
cas9	Caspase 9	AAATACATAGCAAGGCAACC	CACAGGGAATCAAGAAAGG
sod2	Superoxide Dismutase 2	CTTGGGATAGATGTCTGGG	GTGGTCTGATTAATTGTGCG
cat	Catalase	AACCAACAACCCTCCAGACAG	TCCGCTCTCGGTCAAAATGG
hsp70	Heat Shock protein 70	CATCGACGCCAACGGG	CCAGGGAGTTTTTAGCAGAAATCTT
hsp90	Heat Shock protein 90	GCTGGAGACACTCACTTGGG	CCACGGAAGAGGTCAGCATT

Figures

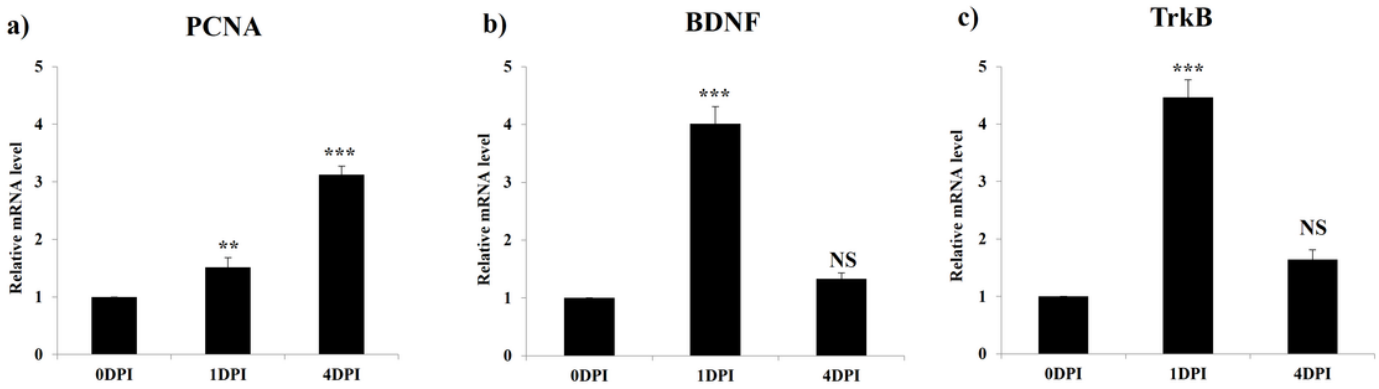

Figure 1

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.

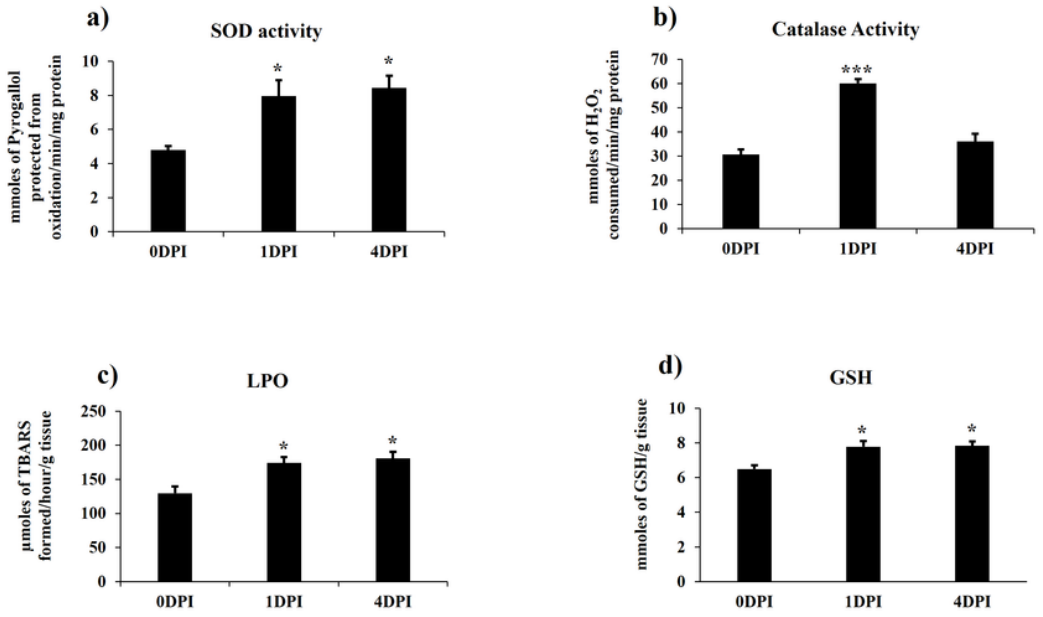

Figure 2

Changes in the oxidative stress parameters after stab injury in the telencephalon of adult zebrafish brain. Stab injury tends to enhance the activity of anti-oxidant enzymes SOD (a) and catalase (b). It also results in the elevated levels of LPO (c) and GSH (d). ***=P≤0.001, **=0.001<P<0.01, *=0.01<P<0.05.

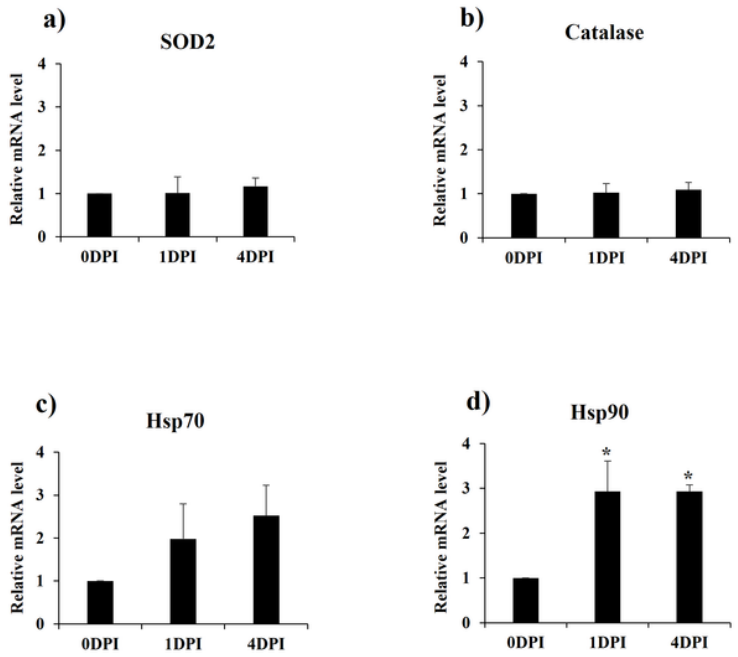

Figure 3

Elevated expression of hsp90 gene in the injured zebrafish brain. qRT-PCR results showing the changes in the mRNA levels of sod2 (a), catalase (b), hsp70 (c) and hsp90 (d) genes in the telencephalon of injured adult zebrafish brain. ***=P≤0.001, **=0.001<P<0.01, *=0.01<P<0.05.

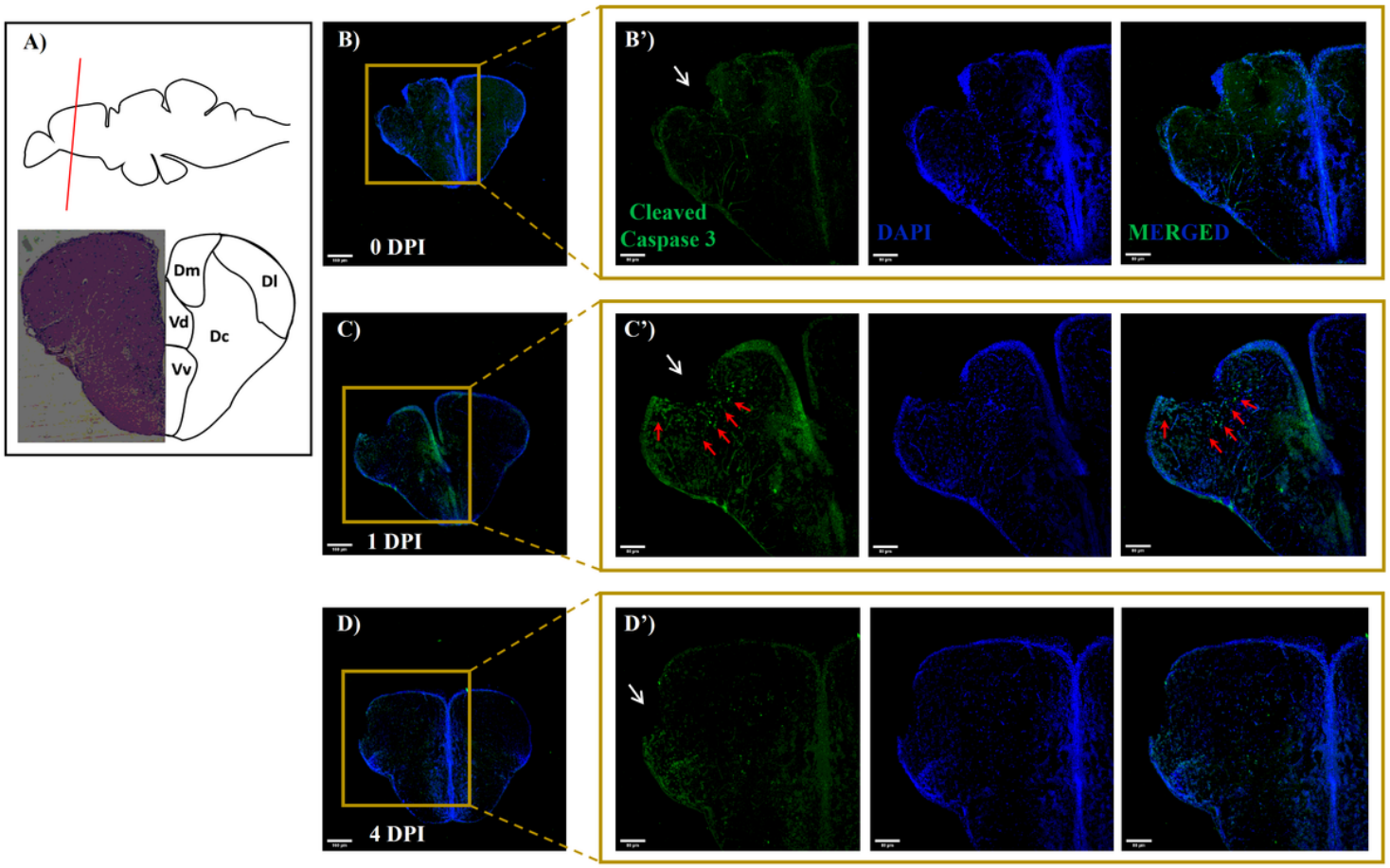

Figure 4

Alterations in the protein level of cleaved caspase3 induced by stab wound injury. A) Schematic representation of the coronal section through mid telencephalic area (point of injury). B)-D') Immuno-staining for cleaved caspase3 in 0DPI (B-B'), 1DPI (C-C') and 4DPI (D-D') experimental fish brain. White arrows indicate the site of injury while the red arrows mark the cells that showed positive immuno-staining for cleaved caspase3 protein. Magnification: 10X for B,C,D and 20X for B',C',D'. Scale bars: 160µm for B,C,D and 80µm for B',C',D'. Dl, Lateral zone of dorsal telencephalic area; Dm, Medial zone of dorsal telencephalic area; Dc, Central zone of dorsal telencephalic area; Dd, Dorsal zone of dorsal telencephalic area; Vd, Dorsal nucleus of ventral telencephalic area; Vv, Ventral nucleus of ventral telencephalic area; Vs, Supracommissural nucleus of ventral telencephalic area; PPa, anterior part of Parvocellular preoptic nucleus.

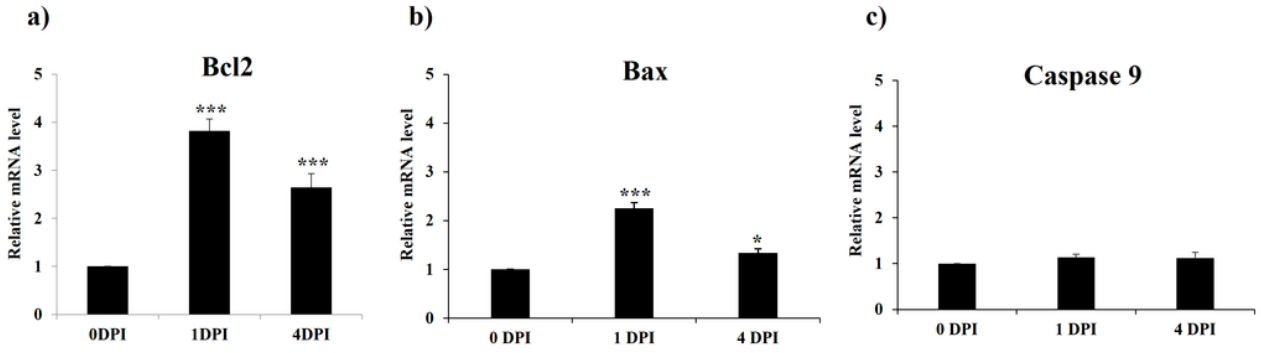

Figure 5

Induction of 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.