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Neural cells and their progenitors in regenerating zebrafish spinal cord

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ABSTRACT The zebrafish (*Danio rerio*), among all amniotes is emerging as a powerful model to study vertebrate organogenesis and regeneration. In contrast to mammals, the adult zebrafish is capable of regenerating damaged axonal tracts; it can replace neurons and glia lost after spinal cord injury (SCI) and functionally recover. In the present paper, we report ultrastructural and cell biological analyses of regeneration processes after SCI. We have focused on event specific analyses of spinal cord regeneration involving different neuronal and glial cell progenitors, such as radial glia, oligodendrocyte progenitors (OPC), and Schwann cells. While comparing the different events, we frequently refer to previous ultrastructural analyses of central nervous system (CNS) injury in higher vertebrates. Our data show (a) the cellular events following injury, such as cell death and proliferation; (b) demyelination and remyelination followed by target innervation and regeneration of synaptic junctions and c) the existence of different progenitors and their roles during regeneration. The present ultrastructural analysis corroborates the cellular basis of regeneration in the zebrafish spinal cord and confirms the presence of both neuronal and different glial progenitors.

KEY WORDS: *regeneration, spinal cord, zebrafish, astroglia, neural progenitor*

Introduction

Teleost fish like *Danio rerio* are unique among the adult vertebrates in their capacity to regenerate lost or damaged parts of body including their central nervous system (CNS). Spinal cord injury (SCI) in mammals leads to decline in various functions because of progressive patho-physiological disturbances which cause secondary injury (Balentine, 1978a, b; Blight, 1985; Popovitch *et al.*, 1997; Guth *et al.*, 1999). Both acute and secondary injury of spinal cord involves complex cellular and molecular mechanisms which eventually lead to permanent irreparable damages to the cord and resultant functional deficit. In contrast to mammals, zebrafish can replace its lost cells, both neuron and glia, repair its severed axons with appropriate target innervations, thus achieve functional recovery after an injury. In this context, adult zebrafish represents an excellent model to study CNS regeneration addressing all different cellular aspects of this complex process and provide more insights that may be extrapolated to induce regeneration in higher vertebrates.

The primary requirements for successful regeneration to occur

after SCI include: replenishment of lost cells which involve glia and neuron, survival and re-growth of severed axons, remyelination and appropriate target innervations of the regenerating axons. In order to understand the similarities and differences in the injury response between mammalian and zebrafish spinal cord, we undertook ultrastructural analysis which may further strengthen our understanding of cellular and molecular basis of regeneration. Early injury response in zebrafish spinal cord includes infiltration of blood cells predominantly macrophages, cell death and demyelination, which are followed by injury induced proliferation and neurogenesis. The late response after injury appears to be axonal re-growth and appropriate target innervations (Hui *et al.*, 2010). Previous studies on teleost fish also suggested that proliferation and neurogenesis can occur in different areas of zebrafish brain

Abbreviations used in this paper: BBB, blood brain barrier; BLBP, brain lipid binding protein; CNS, central nervous system; GFAP, glial fibrillary acidic protein; GLAST, glutamate aspartate transporter; NSC, neural stem cell; OPC, oligodendrocyte progenitor; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; RER, rough endoplasmic reticulum; SCI, spinal cord injury.

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and spinal cord (Zupanc *et al.*, 2005; Adolf *et al.*, 2006; Reimer *et al.*, 2008; Hui *et al.*, 2010; Ito *et al.*, 2010). Proliferating progenitor cells are also present in different compartments of CNS similar to the neural stem cells (NSC) in mammalian CNS (Johansson *et al.*, 1999; Temple and Alvarez-Buylla, 1999). We observe presence of different proliferating progenitors which are contributing to regeneration of zebrafish spinal cord. The spatio-temporal analysis of these progenitors (both neuronal and glial progenitors) in regenerating spinal cord have been elucidated, as these cells are identified by ultrastructural characters and by using appropriate immunohistological markers.

Any injury to the mammalian CNS induces glial scar formation and because of the fibro-astrocytic scar axons cannot regenerate beyond the lesion. The reactive astrocytes in the glial scar play a prominent role in regeneration failure (Rugde and Silver, 1990; Silver and Miller, 2004) although some evidence exists in favor of a beneficial role of glial scar (Faulkner *et al.*, 2004). Reactive glia may form an extra barrier to severed axons after SCI and it is not just the scar but other cell types may be potentially involved to exert more deleterious effect (Silver, 2016). The role of glial scar and astrocyte is increasingly becoming a complex issue to untangle as reactive glia express markers associated with neurogenic stem cells (Silver and Steindler, 2009) and their stem cell like properties can be recognized both *in vitro* and *in vivo* (Buffo *et al.*, 2008).

Until now, comprehensive ultrastructural characterization of cellular composition in adult zebrafish spinal cord pre-injury and their response to SCI has not been studied in its entire gamut. In the present study, we aimed to unravel the ultrastructural characteristics of different cellular events and identify associated cells involved

in regeneration of zebrafish spinal cord in order to understand the underlying cellular basis of regeneration. Astroglial response is one of the most important aspects of differential outcome after CNS injury in mammals and fish. We have analyzed some of the features of these cells like glial hypertrophy, astroglia- neuron interaction by electron microscopy and immunohistological markers. We also found some of these progenitors are important contributors to the successful regeneration in this species. Our time course analysis have demonstrated some characteristic cellular events which are unique to zebrafish spinal cord while some other cellular events are common to both mammalian and zebrafish cord. The present ultrastructural analysis also corroborates with the cellular and molecular features shown in zebrafish cord before and after injury (Becker and Becker, 2001; Reimer *et al.*, 2008; Hui *et al.*, 2010, 2015; Ohnmacht *et al.*, 2016).

Results

Cellular organization of the uninjured adult zebrafish cord

The anatomy of normal uninjured adult spinal cord in zebrafish has already been characterized using different histological staining (Hui *et al.*, 2010). Our present ultrastructural analyses of uninjured cord reveal the presence of bundles of both myelinated as well as unmyelinated axons in the white matter. Within the axoplasm, there are mitochondria and neurofilaments (Fig. 1A) and myelinated axons show compact array of myelin sheath (Fig. 1A inset). The oligodendrocytes possess electron dense nucleus with aggregates of heterochromatin (Fig. 1B) and are present in the white matter. They also show some of the typical features

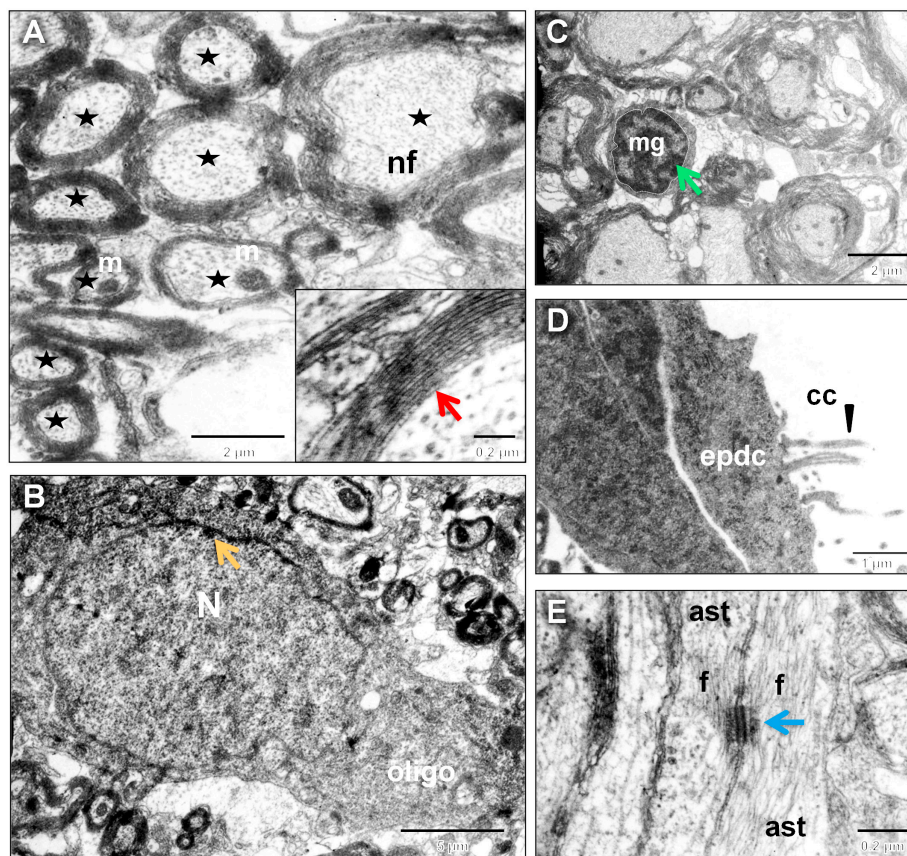


Fig. 1: Ultrastructural characterization of uninjured zebrafish spinal cord. (A) Transverse section of the white matter region of an uninjured spinal cord shows many myelinated axons (stars). The cytoplasm of axons contains neurofilaments (nf) and cell organelles like mitochondria (m). The inset shows the fine structure of myelin sheath (red arrow) in higher magnification. (B) TEM image shows a mature oligodendrocyte (oligo), showing nucleus (N) and peripheral heterochromatin (orange arrow) present in the white matter of spinal cord. (C) TEM image of spinal cord white matter shows the presence of microglia (mg) with little cytoplasm surrounding the nucleus (white dotted circle) and heterochromatin (green arrow). (D) Transverse section through the ependymal region, showing an ependymal cell (epdc) with characteristic microvilli (arrowhead) extended towards the central canal (cc). (E) TEM image shows the junction between two astrocytes (ast) connected through desmosome (blue arrow). Note that the cytoplasm of both astrocytes is filled with intermediate filaments (f). Scale bar, 2 μ m (A) 5 μ m (B), 2 μ m (C), 1 μ m (D), 0.2 μ m (E).

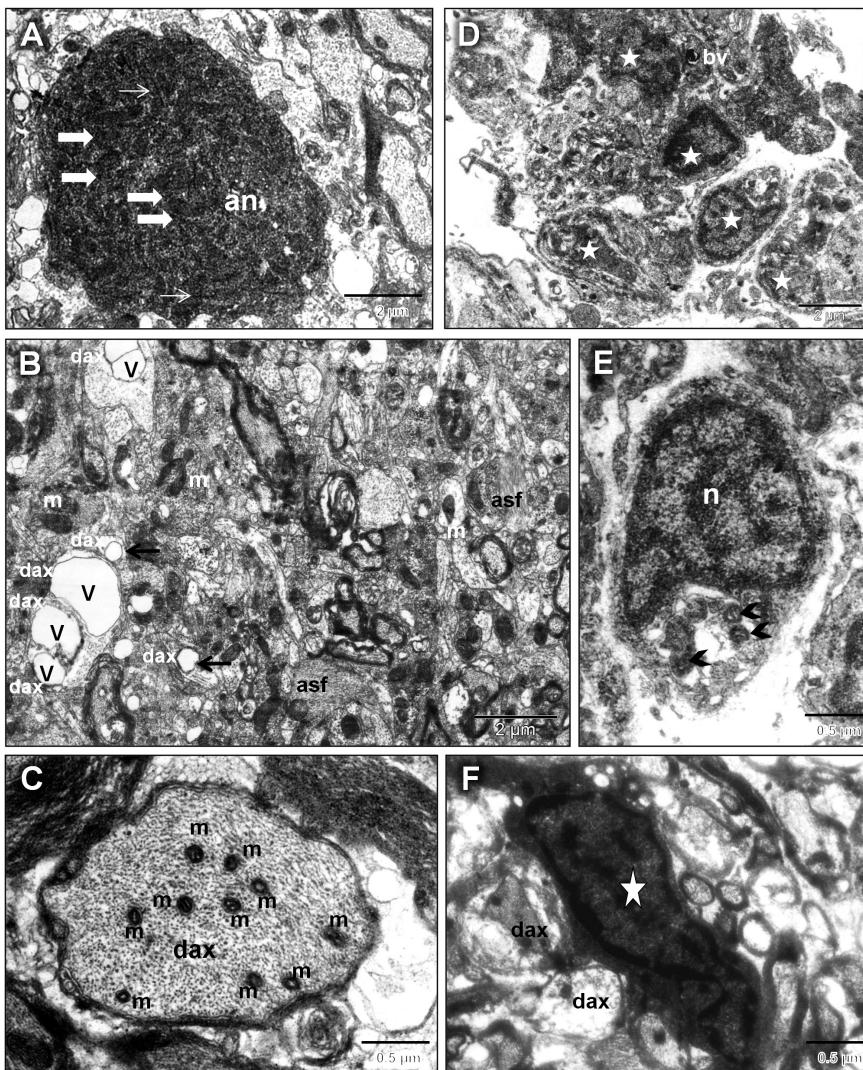


Fig. 2: Ultrastructural characteristics of early response to spinal cord injury. (A) TEM image shows the cytoplasm of an apoptotic neuron in a 3 dpi cord with dark irregular mitochondria (thin arrows), fragmented nucleus (thick arrows) and retracted cytoplasm (adapted from Hui *et al.*, 2010). (B) TEM image of injury epicenter of a 3 dpi cord showing many degenerated axons (dax) with a thin covering of myelin sheath (black arrow). Presence of mitochondria (m) within degenerated axons and hypertrophic astroglial filaments (asf) are obvious. Many void spaces marked as V are present within degenerated axons. (C) Transverse section of 3 dpi cord showing densely packed neurofilaments (fine black dots) and many mitochondria (m) in the cytoplasm of a denuded axon (dax). (D) TEM image of 3 dpi spinal cord section showing disorganized tissue in the injury epicenter, accumulation of multiple phagocytic cells (white stars), and an adjacent blood vessel (bv) that is spared after injury. (E) TEM image in higher magnification shows a microglial cell with engulfed myelin debris (arrowheads) and numerous vacuoles in its cytoplasm. 'n' denotes the nucleus of the microglial cell. (F) A phagocytic macrophage (star) in the vicinity of several degenerating axons (dax) in a 3 dpi cord. Scale bar, 2 μ m (A, B and D); 0.5 μ m (C, E and F).

like short rough endoplasmic reticulum (RER) and microtubules in the cytoplasm, as described (Peters *et al.*, 1991). Other glial components like resident microglia can also be observed. These cells have scanty cytoplasm and large nucleus with peripheral heterochromatin and the heterochromatin network is made up of many fused granules as represented in Fig. 1C. The grey matter volume in zebrafish cord is relatively less compared to that of mammals. In the central region of the cord runs the central canal or ependymal canal which is surrounded by ependymal cells. Neurons are present in the subependymal region (data not shown). The ependymal cells (Fig. 1D) are characterized by the presence of extended processes or cilia facing towards the lumen of the central canal. Some of the ependymal cells are radial glial progenitors, as these cells are GFAP positive and soma of them are facing towards central canal and extending branched fibers towards the pial membrane (Supplementary Figure 1). These glia are heterogeneous as different subpopulations can be identified based on expression of combinations of markers such as GLAST, BLBP and GFAP (Hui *et al.*, 2015, Supplementary Figure 2). We also observe desmosomes between the processes of astroglial cells which have electron lucent cytoplasm with glial filaments (Fig. 1E), suggesting the presence of well developed glial networks in

uninjured adult zebrafish cord. Most of the astroglia that we could identify in grey matter are actually radial glia rather than mature astrocytes. Thus, astroglia were identified ultrastructurally by the presence of moderate content of intermediate filaments in their cytoplasmic processes and few dark inclusions in their cell bodies. Their nuclei appeared to be homogeneous in chromatin content. Oligodendrocytes were identified by the presence of a large nucleus, few short cisternae of rough endoplasmic reticulum and microtubules in their broad processes, while microglia appeared with several processes and few lysosome-like granules in their cytoplasm, as described by Peters *et al.*, (1981). In normal uninjured spinal cord, distinct ultrastructural characters of different components are shown in the figure and mentioned in Supplementary Table 1.

Early cellular responses to injury

Following SCI, there is immediate disruption of blood brain barrier (BBB) leading to infiltration of blood cells in the injury epicenter. Other than neutrophils and monocytes, the predominant variety of wandering cells present near the wound site is macrophages or microglia. Early cellular response to injury is very dynamic; it involves the loss of BBB, followed by glial and neuronal loss, and fragmentation of myelin of injured axons, which occur within 2-3

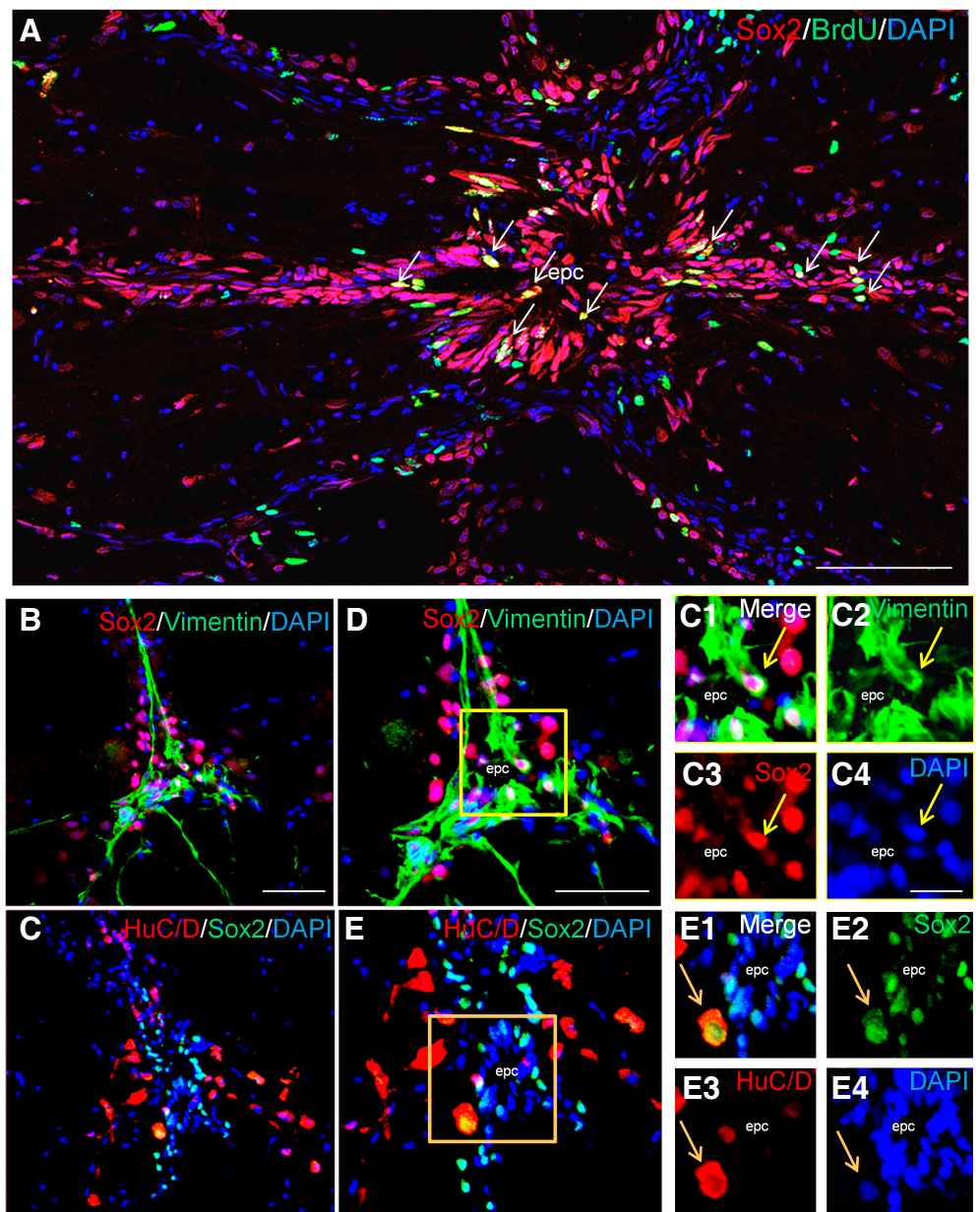


Fig. 3. Glial and neuronal progenitors in injured spinal cord. (A) Longitudinal section of a 7 dpi spinal cord immunostained both with Sox2 and BrdU, showing many proliferating progenitors (white arrows). Nuclei are stained with DAPI. (B,C) Transverse section of a 7 dpi spinal cord immunostained with Sox2 and vimentin, shown in low (B) and high (C) magnification. Nuclei are stained with DAPI. (C1-C4) The higher magnification view of yellow boxed area in C shows a Sox2 and vimentin colocalized glial progenitor (yellow arrow) represented as merge and all individual channels. (D,E) Transverse section of a 7 dpi spinal cord immunostained with Sox2 and HuC/D in low (D) and high (E) magnification. Nuclei are stained with DAPI. (E1-E4) The higher magnification view of yellow boxed area in E shows a Sox2 and HuC/D colocalized neuronal progenitor (orange arrow) around the ependymal canal (epc) represented as merge and all individual channels. Scale bars, 100 μ m (A); 50 μ m (B-E); 20 μ m (C1-C4, E1-E4).

days of injury. Dying neurons with fragmented nucleus can be seen in the injury epicenter, as shown in a 3 day post injury (dpi) cord (Fig. 2A). The injury epicenter shows inflammation and degenerative changes, and that may continue for few more days after initial response. The injury epicenter is characterized by presence of many degenerating axons, swollen mitochondria, void structures within many axonal cytoplasm and astroglial filaments near denuded axons (Fig. 2B). The damaged axons undergo sloughing off myelin sheaths as a consequence of injury. In these injured axons, increased presence of neurofilaments and mitochondria are also evident (Fig. 2B,C). Dense distribution of neurofilaments and many swollen mitochondria within axonal cytoplasm of the injured axons as well as presence of hypertrophic astroglial processes are all associated with early injury response.

Injury of spinal cord disrupts the BBB and allows blood cells to infiltrate into the lesion. The infiltration of blood-borne macrophages

is evident quite early in 2-3 dpi cord and these cells are phagocytic in nature (Fig. 2D,E). The damaged axons show breakdown of myelin sheaths. The phagocytic macrophages are responsible for engulfing axonal debris (Fig. 2E, F) and often show lipid filled vacuoles.

Proliferative response and progenitors

Early injury response is followed by rapid cellular migration and proliferative response as BrdU incorporation starts in 3 dpi cord, peaks in 7 dpi cord, which gradually decreases at later time points of injury (Hui *et al.*, 2010). Many of the ependymal cells are proliferating progenitor(s) and identified as radial glia (Supplementary Fig. 2), as they express various markers of radial glia. High expression of many of neural progenitor markers is also shown in 7 dpi cord where rate of proliferation is known to be highest. The relative fold change of expressions of all these markers in

7 dpi cord are significantly higher than uninjured cord, suggests increased proliferative response after injury and presence of proliferative progenitors (Fig. 3; Supplementary Fig. 3). The detailed ultrastructural characters of these progenitors are shown here.

Neuronal progenitors

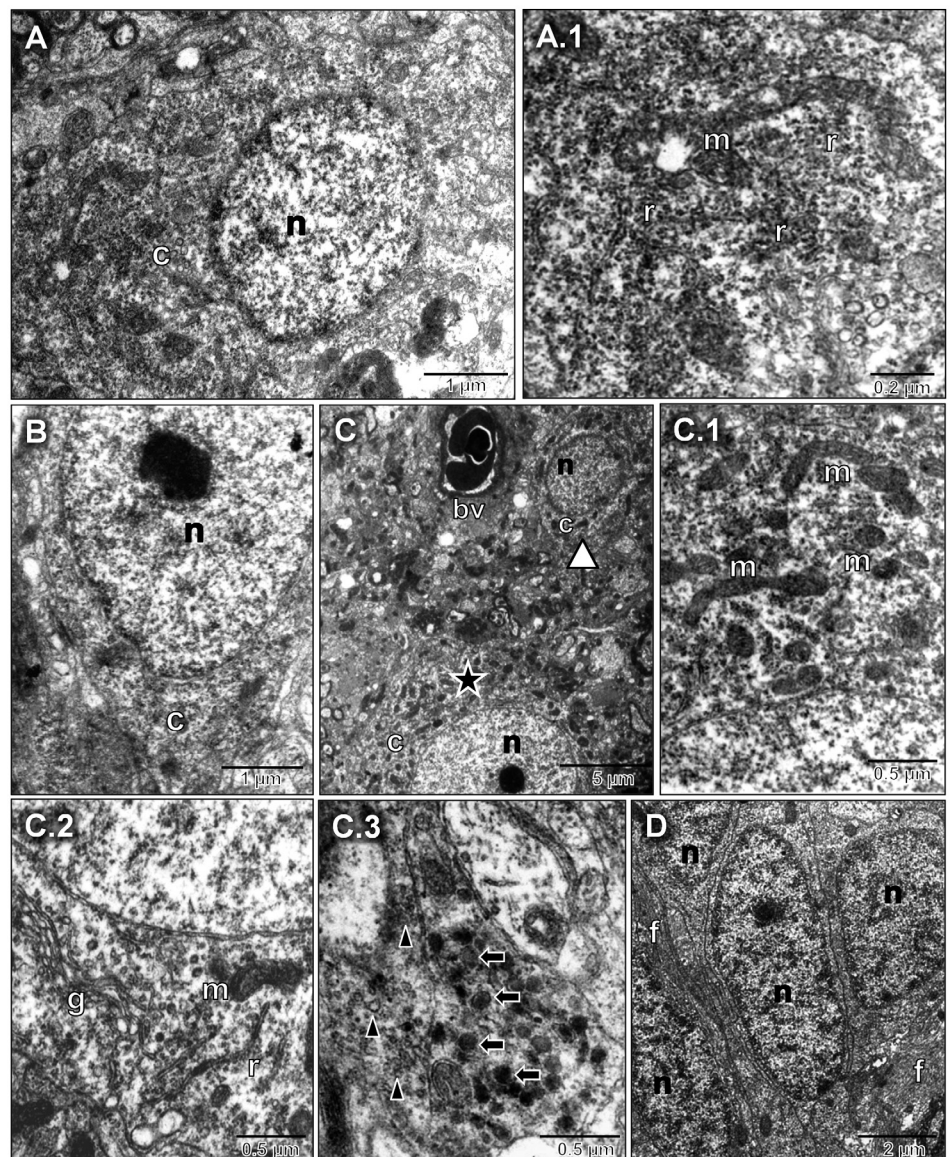
Proliferative response of radial glia may lead to neurogenesis as previously reported in many studies. We corroborate our ultrastructural analysis and immunohistochemical analysis showing presence of neural progenitors by using different neuronal markers like Sox2, nestin, vimentin and HuC/D (Figs. 3, 4, and Supplementary Fig. 3). We have also carried out quantitative analysis of neural progenitor markers by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (Supplementary Fig. 3) and indeed among these Sox2/BrdU positive proliferating progenitors (Fig. 3A) some are neuronal progenitors since these cells are co-labeled with Sox2/HuC/D (Fig. 3D). Ultrastructurally, we confirm the presence of newly born neuronal cells in 7 dpi, 10 dpi cord (Fig. 4 A,B,C) and even in 1 month regenerate showing immature neuron with high nucleo/

cytoplasmic (N/C) ratio with less elaborate cytoplasm having few organelles. While the newly born neurons become more and more mature, they develop characteristic organelles like rosette shaped free ribosome (Fig. 4A.1), well developed yet variably shaped mitochondria (Fig. 4A.1,C.1,C.2) and lysosomes (Fig. 4C.3) within the neuronal cytoplasm in the regenerates. There are neuronal progenitors in the grey matter around the ependyma associated with glial filaments in 10 dpi cord (Figs. 4D, 5F). We see vertically migrating elongated cells closely apposed with glial fibres in many occasion, particularly after proliferation and during neurogenesis.

Glial progenitors

The proliferating progenitor cells in a regenerating cord are of different types as there exist both neuronal (Figs. 3 D,E and 4) and glial progenitors (Fig. 5, Supplementary Figure 2). Fig. 5A shows ultrastructure of an early glial progenitor with dark nucleus and very little cytoplasm in the white matter of 10 dpi cord. Glial progenitor of probable astrocytic lineage can be seen in Fig. 5A, B. The cell has a large nucleus and less cytoplasm (high N/C ratio) with

Fig. 4. Ultrastructural characteristics of newly born neuronal cells after injury in the zebrafish spinal cord. (A) A newly born neuron, with nucleus (n) and cytoplasm (c) in 7 dpi cord section. (A.1) High magnification view of (A) showing organelles like mitochondria (m) and ribosomes (r). (B) An immature neuron with a large nucleus (n) and few organelles in the cytoplasm (c) in a 10 dpi cord. (C) An immature neuron (triangle) with a large nucleus (n) with little cytoplasm (c) having fewer organelles and a newly born yet specified neuron (asterisk) with higher number of organelles and a blood vessel nearby (bv) in a 10 dpi cord. The cytoplasm (c) of the specified neuron (star) is showing organelles and a large nucleus of the same neuron marked as 'n'. (C.1-C.2) High magnification images showing organelles like mitochondria (m), Golgi bodies (g), ribosomes (r) within the cytoplasm of neurons in 10 dpi cord. (C.3) Accumulation of lysosomes (arrows) in a neuron of 1 month old regenerated cord, the adjacent neuronal axon terminal shows immature synaptic vesicles (arrowheads). (D) A representative picture of proliferating neurons along with associated glial fibres with plenty of astrocytic filaments (f) in a regenerating 10 dpi cord. Note at least four elongated cells and their close association with glial fibres present near the ependyma. 'n' denotes nucleus of the proliferating neurons. Scale bars, 1 μ m (A and B); 0.2 μ m (A.1); 5 μ m (C); 0.5 μ m (C.1, C.2 and C.3); 2 μ m (D).



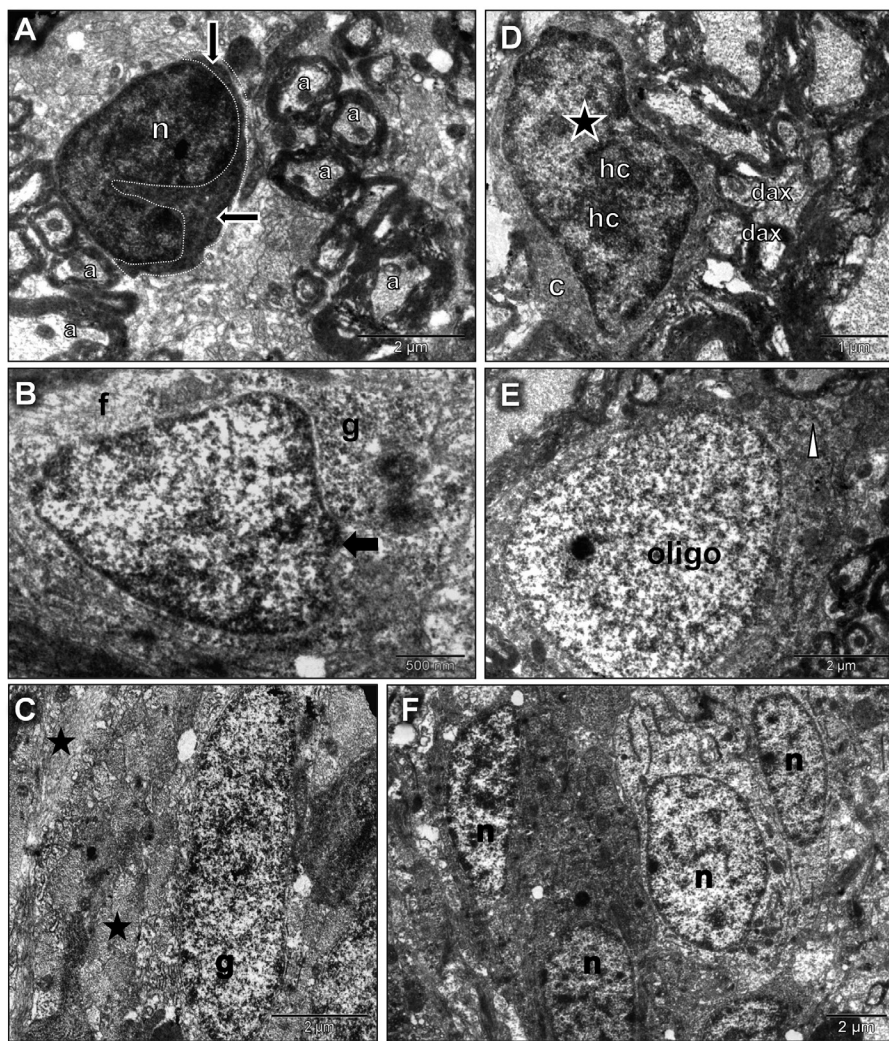


Fig. 5. Ultrastructural characteristics of different glial progenitors after injury in the zebrafish spinal cord. (A) TEM image showing several axons (a) and an early glial progenitor cell having a dark nucleus (n) and scanty cytoplasm (arrow) in a 7 dpi cord. The white line demarcates the boundary of nucleus (n). (B) An immature astrocyte (black arrow) showing intermediate filaments (f) and glycogen granules (g) in the cytoplasm. (C) Section from 10 dpi cord. Another representative picture of an immature glial cell (g) in the white matter showing glial processes (stars). (D) An immature oligodendrocyte (star) with clumps of heterochromatin (hc) and scanty cytoplasm (c) surrounded by demyelinating axons (dax). (E) An oligodendrocyte (oligo) with semi-differentiated cytoplasm with few short cisternae of RER (white arrowhead). (F) 10 dpi cord section shows proliferating neuronal precursor cells (n) in the grey matter of the cord. Scale bar, 2 μ m (A, C, E and F); 1 μ m (D); 500 nm (B).

glycogen granules and intermediate filaments (Fig. 5B). It is an immature astroglial cell, and we do not see mature astrocytes in the white matter. Similarly, few immature oligodendrocytes can be seen; these cells contain typical clumps of heterochromatin in the large nucleus, peripheral heterochromatin near nuclear membrane and small volume of cytoplasm. Small cisternae of RER are also visible in the cytoplasm of the immature oligodendrocytes (Fig. 5D,E). The particular immature oligodendrocyte is seen in the vicinity of few demyelinated axons in white matter (Fig. 5D) in a 10 dpi cord. The predominant glial progenitor in zebrafish is radial glia (Hui *et al.*, 2010), which is ependymoglia and can acquire bipolar shape after injury (Goldshmit *et al.*, 2012). They appropriately express multiple glial markers like GLAST/BLBP/GFAP (Supplementary Figs. 1, 2, Hui *et al.*, 2015) and also coexpress progenitor markers, Sox2 and vimentin (Fig. 3). Moreover, qRT-PCR analysis showed the upregulation of intermediate filament genes *gfap*, *vim* and *nes* at 7 dpi cord (Supplementary Fig. 3), when we see highest rate of progenitor proliferation. Although, the heterogeneity is observed with radial glia (Supplementary Fig. 2), however, the contributions of the particular radial glial subpopulation with respect to their specific functions have not been studied.

Response of astrocytic glia to injury

Astrocytes are a particular type of glial cells in CNS and are remarkably complex. Astrocytes of mammalian post injury CNS are considered to be an important player in initiating inflammatory response. The ultrastructure of astroglial cells of injured cord at different time points are as follows: After injury, glial hypertrophy is a key feature in the spinal cord. We have observed increased number of desmosomes between different astroglia in 7-, 10- and 30 dpi cord (Fig. 6A,B,C). The cells are mostly light astroglia with swollen mitochondria. The cells contain long cisternae of granular endoplasmic reticulum, numerous free ribosomes, rosettes of polyribosomes, many Golgi cisternae, lysosomes and neuropeptide vesicles (Fig. 6D,E,F,G,H). Other common features of activated astroglia are increased presence of intermediate filament bundles, dense cytoplasm containing numerous glycogen granules and lysosomes (Figs. 6B,G, 7A,B,C). Hypertrophic morphology of astroglia is confirmed by presence of increased extension processes containing many mitochondria and swelling of cell bodies. They are characterized by thick bundles of intermediate filaments after injury (Fig. 7A,B). Astrocytic processes are connected by a large number of gap junctions (Figs. 6B,D, 7E). The length of gap junctions may vary considerably from tiny contact to segments up to 1mm. These

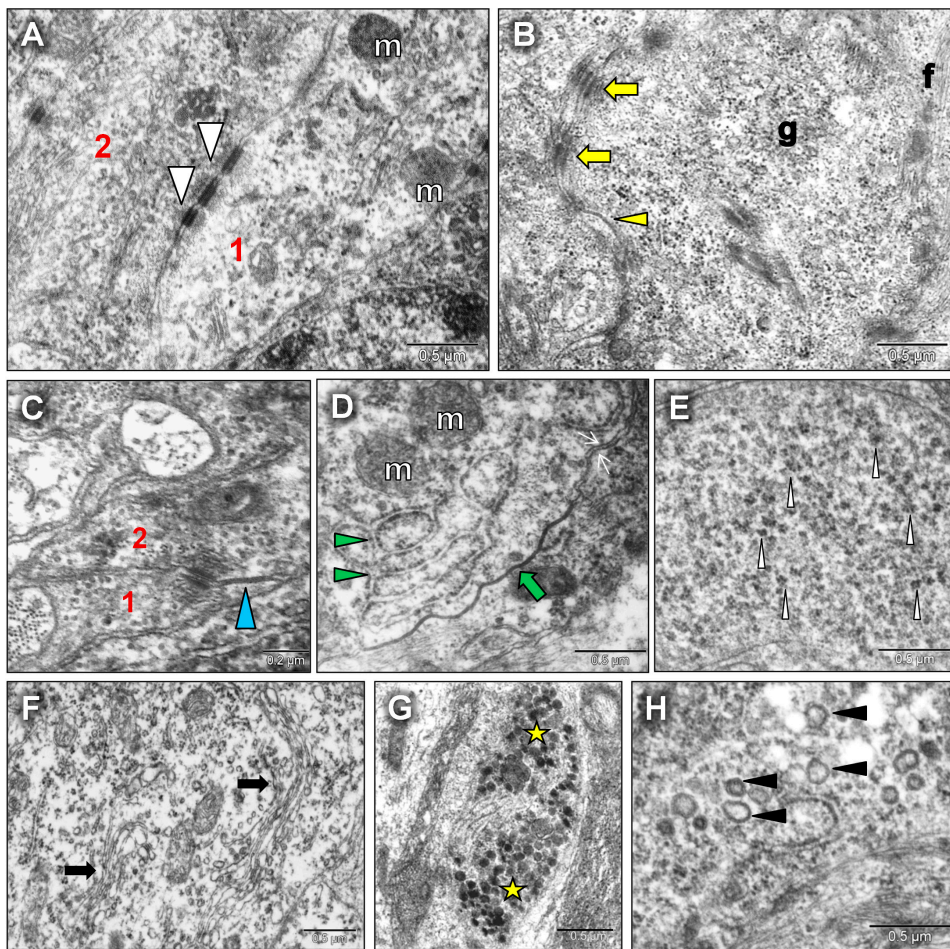


Fig. 6. Ultrastructural features of astroglia in response to injury. (A) TEM image of 7 dpi cord section showing two astroglial cells (1, 2), mitochondria (m) and are connected by gap junctions (white arrowheads). (B) Section of 10 dpi cord showing many desmosomes (yellow arrows) and one gap junction (yellow arrowhead) in an astroglial cell with glycogen granules (g). 'f' denotes intermediate filaments. (C) High magnification image showing gap junction (blue arrowhead) between two astroglial processes (1 and 2) is evident. (D) Section of 10 dpi cord showing the cytoplasm of an astroglial cell with long rough endoplasmic reticulum (green arrowheads), swollen mitochondria (m). The same astroglial cell is connected by a long gap junction (green arrow) with another astroglial cell and the plasma membranes (white arrows) at both ends of the cytoplasmic boundary are also visible. (E) 10 dpi cord section showing astroglial cytoplasm is packed with many rosette shaped polyribosomes (white arrowheads). (F) An astroglial cell contains multiple well-developed Golgi bodies (arrows) in the cytoplasm of a 10 dpi cord. (G) An astroglial cytoplasm with many of lysosomal vesicles (yellow star), indicating their known secretory function of growth factors in response to injury. (H) High magnification image of an astroglial cytoplasm in 10 dpi cord showing the presence of many neuropeptide vesicles (arrowheads) in the cytoplasm, probably suggesting their role in neuropeptide mediated signal transmission with adjacent neurons. Scale bar, 0.5 μm (A, B, D-H); 0.2 μm (C).

characters are visible in injured cord at different time points (3-, 10-, 15 dpi and even after 1 month of injury). Thus, many intra-astrocytic gap junctions and desmosomes are also present between different astroglia as well as their processes in injured cord (Figs. 6A, 7A,E). These astroglial processes can also be seen around myelinated axon and immature neurons (Fig. 7E).

All the different cell types involved in injury response and their specific ultrastructural characters are summarized in the Supplementary Table 1. Briefly, in injured spinal cord, astroglial cells showed a nucleus with moderately rich heterochromatin and ample proliferation of glial intermediate filaments in their cytoplasmic processes as well as dark inclusions. The junctional complexes (gap junctions and desmosomes) appeared numerous and extensive between their processes, indicating remodeling after injury due to possible increase in their cytoplasmic area after significant injury-related axonal loss. No significant changes in the features of oligodendrocytes were found in the injured spinal cord, though they appeared somewhat swollen in appearance. Microglia are round-to-oval in shape whereas peripheral macrophages showed amoeboid arms with elongated nuclei, clumps of patchy chromatin near nuclear envelope, relatively dense cytoplasm with rich accumulation of granules, several lysosomes and autophagosomes. Myelin and axonal debris was often encountered in their cytoplasm. They were mostly centered near the site of injury in injured spinal cord than their widespread location in the intact spinal cord of uninjured animals.

Synapse regeneration

Identification of both excitatory and inhibitory neurons in zebrafish cord has been previously studied (Higashijima *et al.*, 2004a). Based on the neurotransmitter phenotype, neurons can be categorized in larval zebrafish cord. The vesicular glutamate transporter (VGLUT) is present in various glutamatergic neurons that include commissural CoPA, MCoD, UCoD and some of CoSA neurons, whereas cells positive for GLYT2 include CoLA and some of the CoBL and CoSa neurons. GAD expressing neurons are DoLA and KA interneurons. These data provide a map of spinal network active in zebrafish larvae and adult (Higashijima *et al.*, 2004b). We tried to identify some of the types of synapses that are present in regenerating cord at the injury epicenter, based on their morphological characters (Fig. 8).

Generation of different synapses, both axo-axonic or axo-dendronic synapse can be observed in regenerating cord. A 7 dpi cord shows a hyperactive axo-axonic synapse where presynaptic unit contain both clear and dense core vesicles (Fig. 8A). The regenerated axon terminals usually contain plenty of synaptic vesicles, swollen mitochondria and glycogen granules (Fig. 8B, D). Astroglia are active participants in synapse formation and are very often present around excitatory synapses as seen in Fig 8E. The axoplasm of the injured axons contains glycogen granules and mitochondria (Fig. 8C). Presence of both GABAergic (Fig. 8G) and glutamatergic synapses (Fig. 8H) can be demonstrated in regenerated cord 1 month after injury.

Late regenerative response

To elucidate the late response to injury, we have chosen different late time points after injury such as 10-, 15- and 30 dpi cord for ultrastructural analysis. In 15 dpi cord, we see presence of both unmyelinated and myelinated axon bundles (Fig. 9A, B). Although at this time point the number of microglia and macrophage present in the injury epicenter is low, phagocytic macrophages play a very important role in clearing toxic myelin debris in earlier stages of regeneration (Fig. 2). Macrophages with engulfed degenerated myelin could still be present in 15 dpi cord, suggesting the importance of macrophages in removal of myelin debris (Fig. 9D). In the late regenerate, we showed that even after 1 month many axons are not myelinated whereas others are myelinated / remyelinated (Fig. 9). At least some of these axons have regenerated since they are present at the injury epicenter and may have been severed during injury. Surprisingly, there is presence of glial progenitors like Schwann cell progenitor(s) in regenerating cord. Role of Schwann cell in remyelination of axons is very remarkable. We observed presence of Schwann cell progenitors, and Schwann cell remyelinating the injured axons (Fig. 9I). We expected to see myelinating oligodendrocytes in the regenerates but so far we have not recorded any oligodendrocyte myelinating damaged (demyelinated) axons,

although we found presence of oligodendrocyte progenitors (OPC) in 10 dpi cord (Fig. 5 D-E). Identity of these cells was earlier showed to be NG2 positive (Hui *et al.*, 2015).

Discussion

Spinal cord in zebrafish evokes several cellular responses after injury which is crucial for complete regeneration, yet their causal basis is not well understood. There are some similarities and differences in the cellular response between mammals and fish. In the present study, we used electron microscopy to provide unprecedented view to understand the basis of different cellular events based on the morphological characters and expression of verifiable markers. Thus, ultrastructural analysis facilitates our understanding of different cellular events underlying the extraordinary regeneration process. We have studied the various cellular response(s) after SCI in a time dependent manner and documented outcome of both early and late events and contribution of several neural progenitors in this remarkable process of CNS regeneration.

Early injury response

Immediate response after injury is breach of BBB resulting in infiltration of blood cells, cell death which involve both glia and neuron and demyelination of damaged axons. We show initial recruitment of blood borne macrophages in the injury site at very early time points. Although presence of other blood cells like neutrophils and monocytes has been shown in previous analysis (Hui *et al.*, 2010), macrophage remain by far the most important and predominant cell type involved in early injury response and tunes tissue inflammation. The other early response to injury is neuronal cell death. We observed nuclear fragmentation into several highly condensed chromatin bodies,

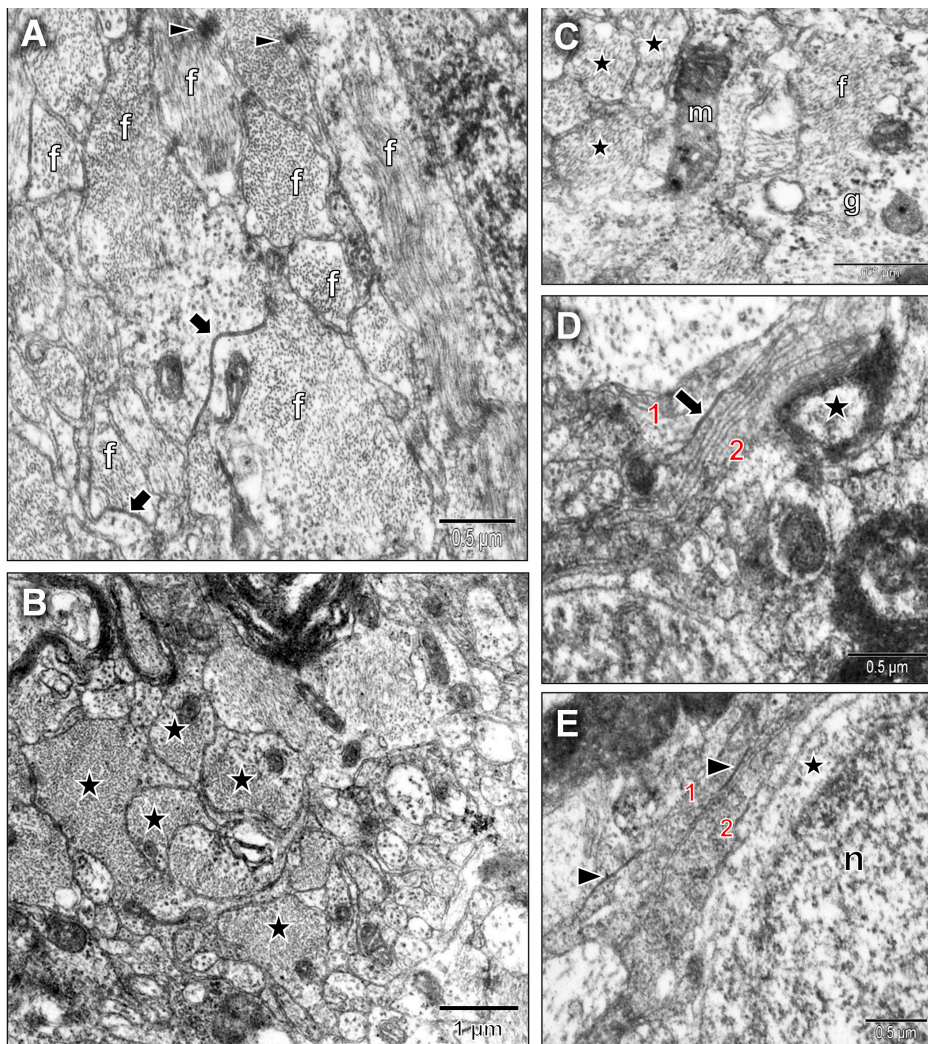


Fig. 7. Ultrastructure of hyperactive astroglia and astroglia-neuron relationship. (A) TEM image of 10 dpi cord showing astroglia after injury with increased synthesis of glial filaments (f) in astroglial processes that are connected by desmosomes (arrowheads) and gap junctions (arrow). (B) Hypertrophic astroglial processes (stars) in 1 month regenerated cord. (C) 10 dpi cord showing astroglial process with filaments (f) and glycogen (g), swollen mitochondria (m). Few glial processes (stars) with intermediate filaments are also visible. (D) Another 10 dpi cord showing a gap junction (black arrow) between two astroglial processes (1, 2), the second astroglial cell (2) has invested the myelinated axon (star). (E) Multiple gap junctions (arrowheads) between two astroglial processes (1, 2). Note presence of one immature neuron (with scanty cytoplasm, star) showing a euchromatic nucleus (n) adjacent to glial process. Scale bar, 0.5 μ m (A, C, D and E); 1 μ m (B).

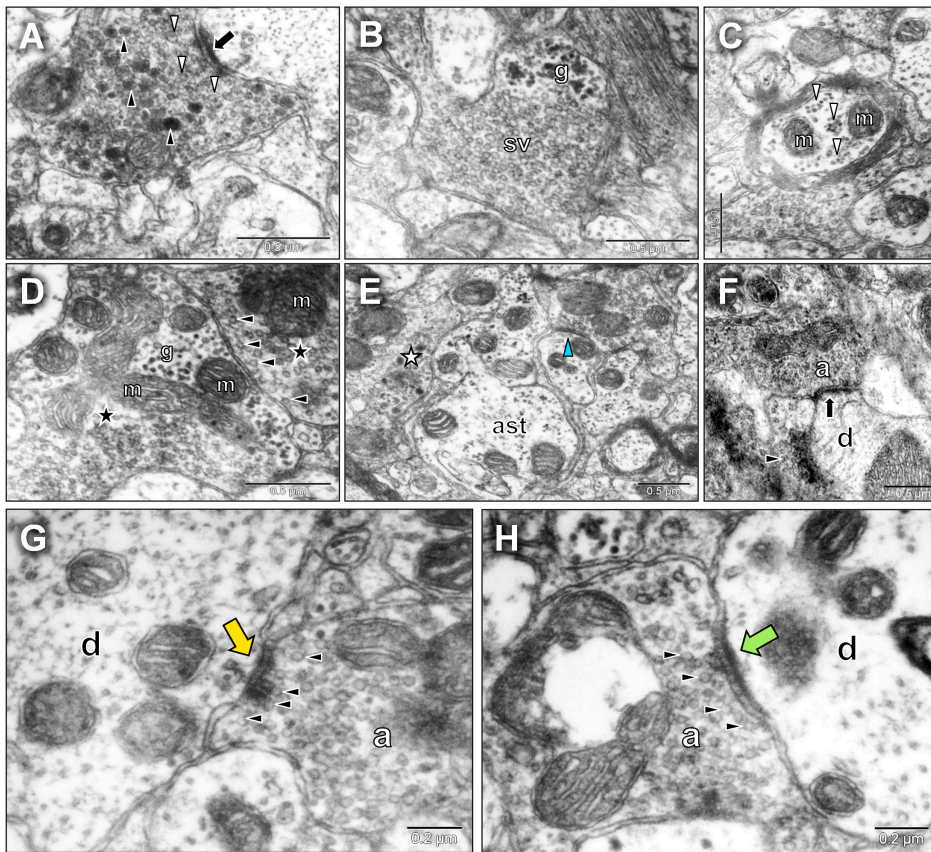


Fig. 8. Ultrastructural features of the regenerating synapse in the spinal cord. (A) TEM image of a 7 dpi cord section showing hyperactive axo-axonic synapse, with presynaptic unit containing clear vesicles (white arrowheads) and few dense-core vesicles (arrowheads), the postsynaptic unit shows neurofilaments (fine black dots) and postsynaptic densities (PSD, arrow). (B) An axon terminal containing clear synaptic vesicles seen as open circles (sv) and numerous glycogen granules (g) in a 1 month regenerate. (C) One month old regenerate showing several axons in cross section, Note the presence of mitochondria (m) and glycogen granules (white arrowheads) in the axoplasm. (D) Two axon terminals (stars) with synaptic vesicles (arrowheads), glycogen (g) and mitochondria (m) in 1 month regenerating cord. (E) One month old regenerate showing an excitatory synapse (blue arrowhead), astrocytic process (ast) which is in close contact with a neuronal process containing clear vesicle and dense core vesicle (star). (F) Another 1 month old regenerate showing axon (a) synapsing with a dendrite (d) that shows postsynaptic density (arrow). The same dendrite synapses with a presynaptic

axon showing clusters of vesicles (arrowhead). (G) 1 month old regenerate showing GABAergic synapse (orange arrow) in axo-dendritic (a-d) junction, with many synaptic vesicles (arrowheads). (H) 1 month old regenerate showing glutamatergic synapse (green arrow) in axo-dendritic junction, with many synaptic vesicles (arrowheads) 'a' denotes pre-synaptic axon and 'd' indicates a post-synaptic dendrite. Scale bar, 0.5 µm (A- F); 0.2 µm (G, H)

shrunken cell cytoplasm often containing irregular mitochondria and having intact infolded cell membrane –the characteristic features of apoptotic neurons. Similar apoptotic cell death has previously been observed in mammalian SCI (Liu *et al.*, 1997; McEwen and Springer, 2005), although much more widespread compared to injured zebrafish cord. Increased presence of macrophages surrounding apoptotic neurons in the injury epicenter in zebrafish cord probably indicates their important role in phagocytosing these dying neurons (Hui *et al.*, 2010). Similar role of resident microglia and peripheral macrophages in clearing damaged and dying neurons has also been reported in zebrafish brain after targeted ablation of neurons (van Ham *et al.*, 2014). Damage to white matter causes loss of glia and myelin. As many axons were severed due to injury, we observe rapid demyelination of damaged axons. During this process, when the myelin sheaths were found to be sloughing off, denuded axons had a very compact array of neurofilaments and increased numbers of mitochondria in the axoplasm compared to the axons of uninjured cord. The blood borne phagocytic macrophages often encroach the demyelinated axons and were involved in devouring the myelin debris, showing lipid filled vacuoles within the cytoplasm. There is a link between macrophage mediated inflammatory response and remyelination, and similar phenomenon seem to be conserved across other species (McMurran *et al.*, 2016). Early inflammatory response is exhibited by microglia

and macrophages as they invade injury epicenter very early after injury and play a major role in controlling the detrimental secondary pathophysiological response. In this context, it is important to mention that macrophages express more anti-inflammatory molecules (M2 type genes) other than inflammatory cytokines and remove the toxic breakdown product of myelin after zebrafish SCI (Hui *et al.*, 2014). As a consequence, macrophages play a major role in removing the effect of myelin derived inhibitory factors and thereby enhance remyelination. Inflammatory response after SCI in zebrafish cord is brief unlike mammals. Very few microglia and macrophages were present at later time points beyond 10 dpi and if present, the macrophages show engulfed myelin debris and microglia had fewer lysosomes which are known to control inflammation. Macrophage/microglia may play multiple roles during regeneration and neurogenesis. These cells play important role in promoting neurogenesis in zebrafish brain and spinal cord (Kyritsis *et al.*, 2012; Ohnmatch *et al.*, 2016). Thus, they can be employed in effective reconstruction of neural circuitry by a) providing a niche for generating neurons, b) elimination of unnecessary cells, c) engulfing pruned or degenerating axons and d) involvement in remodeling of synapses (Jin and Yamashita, 2016). Our ultrastructural observation indicated that macrophages during CNS regeneration in zebrafish fulfill many of these roles mentioned above in creating a regeneration permissive niche.

Proliferative response and involvement of different progenitors

SCI invokes early injury response followed by active proliferation of neuronal and glial progenitors in zebrafish cord. In order to replace the lost cells, both glia and neurons, the proliferative response after injury is important and is the key to successful regeneration. Newly born neurons can be seen mostly in 7 dpi, 10 dpi cord and to a lesser extent in 15 dpi and 1 month regenerate, which confirmed the time frame of neurogenesis as mentioned in previous studies (Reimer *et al.*, 2008; Hui *et al.*, 2010; 2015). Our present ultrastructural analysis indicate that there are both early neuronal progenitor (less mature), with high N/C (Nucleus/Cytoplasmic) ratio and late progenitor (more mature) also with high N/C ratio but having the additional presence of dense chromatin in nucleus and few cytoplasmic organelles like mitochondria, RER and active Golgi apparatus. Morphological evidence of neuron-glia interaction represented here are reflective of rearrangement of newly generated neurons in the ventricular zone to its final destination in subependymal zone (Hui *et al.*, 2010). These cells have remarkable

morphological similarity with neuroblasts in mammalian embryonic brain migrating along radial glial fibres and goldfish retina (Rakic, 1971; Lenkowski and Raymond, 2014), although the mechanism and molecular control of neuronal migration during regeneration deserve further analysis. The presence of proliferative progenitors could be observed both in grey matter and white matter. Glial progenitor cells of both astrocytic and oligodendrocytic lineages were present with high N/C ratio. The progenitors of astrocytic lineage had other characters such as glycogen granules and intermediate filaments and glial processes whereas OPC having semi-differentiated cytoplasm with no intermediate filaments, few cisternae of RER and some heterochromatin in nucleus. Some were present around demyelinated axons in white matter. Therefore, the presence of glial restricted progenitors (GRP) has been further confirmed by the ultrastructural analysis. Similarly, identification and characterization of GRP in developing mammalian cord (Rao *et al.*, 1998) also favors the hypothesis that there is creation of an environment similar to embryonic one in regenerating zebrafish cord.

Presence of multiple progenitors have been characterized ultrastructurally and confirmed by their expression of several progenitor markers like Sox2, vimentin and nestin (Lam *et al.*, 2009). Although some of these markers are expressed in uninjured CNS of zebrafish, significant upregulation of these markers in injured cord suggests that different progenitor(s), both glial and neuronal, are generated after injury. These cells include radial glia, OPC, Schwann cell progenitors with increased proliferation and all contribute to generation of lost cells due to SCI.

We also show here that there are increased expressions of progenitor markers like Sox2 and neuronal markers like HuC/D, suggesting more specifically that there are generations of neuronal progenitors in zebrafish cord after injury. This observation is further confirmed by high level of expression of another stem cell marker *pou5f1* (mammalian homolog of Oct4) after zebrafish SCI, which is essential for proliferation and neurogenesis (Hui *et al.*, 2014). Other important progenitors contributing to regenerating cord are radial glia, which also express several stem cell markers like Sox2 and vimentin. It has been demonstrated

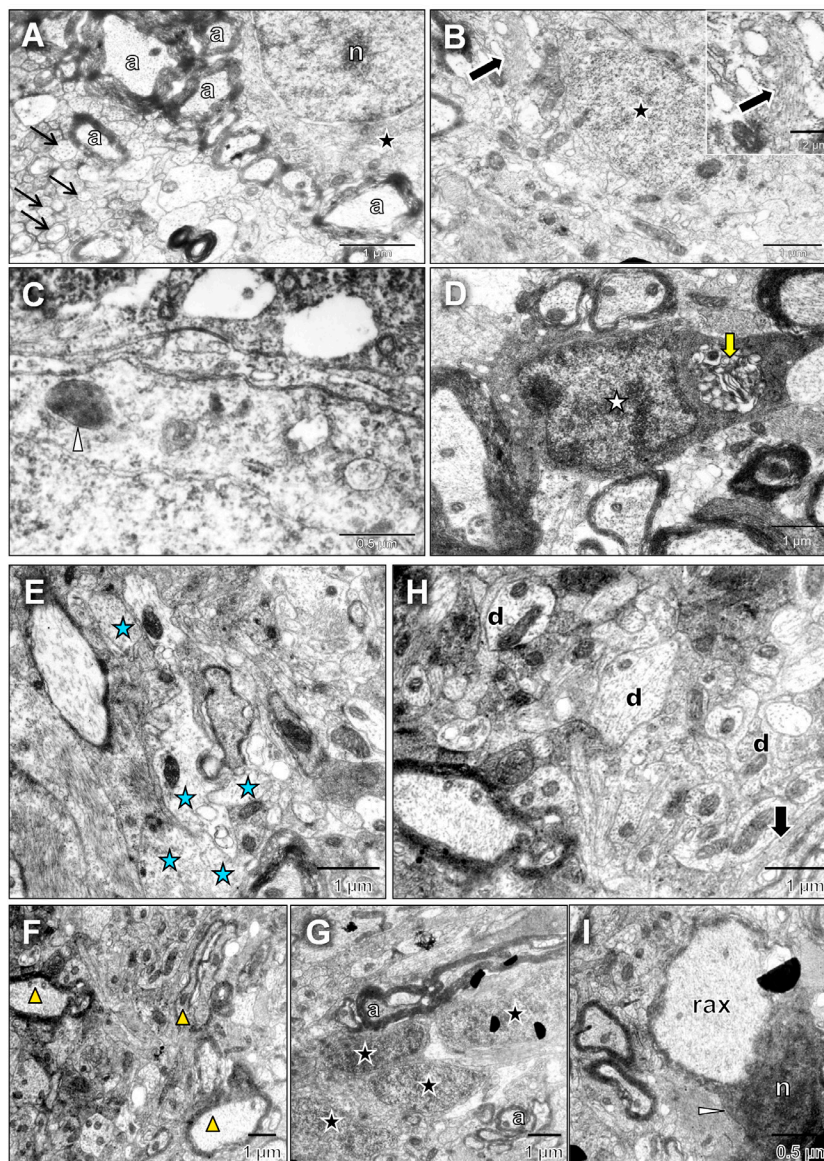


Fig. 9. Late injury response in the regenerated cord. (A) TEM image of a 15 dpi cord section showing white matter with many unmyelinated axons (black arrows), several myelinated axons (a) and an astroglial cell (star) with a pale nucleus (n). **(B)** Another 15 day regenerated cord showing an astroglial cell (star) with filaments (arrow). Inset: High magnification view of filaments (arrow). **(C,D)** 15 day regenerated cord showing (C) a microglial cell with few lysosomes (white arrowhead) and (D) a macrophage (star) with engulfed myelin debris (yellow arrow) in the cytoplasm. **(E,F)** TEM images of one month regenerated cord showing white matter with many unmyelinated axons (E, blue star) and myelinated axons (F, yellow triangle). **(G)** Astroglial progenitors (star) and adjacent axons (a) in the white matter. **(H)** The neuropil region contains many dendritic profiles (d) and an unidentified cell process (arrow). **(I)** The nucleus (n) of a Schwann cell and with the basal membrane (arrowhead) is wrapping around the remyelinating axon (rax). Scale bar, 1 µm (A, B, D, E, F, G and H); 0.5 µm (C and I).

that sustained expression of Sox2 confers the progenitors to regenerative mode as observed in Müllerian glia in zebrafish and medaka retina (Ramachandran, 2010) and similarly radial glia in zebrafish spinal cord (Hui *et al.*, 2014, 2015; Ogai *et al.*, 2014) express Sox2 conferring plasticity to these cells.

Astroglial response to injury

In zebrafish, radial glia persist throughout life, whereas in mammals they are found in developing CNS and are later transformed into astrocytes (Rakic, 2003; Malatesta *et al.*, 2008). The presence of stellate astrocyte in zebrafish CNS is debatable. Although some reported absence of stellate astrocytes in the zebrafish brain (Grupp *et al.*, 2010) others showed presence of small astrocytes with no axons (Kawai *et al.*, 2001) in spinal cord. Lack of parenchymal astrocytes and presence of radial glia as the major form of astroglia in zebrafish CNS is confirmed by the present analysis, as reported by others (Hui *et al.*, 2010; Briona and Dorsky, 2014; Becker and Becker, 2015; Becker *et al.*, 2018). Furthermore, radial glia are spinal neural stem cells known to generate both neuron and glia, and many of these are at a quiescent stage (Kriegstein and Alvarez-Buyella, 2009; Hui *et al.*, 2010; Malatesta and Gotz, 2013; Becker and Becker, 2015; Becker *et al.*, 2018).

The zebrafish spinal cord has unique response to injury where radial glia exhibit both gliotic and regenerative potential. Understanding and manipulating both gliotic changes and regenerative properties of the progenitor(s) would allow us to generate a therapeutic potential for SCI in higher vertebrates. Astrocytes in mammals support neuronal function by promoting synaptic contact thereby regulating the flow of ions and neurotransmitters and hence providing metabolic substrate for neurons and maintaining BBB (Sun and Jakobs, 2012). Secondary injury response in CNS involves astrocytes, which play a key role in reuptake of glutamate from synapses to protect neurons which are highly vulnerable to excitotoxicity (Palmer *et al.*, 1993). Mammalian CNS injury reactions involve microglia, astrocytes and OPC. Astrocytes contribute to neuropathology through astrogliosis when they react to injury by hypertrophy of somata and show increased synthesis of GFAP, re-expression of other markers like nestin and vimentin (Bignami and Dahl, 1976; Sofroniew, 2005; Dimou and Gotz, 2014). Based on the diverse response of astroglia after injury and in disease conditions, two functionally distinct categories of reactive astrocytes have been proposed, namely scar forming reactive astrocytes and hypertrophic reactive astrocytes (Sofroniew, 2009; Khakh and Sofroniew, 2015). Here astroglial cells in zebrafish show hypertrophy after injury, yet are not forming scar and may be involved in protecting neurons and synapses.

In zebrafish CNS, injury response involves limited wound size and inflammation yet no formation of astroglial scar. We show some feature of gliosis after SCI, like marked glial hypertrophy, cell cycle re-entry and increased expression of various markers like vimentin, glutamine synthetase (GS), GFAP in radial glial cells (Hui *et al.*, 2010, 2015). Similar to mammalian reactive Müllerian glia, where these cells may retain their normal metabolic function even when proliferating (Vazquez-chona *et al.*, 2011), zebrafish radial glia may undergo dedifferentiation (Hui *et al.*, 2015) but retain normal glial homeostasis. The observed features of gliosis after zebrafish SCI are similar to that in mammals. Our ultrastructural analysis confirmed the features of gliosis. Many of these features are also observed in other fish (Bernstein and Bernstein, 1967;

Bignami *et al.*, 1974; Kalman, 2004).

Present analysis revealed presence of hypertrophic swollen astroglia with conspicuous nucleoli and many processes containing increased cellular organelles, like mitochondria, Golgi bodies with more cisternae and RER. Several other features observed are increased content of intermediate filaments and glycogen granules and many junctional complexes. These features may be suggestive of their contribution to the energy metabolism of neurons (Koizumi, 1974; Magistretti *et al.*, 1993). Both hypertrophic astroglia in injured cord and normal astroglia in uninjured cord are connected to each other by desmosomes and gap junctions, similar to what has been observed in mature mammalian astrocytes. Presence of neuropeptide vesicles in astroglia also indicates their role in neurotransmitter recycling. Association of these cells around excitatory synapses may point towards their function in the maintenance of synapses.

Unlike mammals, zebrafish have remarkable capacity to regenerate neurons in many parts of the CNS. As mentioned previously, the radial glia act as stem cells to generate appropriate progenitors for regeneration (Reimer *et al.*, 2008; Briona and Dorsky, 2014; Hui *et al.*, 2015; Becker and Becker, 2015; Becker *et al.*, 2018). Under normal conditions, these glia are responsible for support and maintenance of neurons as they regulate glucose metabolism and control recycling of the neurotransmitter glutamate, blood flow and water homeostasis (Nagelhus *et al.*, 1998; Grupp *et al.*, 2010; Kettenmann and Ransom, 2012). These cells are indispensable for GABAergic and glutamatergic synaptic transmission as they control the supply of neurotransmitter precursor molecules (Verkhatsky *et al.*, 2015). Neurotransmitter mediated regeneration of zebrafish retina (Rao *et al.*, 2017) involve Müllerian glia and inhibition of GABA signaling facilitate Müllerian glia proliferation. Whereas dopamine and serotonin from brain induce spinal motor neuron regeneration in zebrafish (Reimer *et al.*, 2013; Barreiro-Iglesias *et al.*, 2015) by modulating regenerative neurogenesis. We observed successful regeneration of GABAergic and glutamatergic synapses and glia-neuronal interaction is also evident.

Late response to injury

The late response during regeneration consists of regrowth and remyelination of axons. One of the injury responses is denudation of damaged axons that we have seen immediately after injury. Ultrastructural characters shown here include extensive degenerative changes with many damaged axons containing very dense neurofilaments and ample mitochondria. The role of mitochondria in controlling the oxidative energy metabolism during CNS regeneration has not been elucidated and needs future attention. There is functional recovery after SCI in zebrafish, although not all the damaged axons can regrow and remyelinate. The volume of white matter in regenerated cord does not reach 100 % (Hui *et al.*, 2010). Thickness of myelin sheath in remyelinated axons are always less compared to uninjured axons, as confirmed by G-ratio. Schwann cells, the PNS glia, are highly versatile and may perform similar function like myelin formation, synaptic junction ensheathing, etc in zebrafish CNS. We confirmed the presence of Schwann cell progenitors and involvement of these cells in remyelination as shown by Nona *et al.*, (1992) during goldfish optic nerve regeneration. Although synapse regeneration is important in rescuing neuronal function after SCI, very little is known about the structures and molecular identity of regenerated synapse. There are some reports on synaptic regeneration in various other species in

lampreys, leech and *Drosophila* (Miladinic *et al.*, 2009; Oliphint *et al.*, 2010) showing that regenerating CNS axons form appropriate synapses. Our data represent regeneration of different types of synapses such as GABAergic and glutamatergic synapses. We locate ultrastructure of these structures in injury epicenter, in particular GABAergic synapse of Kolmer-Agduhr interneurons, which are located near the ependyma, as observed by others (Bernhardt *et al.*, 1992). However, more exhaustive quantitative analysis like number, axonal distribution and molecular composition along with ultrastructural features need to be studied in this species. We showed basic structural characteristic of regenerated synapses that may have contributed to functional recovery after SCI.

In conclusion, we suggest that there should be a combinatorial approach to understand the injury response in the zebrafish CNS. Future studies may address and emphasize on uncovering various aspects viz. a) the specific role of neuronal as well glial progenitors in generating new neurons and glia, b) in-depth analysis of the role of macrophages in controlling deleterious role of neuroinflammation and enhancement of regeneration, c) how the beneficial role of astrogliosis could be augmented and d) elucidating the molecular and cellular control underlying induction of axonal regrowth and remyelination. Here, we gave a most comprehensive ultrastructural account of different cellular events during regeneration of zebrafish cord, which may shed more light on our present understanding and generating therapeutic strategies. However, more attention has to be given to study the intricate details of intrinsic regeneration capability in lower vertebrate models like urodeles and teleost fish in order to successfully induce spinal cord regeneration in higher vertebrates.

Materials and Methods

All animal experiments mentioned in this study were cleared by Institutional Animal Ethics Committee, Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, Kolkata under CPCSEA registration of Govt. of India (Registration Number CPCSEA/ORG/CH/Reg. No 925/295).

Zebrafish maintenance

Adult zebrafish of both sexes with age ranging from of 4-6 months were maintained in the Aquatic system in the animal house facility with a 14 hr light/10 hr dark cycle. They were housed in separate groups of 10-12 in each tank and maintained at a constant water temperature of 28°C along with a standard feeding regime.

Surgical procedures

Adult fishes were anaesthetized for 5 min in 0.02% tricaine methanesulfonate (MS222; Sigma-Aldrich Corporation, St. Louis, MO) before performing any surgical procedures. Spinal cord injury was performed as described in Hui and Ghosh (2016). Briefly, a skin incision was made laterally at the level of the dorsal fin to expose the vertebral column, which corresponds to the 15–16th vertebrae as confirmed previously (Hui *et al.*, 2010). The spinal cord was crushed dorso-ventrally for about 1 sec with a number-5 Dumont forceps. A fine suture was used to close the muscle and cutaneous wound and fishes were immediately transferred to aquarium water and allowed to regenerate at 28°C. Regenerating spinal cord tissues (from 5-8 samples) at different time points viz. 3 day post injury (dpi), 7 dpi, 10 dpi, 15 dpi and 30 dpi were collected after terminal anaesthesia was applied to the fishes and only spinal cord tissues were dissected out by opening the bony encasing. About 2 mm-length of spinal cord including the regenerating tissue with adjacent uninjured tissue on either side was excised from the whole spinal cord.

Transmission electron microscopy

Both injured and uninjured spinal cord tissues were immediately fixed in 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.3) for 1 h at room temperature and for another 18 hs in the same fixative at 4°C. After washing with buffer, tissues were osmicated in 1% OsO₄ in 0.1M phosphate buffer and embedded in Araldite CY212. Ultrathin sections (70-80 nm) were cut using an ultramicrotome (Leica, Wetzlar, Germany) and stained with uranyl acetate and lead citrate and viewed under a Morgagni 268D electron microscope (FEI Company, Eindhoven, The Netherlands). Data represented here come from analysis of at least three tissue samples at any particular time point. Time course analysis of regeneration was performed using 5 different time points of injury, along with uninjured cord.

Immunohistochemistry

Immunostaining was performed as reported earlier (Hui *et al.*, 2010) by using primary antibodies shown to specifically recognize zebrafish. Briefly, sections were rehydrated and rinsed in phosphate buffered saline (PBS) with 0.1% Tween-20 (PBSTX). Then sections were incubated with blocking solution for 1hr (5% goat/rabbit/donkey serum, 1% BSA in PBS) at room temperature and after that with primary antibody for overnight at 4°C. All primary antibodies were diluted in PBSTX. To unmask some antigens in paraformaldehyde fixed tissues, antigen retrieval has been performed by incubating the slides in 95°C warm water bath for 15 mins in sodium-citrate buffer (pH 6.0) before incubation with the blocking buffer. The following primary and secondary antibodies were used for this study: Anti-glial fibrillary acidic protein (GFAP; dilution: 1:500, DAKO, USA, Catalogue no. 20334); Anti-BrdU (dilution: 1:300, Sigma, USA, Catalogue no. B-8434); Anti-brain lipid binding protein (BLBP; dilution: 1:400, Abcam Plc, USA, Catalogue no. Ab-27171); Anti-glutamate aspartate transporter (GLAST; dilution: 1:400, Chemicon, USA, Catalogue no. AB1782); Anti-vimentin (dilution: 1:300, Millipore, USA, Catalogue no. MAB3400); Anti-Sox2 (dilution: 1:400, Abcam USA, Catalogue no. Ab15830); Anti-HuCD (dilution: 1:50, Molecular Probes, USA, Catalogue no. A-21271); TRITC-conjugated goat anti-rabbit IgG antibody (dilution: 1:400, Jackson ImmunoResearch Laboratories, USA); FITC-conjugated goat antirabbit IgG antibody (dilution: 1:400, Jackson ImmunoResearch Laboratories, USA); FITC-conjugated goat anti-mouse (dilution: 1:200, Santa Cruz Biotechnology, CA, USA); TRITC-conjugated goat anti-mouse (dilution: 1:400, Jackson ImmunoResearch Laboratories, USA). Nuclei were stained either with 4',6-diamidino-2-phenylindole (DAPI) or with Hoechst (Sigma, USA). BrdU immunohistochemistry was performed according to previous communication (Hui *et al.*, 2010).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent, and cDNA was subsequently synthesized either with the Transcriptor first strand cDNA synthesis kit (Roche, Basel, Switzerland) or SensiFAST cDNA Synthesis Kit (Bioline, Australia). qRT-PCR was performed using a LightCycler 480 system (Roche, Basel, Switzerland). The amount of cDNA was normalized according to *actb2* amplification in qRT-PCR. All qRT-PCR were performed using SYBRTM Select Master Mix (Applied Biosystems, CA, USA) and results obtained from at least 5 biological replicates. The fold induction was calculated by setting control conditions to 1. The following primer sequences were used for the qRT-PCR analysis *vim*: Forward [5'-aggaggccaatgattaccg-3'], Reverse [5'-acgctccagagactcattcg-3']; *gfap*: Forward [5'-aaatgccacactacaggaag-3'], Reverse [5'-tctgcaccggaacagtgtat-3']; *nes*: Forward [5'-agaccaacac-caaacaagaccg-3'], Reverse [5'-acacagaactctgagaaggca-3'].

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