

Histological and molecular analysis of cerebellar regeneration in zebrafish (*Danio rerio*): Correlation of inflammatory response with activation of developmental pathways

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ABSTRACT

Objective(s): This study aimed to characterize the temporal relationship among inflammatory responses, developmental gene reactivation, and structural restoration during cerebellar regeneration after mechanical injury in adult zebrafish.

Materials and Methods: A focal cerebellar lesion was induced in 300 adult zebrafish using a stereotaxic approach with a 30-gauge needle. Histological analyses were performed at 1, 7, 14, 21, and 28 days post-injury (dpi) using hematoxylin and eosin staining. Quantitative RT-PCR was used to assess the expression of inflammatory genes (*tnf-α*, *il-1β*, and *il-10*) and regeneration-associated genes (*shha*, *pax5*, *neurod*, *slc17a7a*, *ca8*, and *aldoca*) at defined post-injury intervals.

Results: Histological examination revealed severe tissue disruption and inflammatory cell infiltration at 1 dpi, followed by progressive reorganization of cerebellar architecture by 28 dpi. Expression of *tnf-α* and *il-1β* increased transiently during the acute phase, peaking at 18-24 hr post-injury before returning to basal levels. In contrast, *il-10* showed a delayed but robust induction, reaching maximal expression at 18 hr and remaining elevated during the resolution phase. Developmental and neurogenic genes displayed distinct temporal profiles: *shha* and *neurod* peaked at 7 dpi; *pax5* and *slc17a7a* reached maximal expression at 14 dpi; whereas *aldoca* showed the strongest early induction. Quantitative analysis further confirmed a significant reduction in lesion size throughout the regeneration period.

Conclusion: Zebrafish cerebellar regeneration involves a coordinated sequence of inflammatory, developmental, and neurogenic events. The temporal integration of immune modulation with reactivation of developmental pathways appears to be essential for successful restoration and provides insights into strategies for neural repair in vertebrates.

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Introduction

Regeneration has long been recognized as the ability to regenerate part or all of a lost organ in some animals (1). Repair capacity varies widely among vertebrates: some fish—including zebrafish—and some amphibians show robust repair capabilities. At the same time, mammals have limited regenerative capacity (except in cases such as antler growth or partial liver repair). This difference has allowed comparative studies between species to provide valuable insights into conserved molecular mechanisms such as the Wnt, FGF, Hedgehog, and JAK/STAT pathways (2, 3).

The zebrafish, also known as *Danio rerio*, is a small freshwater bony fish that inhabits streams and ponds across the Himalayan region of South Asia, including Myanmar, Bangladesh, Pakistan, India, and Nepal. It is a member of the family Cyprinidae (carps), which includes a large group of freshwater bony fishes (4, 5), and has been introduced as a vertebrate model for biomedical research (6, 7). Zebrafish

have the potential to regenerate almost all organs and tissues, including the heart, fins, retina, brain, liver, spinal cord, kidney, notochord, gills, pancreas, lateral line (hair cells), and central nervous system (2, 8, 9, 10).

Studies on brain repair in zebrafish after injury have shown that complex, multi-step neural reprogramming occurs in this fish (11), followed by activation of radial glial cells in the brain, proliferation, and generation of new neurons (12, 13). These findings indicate that zebrafish is a suitable model for studying central nervous system regeneration, and that repair studies on this fish can provide valuable information about nervous system regeneration through interspecies comparison (9, 14).

The cerebellum is a distinct region of the brain and an important motor and sensory projection structure found in jawed vertebrates (gnathostomes)(11, 15). The zebrafish cerebellum has three major divisions: the Corpus cerebelli, the Valvula cerebelli, and the Lobus caudalis cerebelli, which

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have a layering pattern similar to that of other vertebrates but simpler than that of mammals. In these fish, the corpus cerebellum is thought to be homologous to the vermis of other vertebrates in the midline (16). In this species, the corpus cerebelli extends mainly downward and ventrally (17, 18). The cerebellar valve in zebrafish is a prominent structure that extends into the mesencephalic ventricle and covers part of the optic tectum (19, 20). According to previous studies, during the stages of cerebellar development in zebrafish, key genes such as *atoh1a/atoh1b* for the differentiation of granule neurons, *ptf1a* for the Purkinje layer, and *fgf8* as a center of tectum-cerebellar signaling play essential roles (21, 22). In addition, the genes *shha* and *pax5* are key regulators of patterning and early development of brain regions in zebrafish; *Shh* protein is involved in patterning and neurogenesis pathways and initiates early signaling, while the *Pax5* protein functions in identifying intermediate regions and demarcating posterior brain structures, and also supports the maintenance of precursors (22, 23). Genes such as *neurod*, *slc17a7a*, *aldoca*, and *ca8* are also among the genes that have been identified in transcriptomic studies related to posterior brain structures in this species. *Neurod* drives robust neuronal differentiation, and *Slc17a7a*, *Ca8*, and *Aldoca* are involved in reestablishing synaptic and metabolic functions (24). These genes are important indicators of neuronal differentiation pathways, synaptic activity, and functional organization of granule layers and Purkinje cells in the cerebellum.

According to previous studies, the zebrafish cerebellum has a well-established regenerative capacity after injury, which is mainly carried out by neural progenitor cells and radial glia (25). The stem cells active in this area mainly include cerebellar radial glia and progenitors of the Proliferative Cerebellar Zone (PCZ), which retain the ability to proliferate, self-renew, and differentiate into new neurons. Studies have shown that radial glia in zebrafish not only have a structural role, but are also recognized as adult stem cells by expressing markers such as *GFAP*, *SOX2*, and *HER4.1*, and enter the cell cycle and activate neuronal pathways after injury (12, 26).

After tissue injury, DAMPs (damage-associated molecular patterns) are released. The immune system is stimulated and inflammatory responses occur. Proinflammatory cytokines (*IL-1 β* and *TNF- α*) stimulate the synthesis and release of inflammatory mediators, including chemokines (27). Based on the chemokine concentration gradient, leukocytes migrate to the site of inflammation to treat the damaged tissue (28). Anti-inflammatory responses (*IL-10*) should inhibit inflammation, because prolonged and unresolved inflammation increases fibrotic responses and reduces functional compensation and regeneration. As a result, inflammatory processes are actively interrupted by functional reprogramming of the involved cells, leading to the restoration of homeostasis (27). At this stage, the response enters the resolution phase, which is accompanied by apoptosis and reverse migration of neutrophils to the site of injury (29).

Studying the mechanisms of tissue regeneration in the zebrafish cerebellum is particularly important, as the zebrafish cerebellum can provide a valuable model for understanding the intrinsic mechanisms of neural regeneration in vertebrates. Identifying the molecular pathways that drive regeneration in this model could

provide new insights into how stem cells are activated, neurons regenerated, and neural circuits rewired.

Given that the repair process in zebrafish occurs widely in many tissues, limited studies have been conducted on cerebellar tissue repair in this species. Therefore, in this study, we examined the repair process in the damaged area by preparing tissue sections at different days after physical injury to the cerebellum. Because tissue repair can resemble a resumption of developmental processes in the damaged area, the aim of the present study was to investigate repair responses in the zebrafish cerebellum after injury using molecular approaches. Therefore, the quantitative expression levels of *shha*, *pax5*, *neurod*, *ca8*, *aldoca* (*vglut1*), and *slc17a7a* genes were measured at different days after injury. The *shha* and *pax5* genes are key regulators of cerebellar patterning and demarcation in early development and play an important role in controlling progenitor cell proliferation. In later stages of development, *neurod*, *ca8*, and *aldoca* genes direct the differentiation of granule and Purkinje neurons and the functional maturation of cerebellar circuits, while *slc17a7a* (*vglut1*), as an indicator of glutamate transmission, indicates the expression of mature granule neurons and the formation of synaptic circuits. On the other hand, since early inflammation plays a vital role in the initiation of the neural regeneration process in zebrafish, in this study, the expression of inflammatory genes *tnf- α* and *il-1 β* , as well as anti-inflammatory *il-10*, was also examined during the repair process at different hours and days. The results revealed interactions among neurogenesis, tissue repair, and inflammation and showed that precise coordination between pro-inflammatory and anti-inflammatory responses is essential for initiating and promoting effective regeneration.

Although previous studies have independently described inflammatory responses, neural progenitor activation, or Purkinje cell regeneration in the zebrafish cerebellum, a comprehensive temporal analysis integrating histological recovery with inflammatory and developmental gene expression during mechanical cerebellar injury remains limited. In contrast to previous studies that focused on specific cellular populations or single pathways (20, 30, 31), the present study simultaneously characterizes inflammatory, developmental, neurogenic, and metabolic gene responses alongside structural regeneration throughout the entire repair period. This integrated approach provides a temporal framework linking immune modulation with development.

Materials and Methods

Animal phase and fish maintenance (Stereotaxic lesion in adult zebrafish cerebellum)

A total of 300 adult zebrafish with an average age of 6 months were purchased from ornamental fish breeding centers and transferred to tanks containing filtered and dechlorinated water under standard conditions at a temperature of $28 \pm 1^\circ\text{C}$ and a photoperiod of 14 hr light - 10 hr dark to simulate the natural life cycles of fish. Feeding was done twice a day using a combination of commercially available dry food and freshly hatched brine shrimp. Before using the fish in the experiment, they were acclimatized to the environment for one week (32).

Of the total number of fish, 150 were assigned to the injured groups and 150 served as uninjured controls.

Injured and control fish were housed in separate tanks under identical environmental conditions. All experimental procedures were conducted in accordance with institutional and national guidelines for the care and use of laboratory animals and were approved by the local Ethics Committee (IR.GUILAN.REC.1400.20).

To induce cerebellar injury, the fish were anesthetized by adding tricaine methanesulfonate (MS-222; Sigma, USA) to the water in the tanks containing the fish. After complete elimination of the reflex response, a focal lesion was induced in the cerebellum of adult zebrafish using a stereotaxic approach with a 30 G insulin syringe (Figure 1). Fish were positioned and stabilized, and coordinates were defined relative to the midpoint between the eyes, which served as the reference point. Based on preliminary atlas-guided targeting (19), the lesion was directed toward the cerebellar region, primarily affecting the corpus cerebelli (CCe) and lobus caudalis cerebelli (LCa). Approximate stereotaxic coordinates were AP: -1.5 mm (posterior to the reference point), ML: 0-0.3 mm (lateral from the midline), and DV: 0.8-1.0 mm (from the skull surface), adjusted according to fish size.

Lesion induction was verified macroscopically at the site of injury and further confirmed by histological analysis using serial brain sections. The anatomical location of the lesion was validated by comparison with the adult zebrafish brain atlas, ensuring accurate targeting of cerebellar subregions.

After injury, the fish were placed directly into recovery tanks with fresh, oxygenated water and continuously monitored for behavior until they returned to normal swimming activity. They were then kept under normal rearing conditions until sampling. Control groups did not receive any cerebellar lesions.

Lesion diameter was quantified using ImageJ software (NIH, USA) after calibration with the scale bar and expressed as mean $\pm$ SD.

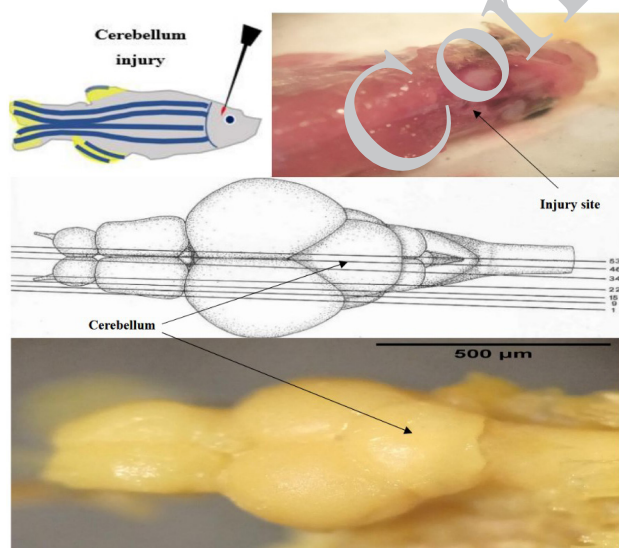


Figure 1. Schematic and anatomical orientation of the dissected zebrafish brain after cerebellar injury

A) Schematic illustration of the insulin syringe entry site into the zebrafish brain. B) The cerebellar injury site is clearly visible, as indicated by the arrow. C) Schematic and macroscopic images showing the position of the cerebellum in the zebrafish. Scale bar: 500 μ m

Behavioral assessment

Behavioral recovery was qualitatively assessed using swimming depth preference and social interaction patterns. Swimming depth was monitored as an indicator of post-injury motor control and anxiety-related behavior, while social behavior was evaluated based on the tendency of injured fish to swim individually or in groups.

Histological examination of cerebellar tissue repair at different days after injury

For histological analysis, 50 fish were subjected to cerebellar injury and maintained in a dedicated tank. In parallel, 50 control fish were anesthetized but not injured and were maintained under identical conditions in a separate tank. At each experimental time point (1, 7, 14, 21, and 28 days post-injury), 10 injured fish were randomly selected for tissue collection and histological processing. In addition, 10 control fish were analyzed for baseline comparison.

Fish were first anesthetized on different days after injury (1 day, 7 days, 14 days, 21 days, and 28 days), and then their brain region was separated (11, 20).

The brain tissue was transferred to fresh 4% paraformaldehyde fixative (Merck, Germany) and fixed overnight at 4 °C. The tissues were then washed with phosphate-buffered saline (PBS) and subjected to ascending concentrations of ethanol (70% to 100%) for one hour each. After clearing with xylol, the tissue was embedded in paraffin, and paraffin blocks were prepared from the brain tissue. The brain tissue was then cut sagittally using a rotary microtome (Leica, Germany), and 5 μ m sections were prepared.

Tissue sections were placed on poly-L-lysine-coated slides to enhance adhesion, air-dried at 37 °C overnight, and routinely stained with hematoxylin and eosin (H&E). H&E staining provided a simple method for observing overall cerebellar cellular structure, injury boundaries, cellular infiltration, and post-repair events. Therefore, stained slides were photographed with a light microscope equipped with a digital imaging system (Olympus, Japan), and morphological comparisons were made between the control and treatment groups. Lesion size was quantified by measuring the longitudinal diameter of the injured area using ImageJ software (NIH, USA) on calibrated images based on a 100 μ m scale bar.

RNA extraction from cerebellar tissue in treatment and control groups

For gene expression analyses, a total of 102 injured fish and 102 control fish were used. Injured and control fish were maintained in separate tanks under identical conditions. At each analyzed time point, cerebellar tissue was collected from three injured fish to generate three independent biological replicates. Similarly, three control fish were sampled at each corresponding time point to serve as controls.

Since repair processes at the molecular level can include the resumption of expression of genes related to early stages of development in zebrafish, in this study, the expression of some developmental genes (*shha*, *pax5*, *aldoca*, *slc17a7a*, *neurod*, *ca8*) relative to the *gapdh* reference gene in cerebellar tissue of fish from both injured and control groups was analyzed at the same time points after injury (developmental genes on days 1, 7, 14, 21 and 28).

In contrast, after injury, proinflammatory factors (tnf- α ,

il-1 β) and, finally, anti-inflammatory factors (il-10) also changed in fish. In this study, the expression of related genes was also analyzed during different days after injury for *tnf- α* and *il-1 β* genes at 2, 4, 6, 12, 18, 24, and 72 hr, and for the il-10 gene in the presence and absence of metronidazole at 6, 12, 18, 24, and 72 hr.

In this study, metronidazole treatment was also included to evaluate its influence on anti-inflammatory responses during regeneration, based on previous studies employing metronidazole-mediated immune cell modulation in zebrafish models. In fact, the rationale for metronidazole administration was to investigate its modulatory effect on inflammatory resolution during cerebellar regeneration.

For this purpose, all samples were lysed and homogenized in Vitisol, and RNA was isolated using a phenol-chloroform-based reagent (Sinaclone, Iran) according to the instructions. To determine the integrity of RNA in the extracted samples, the concentration of the samples was first measured spectrophotometrically (NanoDrop, Thermo Fisher, USA). Then, the appearance of distinct 18S and 28S rRNA bands was tested on a 1% agarose gel. Three distinct bands corresponding to the 5.8S, 18S, and 28S ribosomal RNA subunits were identified on the gel, indicating intact and high-quality RNA. qRT-PCR products for *gapdh*, *slc17a7a*, *pax5*, and *shha* genes were determined using agarose gel electrophoresis 2%)(Figure 2).

cDNA Synthesis

For cDNA synthesis, a commercial cDNA synthesis kit (Bioedit, Iran) was used with OligodT primers. According to the RNA concentration for each sample, the appropriate amount of RNA was transferred to a 0.2 ml sterile microcentrifuge tube. The final volume was adjusted to 20 μ l with nuclease-free water by adding the items mentioned in the kit to this tube. The tube contents were mixed carefully and incubated for 60 min at 55 $^{\circ}$ C to perform reverse transcription. Enzyme inactivation was subsequently performed by heating the reaction mixture at 95 $^{\circ}$ C for 10 min. The synthesized cDNA was immediately stored at -20 $^{\circ}$ C until used in qRT-PCR experiments.

Quantitative RT-PCR and expression of targeted genes

We quantified the expression of genes related to cerebellar repair using qRT-PCR with a Bio-Rad iCycler (Bio-Rad, USA). The reaction mixture consisted of 1 μ l cDNA, 0.5 μ l

forward primer, 0.5 μ l reverse primer, 6.25 μ l SYBR Green Master Mix, and the volume was made up to 12.5 μ l with nuclease-free water.

To perform qRT-PCR, the initial DNA denaturation was performed at 95 $^{\circ}$ C for 5 min. Then, the device was set for the following steps during 40 cycles: 95 $^{\circ}$ C for 15 seconds, 56-60 $^{\circ}$ C for 30 seconds, and 72 $^{\circ}$ C for 30 seconds. Finally, in the last cycle for melting curve analysis to confirm specificity, the device was heated to 95 $^{\circ}$ C for 10 seconds, then to 65 $^{\circ}$ C for 60 seconds, and then to 97 $^{\circ}$ C for 1 second (33).

In this study, we assessed the expression of developmental genes (*shha*, *pax5*, *neurod*, *slc17a7a*, *ca8*, *aldoca*) and inflammatory genes (*tnf- α* , *il-1 β* , *il-10*) after injury using this technique. Each gene expression analysis was performed using three biological replicates per time point, with two technical replicates per sample.

Data were normalized against housekeeping genes and quantified using the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in Table 1.

Table 1. Primer sequences used in quantitative real-time polymerase chain reaction (qRT-PCR)

	Primers	Sequence	Length	Tm	GC (%)
<i>il10</i>	Forward	CGGGATATGGTGAAATGCAAG	21	57.37	47.62
	Reverse	AAAGAGCAAATCAAGCTCCC	20	56.30	45.00
<i>il-1β</i>	Forward	CGCTCCACATCTCGTACTCA	20	59.27	55.00
	Reverse	CATCTCCACCATCTGCGAAT	20	57.75	50.00
<i>tnf-α</i>	Forward	GGAGAGTTGCGCTTTACCGCT	20	60.04	55.00
	Reverse	TGTTGATTGCCCTGGGTCTTA	21	59.29	47.62
<i>aldoca</i>	Forward	GGGCTCTTACCTTCTCATTG	21	58.17	52.38
	Reverse	ACTTCCCTTGAGATGCCAGAC	21	59.72	52.38
<i>slc17a7a</i>	Forward	GGCGCATTTCTTTACCTCC	20	59.26	55.00
	Reverse	GTTGTCTCCGATTGGCACA	20	60.61	55.00
<i>neurod</i>	Forward	GCCACACTAGACTTCAATCCG	21	58.73	52.38
	Reverse	TTCTCGCTGTATGACTTCGT	21	59.18	47.62
<i>shha</i>	Forward	AAAGCCCACATTCATTGCTCT	21	58.47	42.86
	Reverse	ATGATGAAGTCGCTGAACACCA	22	60.29	45.45
<i>ca8</i>	Forward	GCAGCCTTCCAGTAACACC	19	58.44	57.89
	Reverse	TTACATAGCCAGGATGTTGT	21	57.28	42.86
<i>pax5</i>	Forward	TCTGGCAGTCCTTATTTCCCATC	22	59.56	50.00
	Reverse	TGACCAGGAAATAGTCCGAT	21	58.24	47.62
<i>gapdh</i>	Forward	ATCCATCTTTGACGCTGGT	19	56.73	47.37
	Reverse	TCAGGTCACATACACGGTTG	20	57.55	50.00

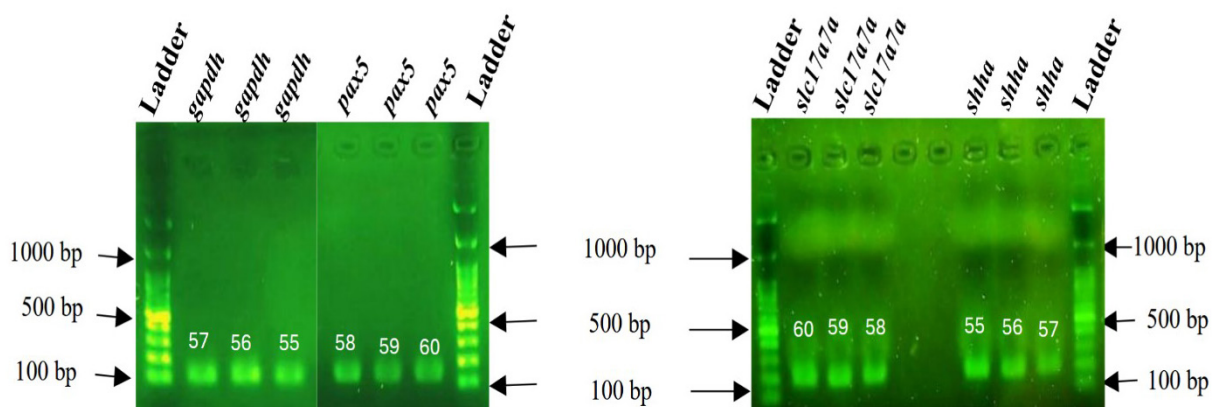


Figure 2. Visualization of qRT-PCR products for *gapdh*, *slc17a7a*, *pax5*, and *shha* genes was performed using 2% agarose gel electrophoresis. The gel was run with a 100 bp DNA ladder as a molecular marker to confirm the size of the amplified products. The temperatures shown in the figure are expressed in degrees Celsius ($^{\circ}$ C).

Data analysis

We analyzed the data using GraphPad Prism version 10.3.1 (GraphPad Software, USA). Data related to gene expression were presented as mean±standard deviation. Statistical differences between groups and at different time points were compared using one-way analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered significant.

Results

Examination of cerebellar tissue response to injury and its repair from a histological perspective. Injury was physically induced in the cerebellar tissue of fish using a vertical insulin syringe (30 G needle). Injured fish showed reduced swimming stability and a tendency to remain at the bottom of the tank during the early post-injury period. Swimming coordination gradually recovered during later stages.

After injury, the cerebellar tissue was fixed in fresh paraformaldehyde fixative solution, and serial sections of cerebellar tissue were carefully examined on different days after injury (7 dpi, 14 dpi, 21 dpi, and 28 dpi) and compared with the control group.

The results showed that cerebellar structure appeared intact in control samples. In the present study, the lesion size was approximately 0.5-0.7 mm in diameter at the microscopic level, with histological sections showing an area of ~200-400 μm at 1 hour post-injury. The lesion size was estimated using ImageJ software.

There was a clear distinction between the corpus cerebellum (CCe) and the caudal cerebellar lobe (LcA), indicating a highly ordered arrangement of neural layers and uniform cellular architecture (Figure 3A, B, C). On the first day after injury, the injury site showed obvious histological disruption, including cellular disorganization, necrosis

(based on histological indices and neuronal architectural disintegration), and inflammatory cell infiltration, with the observation of accumulation of mononuclear cells with hyperchromatic nuclei (mononuclear inflammatory cells morphologically consistent with activated microglia/macrophages) around the lesion in both the CCe and LcA areas, as indicated by arrows (Figure 3D, E). At this stage, injured fish predominantly remained near the bottom of the tank and exhibited markedly reduced swimming speed. Some individuals displayed transient circular swimming patterns, consistent with impaired motor coordination.

At day 7 after injury, in addition to long-lasting inflammation, there was evidence of early structural regeneration, with increased cell density and proliferating cells characteristic of activated radial glia around the injured area (Figure 3F, G). Concurrently, swimming activity gradually improved, with reduced incidence of abnormal movement patterns, although fish still preferentially occupied the lower regions of the tank.

At day 14 after injury, partial repair of the damaged tissue was observed, leading to delamination of neurons and reduced infiltration of inflammatory cells. Finally, as the repair process progressed, cerebellar architecture improved continuously (Figure 4A, B). At this time point, the fish showed more stable and coordinated swimming behavior and began to explore a wider range of water depths, indicating the onset of functional recovery. At day

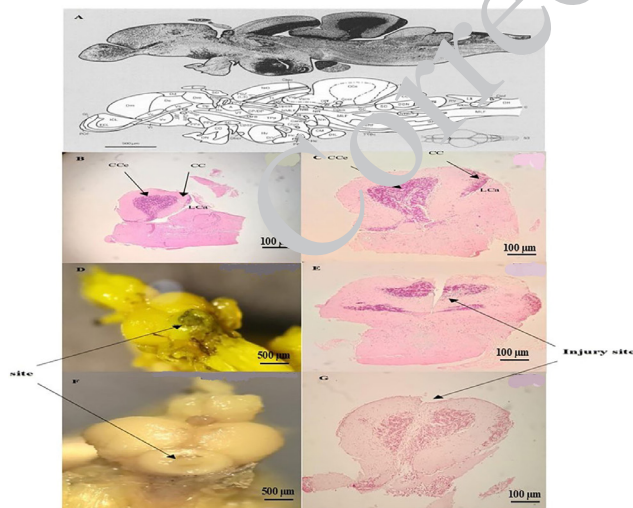


Figure 3. Tissue sections of healthy zebrafish cerebellum (control), day 1 and day 7 after injury

A: Schematic image taken from the Zebrafish Atlas showing the healthy structure of the entire brain. B and C: Tissue sections of zebrafish cerebellum showing the differentiation between CCe and LcA with well-preserved neuronal layers in a highly regular pattern. (D, E) Day 1. (D) Macroscopic image showing the location and severity of the injury. (E) Tissue section of zebrafish cerebellum showing the extent and severity of the injury. The cerebellar tissue was slightly rotated during molding, resulting in a dorsal view in the section. (F, G) Day 7. (F) Macroscopic image of the zebrafish brain under a loupe showing improved cerebellar injury compared with the day 1 sample. (G) Tissue section showing early regeneration, with increased cell density and proliferating cells consistent with radial glial activation around the lesion area. Some reorganization of tissue architecture and residual mild inflammation are also visible. Scale bars: 500 μm (macroscopic images), 100 μm (histology). H&E staining

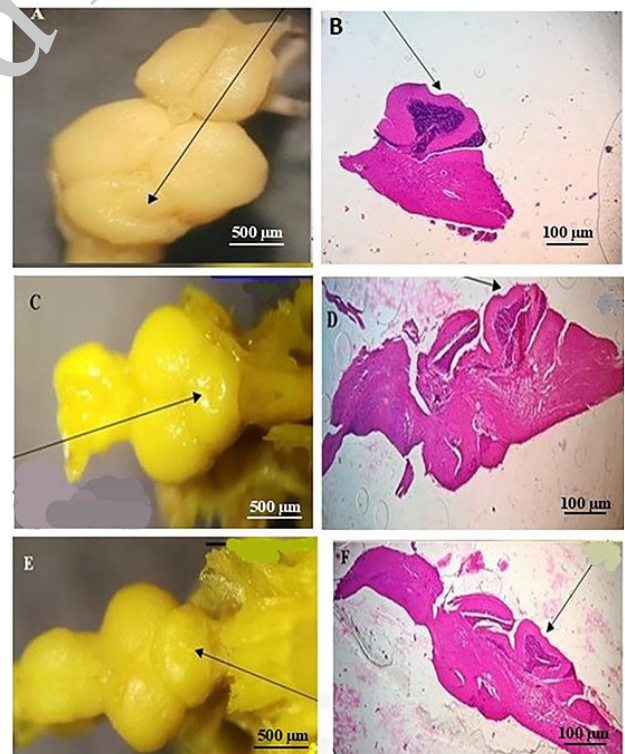


Figure 4. Macroscopic and histological evaluation of zebrafish cerebellum at days 14, 21, and 28 after injury

(A, B) Day 14: Macroscopic image (A) shows partial recovery, and a slight depression remains at the injury site. Histological section (B, H&E staining) shows partial restoration of neuronal layering and reduction of inflammation, indicating the ongoing repair process. (C, D) Day 21: External view (C) shows advanced repair with minimal surface roughness. Histological section (D) shows extensive rearrangement of cerebellar architecture and near-complete structural restoration of neuronal layers. (E, F) Day 28: Macroscopic image (E) is almost identical to control samples with no visible signs of injury, and histological section (F) confirms extensive structural tissue recovery, complete reconstruction of layers, and structural integrity. Scale: 500 μm (macroscopic images), 100 μm (histology). H&E staining

21 after injury, the injured site showed extensive healing, with cellular reorganization and minimal scarring. In fact, 28 days after injury, both functional and structural regeneration were observed in zebrafish. This stage is not observed in mammals such as mice, according to previous studies. This area was morphologically very close to the control tissue (Figure 4C, D). Behaviorally, swimming speed and depth preference had largely returned to normal, and the injured fish increasingly displayed group swimming behavior.

At day 28 after injury, the cerebellar tissue was well-established as regenerative, with structural restoration of the neural layers and an architecture indistinguishable from controls, indicating extensive structural recovery and functional integration.

At day 28 after injury, cerebellar tissue was well established as regenerative, with near-complete structural restoration of the neural layers and an architecture indistinguishable from controls, indicating extensive structural recovery and functional integration (Figure 4E, F). At this stage, no discernible differences in swimming behavior or social interaction were observed between injured and control fish. These results emphasize the strong capacity of zebrafish cerebellar tissue to repair from acute injury to structural recovery over the time scale studied.

Fish behavior was recorded for 10 min using a digital camera and analyzed visually to evaluate swimming depth and locomotor coordination.

In addition to qualitative histological observations, we quantitatively assessed the extent of tissue damage by measuring the longitudinal diameter of the lesion area at different time points after injury. Measurements were performed using ImageJ software on H&E-stained sagittal sections. For each time point, five independent sections obtained from five different fish were analyzed. The mean lesion diameter showed a progressive and significant reduction over time, decreasing from $504.1 \pm 5.7 \mu\text{m}$ at 1 dpi to $484.7 \pm 4.0 \mu\text{m}$ at 7 dpi, $230.0 \pm 3.3 \mu\text{m}$ at 14 dpi, $139.6 \pm 2.7 \mu\text{m}$ at 21 dpi, and reaching $67.0 \pm 2.2 \mu\text{m}$ at 28 dpi. This Quantitative analysis showed that lesion size was maximal at day 1 post-injury and gradually decreased during the regeneration period.

Examination of the expression of inflammatory and repair genes during cerebellar regeneration

Quantitative real-time PCR (qRT-PCR) was performed to examine the temporal dynamics of the expression of genes related to inflammation and repair in the zebrafish cerebellum after mechanical trauma induced by an insulin syringe 30. Gene expression was measured at standard time points after trauma, normalized against the housekeeping gene *gapdh*, and compared as fold changes with the uninjured control group. Fold change calculations were extracted from the $2^{-\Delta\Delta\text{CT}}$ method using Microsoft Excel, while statistical analyses and bar graph visualization were performed using GraphPad Prism. Each experimental group consisted of three biological replicates, with two technical replicates per sample.

Expression of inflammatory genes

For the proinflammatory genes *tnf- α* and *il-1 β* , expression was measured at 2, 4, 6, 12, 18, 24, and 72 hr post-injury. The anti-inflammatory gene *IL-10* was measured in two groups: a control group without metronidazole treatment

and a metronidazole-treated group. In both groups, *IL-10* was assessed at 2, 4, 6, 12, 18, 24, and 72 hr post-injury. These time points were chosen to cover the acute phase of the inflammatory response and its decline. In addition, the basal expression levels of the genes in uninjured control samples were also recorded.

il-1 β expression was close to baseline in the early hours after injury, but increased by approximately 1.5-fold at 18 hr after injection ($P < 0.01$). Its expression was also significantly increased at 24 hr post-injection ($P < 0.05$) and decreased towards control at 72 hr post-injection (Figure 5A). *tnf- α* was similar, with a significant increase at 18 hr post-injection (approximately 1.4-fold, $P < 0.01$) and a constant level at 24 hr post-injection (approximately 1.5-fold, $P < 0.01$), and then a decrease to basal levels by 72 hr post-injection (Figure 5B). In contrast, *il-10* induction was slower in amplitude but greater in magnitude. In the absence of metronidazole treatment, expression was significantly increased at 12 hr post-injection ($P < 0.01$), reached its maximum at 18 hr post-injection (>6 -fold, $P < 0.001$), and remained elevated up to 72 hr post-injection ($P < 0.01$) (Figure 6A). With treatment with metronidazole, this profile was similar but intensified, reaching approximately 7-fold at 18 hr post-injection ($P < 0.001$) and then gradually returning to baseline by 168 hr post-injection ($P < 0.05$) (Figure 6B).

Expression of genes associated with regeneration

Regeneration-related genes *shha*, *pax5*, *neurod*, *nc82*, *7a7a*, *ca8* and *aldoca* - were analyzed at 1, 7, 14, 21, and 28 days after injury to track the process of repair and neuronal differentiation. Gene expression in healthy control fish was also measured at these time points. These time points corresponded to histological markers of tissue repair and allowed correlation of molecular and structural activity. Expression of genes associated with inflammation and regeneration after cerebellar injury showed distinct yet coordinated temporal patterns.

shha and *pax5*

Expression of the *shha* gene increased moderately on day

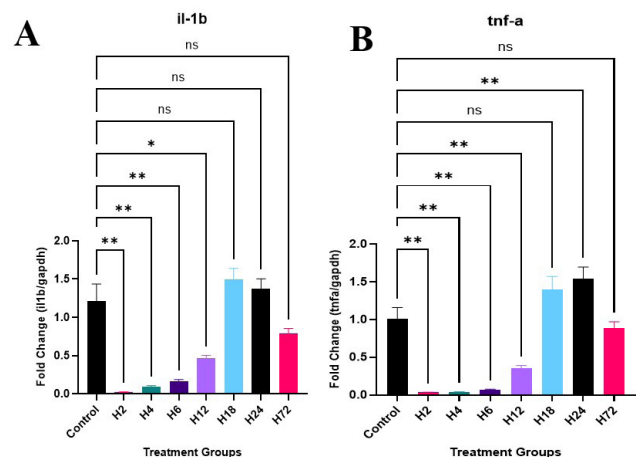


Figure 5. Time course of expression of proinflammatory cytokines in zebrafish cerebellum after injury

A) *il-1 β* showed a delayed increase, peaking at 18–24 hpi and returning to baseline at 72 hpi. B) *tnf- α* showed a similar pattern with significant induction at 18–24 hpi and normalization at 72 hpi. Values are shown as mean $\pm$ standard deviation from three biological replicates. Statistical analysis was performed using one-way ANOVA or Welch's ANOVA followed by appropriate post hoc tests. $P < 0.05$ was considered significant

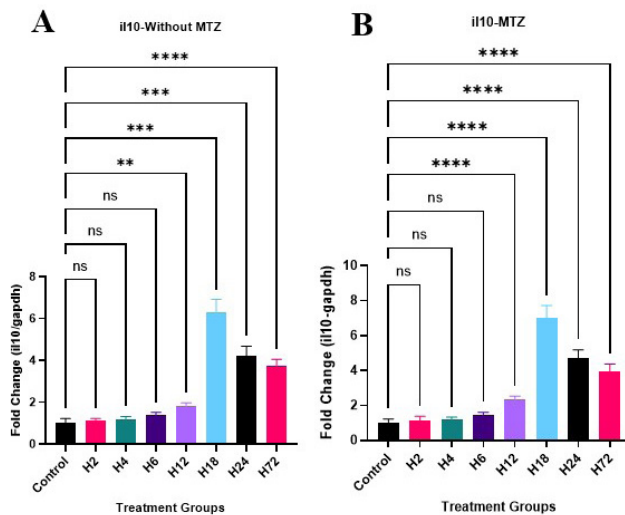


Figure 6. Dynamics of the anti-inflammatory cytokine *il-10* after cerebellar injury

A) In the absence of metronidazole, *il-10* expression increased significantly at 12 hpi, peaked at 18 hpi, and remained elevated until 72 hpi. B) In the presence of metronidazole, the same profile was observed but with a very slight increase above the peak at 18 hpi and a gradual return towards baseline by 72 hpi. Data are reported as mean±standard deviation (n=3). Statistical significance was determined using one-way ANOVA or Welch's ANOVA as appropriate

1 after injury (approximately 1.7-fold, $P<0.05$) and peaked on day 7 after injury (approximately 2.1-fold, $P<0.01$). Then, expression levels gradually decreased, and by day 14 post-injury they were reduced by about 1.6-fold ($P<0.05$). By day 28 post-injury, it fell below the baseline level (~0.2-fold, $P<0.01$)(Figure 7A).

The expression of the *pax5* gene showed a delayed but complementary profile: the expression of this gene remained close to the control on day 1 post-injury, on day 7 post-injury its expression increased significantly (~1.4-fold, $P<0.05$), on day 14 post-injury its expression reached its maximum (~1.8-fold, $P<0.01$) but subsequently decreased,

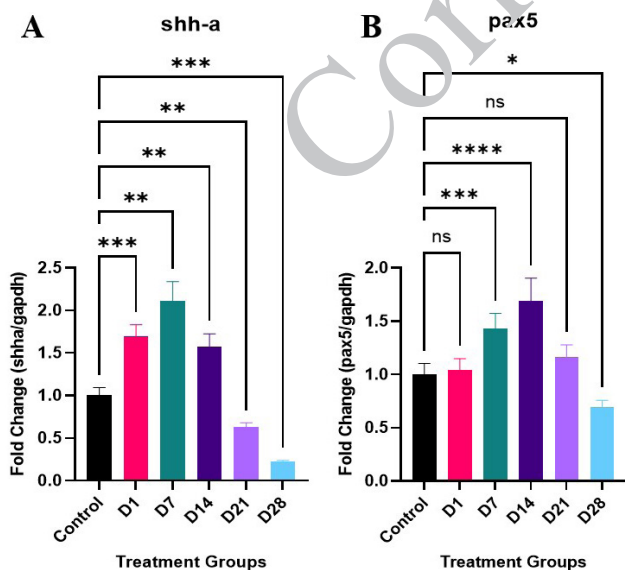


Figure 7. Dynamics of *shha* and *pax5* expression during cerebellar regeneration

(A) *shha* expression increased significantly at 1 dpi (~1.7-fold, $P<0.05$) and peaked at 7 dpi (~2.1-fold, $P<0.01$), followed by a gradual decrease to below baseline by 28 dpi (~0.2-fold, $P<0.01$). (B) *pax5* remained at baseline at 1 dpi but increased at 7 dpi (~1.4-fold, $P<0.05$) and peaked at 14 dpi (~1.8-fold, $P<0.01$). Expression then decreased at 21 dpi and reached below baseline at 28 dpi (~0.7-fold, $P<0.05$)

such that by day 21 post-injury its expression reached close to the baseline level. However, by day 28 post-injury its expression returned to below the control (~0.7-fold, $P<0.05$) (Figure 7B).

neurod and *slc17a7a*

neurod showed one of the strongest responses. Its expression was increased at 1 dpi (approximately 2.5-fold, $P<0.01$), peaked at 7 dpi (approximately 7.6-fold, $P<0.001$), and remained high at 14 dpi (approximately 6.3-fold, $P<0.001$). Its expression then decreased but was still above baseline at 21 dpi (approximately 3.4-fold, $P<0.01$). At 28 dpi, its expression fell below control (approximately 0.3-fold, $P<0.01$)(Figure 8A).

In parallel, *slc17a7a* expression showed a gradual increase, increasing by ~1.4-fold on day 1 post-injury ($P<0.05$), peaking at 14 dpi (~3.7-fold, $P<0.001$) and then slightly decreasing at 21 dpi (~2.7-fold, $P<0.01$), while at 28 dpi its expression remained unchanged (~1.5-fold, $P<0.05$) (Figure 8B).

ca8 and *aldoca*

ca8 showed a transient response, with expression increasing (~3.4-fold, $P<0.001$) at 7 dpi, decreasing (~2.1-fold, $P<0.05$) at 14 dpi, decreasing (~0.6-fold, $P<0.05$) at 21 dpi, and reaching 2.1-fold ($P<0.001$) at 28 dpi, returning to below baseline (Figure 9A). The distinct expression pattern of *ca8* gene after injury, compared to the developmental process of this fish, indicates that the same developmental pathways are used in cerebellar regeneration, but they probably adapt to the environmental conditions and changes in tissue structure at the site of injury. The *aldoca* gene showed the most dramatic induction of all genes examined, with strong expression increases at 1 dpi (~15.8-fold, $P<0.001$) and at 7 dpi (~17.8-fold, $P<0.001$). Expression

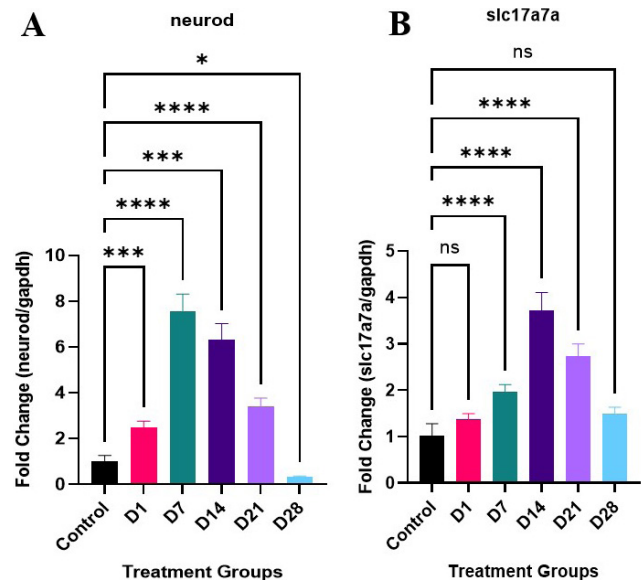


Figure 8. Expression profiles of *neurod* and *slc17a7a* genes at different stages of regeneration

(A) *neurod* showed initial induction at 1 dpi (approximately 2.5-fold, $P<0.01$), peaked at 7 dpi (approximately 7.6-fold, $P<0.001$), and remained elevated at 14 dpi (approximately 6.3-fold, $P<0.001$). Levels decreased at 21 dpi (approximately 3.4-fold, $P<0.01$) and fell below baseline at 28 dpi (approximately 0.3-fold, $P<0.01$). (B) *slc17a7a* gradually increased from 1 dpi (~1.4-fold, $P<0.05$), peaked at 14 dpi (~3.7-fold, $P<0.001$), then decreased at 21 dpi (~2.7-fold, $P<0.01$) but remained elevated at 28 dpi (~1.5-fold, $P<0.05$)

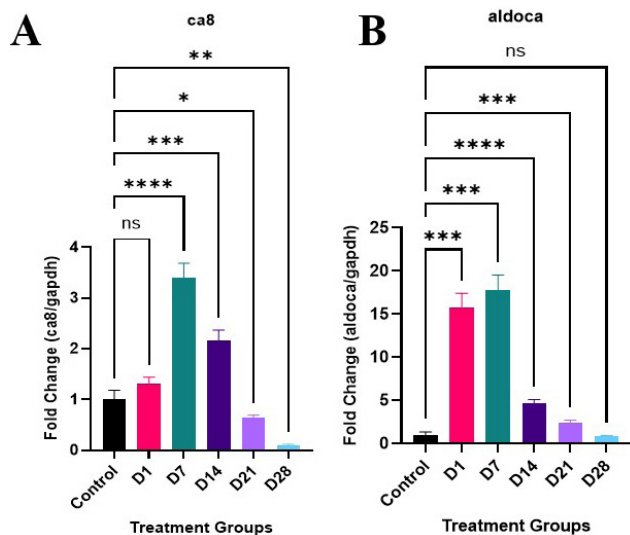


Figure 9. Temporal changes in the expression of *ca8* and *aldoca* genes during regeneration

(A) *ca8* showed a transient increase at 7 dpi (approximately 3.4-fold, $P < 0.001$), a decrease at 14 dpi (approximately 2.1-fold, $P < 0.05$), and a decrease below baseline at 21 dpi (approximately 0.6-fold, $P < 0.05$) and 28 dpi (approximately 0.1-fold, $P < 0.001$). (B) *aldoca* showed the strongest induction, increasing at 1 dpi (~15.8-fold, $P < 0.001$) and 7 dpi (~17.8-fold, $P < 0.001$), followed by a gradual decrease at 14 dpi (~4.7-fold, $P < 0.001$) and 21 dpi (~2.5-fold, $P < 0.01$), and returning to near baseline at 28 dpi (~0.9-fold, ns)

of this gene gradually decreased at 14 dpi (about 4.7-fold, $P < 0.001$) and 21 dpi (about 2.5-fold, $P < 0.01$) and returned to near baseline at 28 dpi (about 0.9-fold, ns) (Figure 9B).

Taken together, these results point to a coordinated sequence of events: *shha* initiates early signaling, *pax5* supports progenitor maintenance, *neurod* drives robust neuronal differentiation, and *slc17a7a*, *ca8*, and *aldoca* contribute to reestablishing synaptic and metabolic functions. This molecular timeline was highly consistent with histological evidence of progressive cerebellar repair. Consistent with these molecular changes, quantitative analysis of lesion size showed a progressive reduction in the extent of tissue damage over time, providing direct evidence that histological repair reflects true structural recovery rather than purely qualitative reorganization.

Temporal expression patterns of inflammatory (*il1b*, *tnfa*, *il10*) and (regeneration-associated) (*aldoca*, *ca8*, *neurod*, *pax5*, *shha*, *slc17a7a*) genes after cerebellar injury in zebrafish were measured and are summarized in two separate column-style scatter plots (Figure 10 and 11).

The temporal pattern of gene expression after injury revealed that some developmental genes behaved similarly to early development. For example, the expression of *neurod* and *slc17a7a*, which are responsible for neuronal differentiation and synaptic maturation during development, increased during the middle days of regeneration, similar to the wave of neurogenesis during embryonic development. In contrast, genes such as *pax5* and *ca8*, which are highly expressed during development, showed decreased expression after injury, which could reflect differences in developmental and repair needs. The *shha* gene also showed an initial increase similar to the progenitor proliferation phase in development.

Discussion

The cerebellum is crucial for motor coordination, balance, and motor learning, but its highly organized

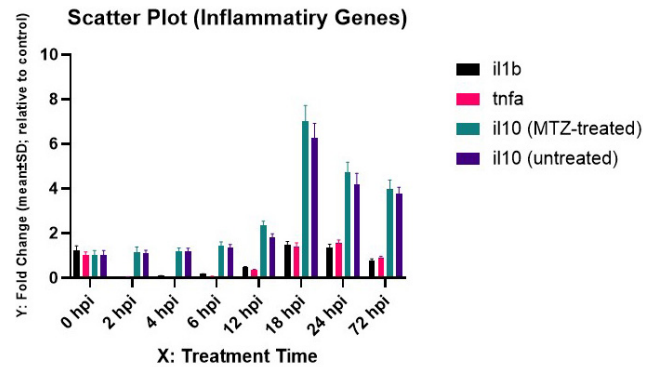


Figure 10. Scatter plot of inflammatory genes (*il1b*, *tnfa*, *il10*)

Y-axis: mean fold change (relative to control); X-axis: post-injury time points (Control/0 h, 2 h, 4 h, 6 h, 12 h, 18 h, 24 h, 72 h). Color legend: black=*il1b*; red=*tnfa*; green=*il10* (MTZ-treated); blue=*il10* (untreated). Data are presented as mean±SD (n=6 biological replicates); each biological replicate is the mean of two technical qPCR replicates (two Ct values). MTZ=metronidazole. Error bars represent SD

structure makes it vulnerable to damage. In mammals, damage to it is often associated with irreversible defects due to its low regenerative capacity. In contrast, adult zebrafish exhibit a high regenerative capacity for cerebellar tissue repair, which contrasts markedly with mammals in which lesions do not heal and develop permanent defects (30). This exceptional regenerative capacity is largely due to the absence of permanent glial scarring and the availability of a highly regulated inflammatory response favorable for tissue repair (34). The aim of the present study was to describe a combination of histological and molecular studies to elucidate the dynamic balance between inflammation, developmental signaling, and neurogenesis during zebrafish cerebellar regeneration.

In this study, we investigated zebrafish cerebellar regeneration after injury from histological and molecular perspectives. This study revealed that there is a tightly regulated series of events in this pathway, starting with initial inflammatory signaling (TNF- α and IL-1 β), followed by the induction of an anti-inflammatory cytokine (IL-10), followed by the activation of developmental pathways (*shha* and *pax5*) and the up-regulation of neurogenic and metabolic markers (*neurod*, *slc17a7a*, *ca8* and *aldoca*). This time-dependent cascade pathway creates a synergistic interaction between immune modulation and neural progenitor

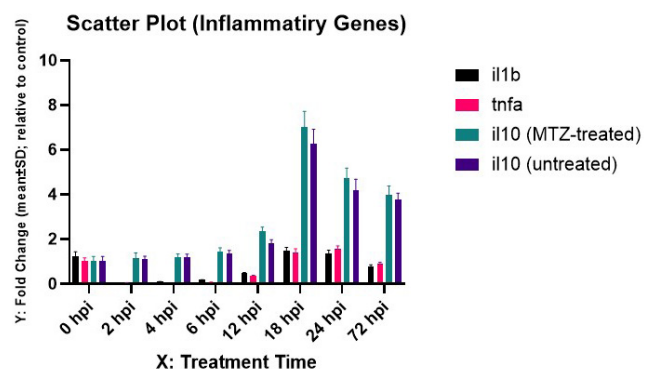


Figure 11. Scatter plot of regeneration-associated genes (*aldoca*, *ca8*, *neurod*, *pax5*, *shha*, *slc17a7a*)

Y-axis: mean fold change (relative to control); X-axis: days post-injury (Control, 1 dpi, 7 dpi, 14 dpi, 21 dpi, 28 dpi). Each color corresponds to one gene (legend as in the figure). Data are presented as mean±SD (n=6 biological replicates); each biological replicate is the mean of two technical qPCR replicates (two Ct values). Error bars represent SD

activation, which in turn enables tissue repair. Such patterns not only confirm the zebrafish as a useful model system for studying brain regeneration, but also provide new insights into how neurogenesis and inflammation are coordinated during cerebellar repair.

Comparison of gene expression in development and repair suggests that cerebellar regeneration in zebrafish is not simply a direct reactivation of the developmental program, but rather a selective rearrangement of molecular pathways. The increase in genes such as *neurod* and *slc17a7a* confirms that the differentiation and formation of granule circuits are reactivated, similar to what is observed in embryonic development. On the other hand, increased expression of *slc17a7a* (a marker of glutamatergic synapse formation) may suggest reintegration of newly generated neurons into functional circuits. Consistent with this molecular change, fish at later post-injury stages exhibited restored forward swimming and improved motor coordination.

In contrast, the decrease in *pax5* and *ca8* expression after injury, contrary to the developmental pattern, likely reflects a reduced need for structural demarcation or a change in cell identity in the early stages of regeneration. The increase in *shha* also suggests a role for this morphogen in inducing cell cycle progression and initiating the proliferative phase. These different gene expression patterns suggest that cerebellar repair combines developmental programs with injury-specific pathways. Based on these findings, cerebellar regeneration appears to represent a continuation of developmental programs in a modified form, rather than a direct recapitulation of the original developmental process.

Our findings are well supported by direct demonstration of cerebellar repair *in vivo* in zebrafish. Consistent with our study, it has been shown that even after induced destruction of Purkinje cells, neurons fully regenerate, electrophysiological reintegration is reestablished, and motor function is restored at all stages of life (30). This ability strongly supports our study of late induction of neural (*neurod*) and metabolic (*aldoca* and *ca8*) markers to demonstrate functional regeneration of cerebellar neuronal circuits.

Tsarouchas and colleagues have shown that $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ are required for spinal cord regeneration in zebrafish larvae, and that blocking these cytokines interferes with neural repair. This is precisely what we observed in our discovery of the early induction of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ as a driver of cerebellar repair (31). Zebrafish IL-10 is a potent anti-inflammatory cytokine that is essential for tissue homeostasis, including gill integrity. Our observed late peak of IL-10 in cerebellar regeneration is consistent with this conserved protective role (35). It has been shown that $\text{TNF-}\alpha$ is secreted by a subpopulation of activated macrophages during the early stages of regeneration and that this signal directly stimulates the proliferation of neural progenitor cells (36). IL-10 is recognized as a significant anti-inflammatory mediator that facilitates repair in zebrafish CNS models (37).

Another study showed that inflammation is not a secondary factor in regenerative neurogenesis, but rather a major driver. According to this study, after injury to the zebrafish retina, the $\text{IL-1}\beta$ signaling pathway is activated and subsequently converts Müller glia into neural progenitors capable of replacing the lost neuronal population (38). Parallel findings obtained during telencephalon repair

reinforce the key role of microglia in modulating inflammation and supporting neural regeneration. Inhibition of microglia prolongs inflammatory responses, followed by impaired progenitor cell proliferation and repair signaling. Our results on the sequential kinetics of $\text{TNF-}\alpha$ / $\text{IL-1}\beta$ and IL-10 are consistent with the above studies (34). Unlike zebrafish, mammalian cerebellar circuits do not regrow after injury under normal conditions, even when they use the same molecular signals. For example, in mouse models of cerebellar injury, long-term up-regulation of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ has been observed, which is associated with chronic neuroinflammation and Purkinje cell loss without functional recovery (39, 40).

Shh signaling has been reported to be reactivated during tissue regeneration throughout zebrafish organs, including the cerebellum (41). Concurrently, Asano and Gruss identified a role for *pax5* in cerebellar patterning during development—our findings of *shh* and *pax5* induction further support the rewiring of developmental programs during repair (42). These findings are directly consistent with our observed temporal cascade—from early $\text{TNF-}\alpha$ / $\text{IL-1}\beta$, to IL-10 , to *shha/pax5*, and ultimately to neurogenic/metabolic markers—and support generalizable principles of inflammation-induced, growth-driven neural repair in the zebrafish cerebellum.

The expression patterns of *aldoca* and *ca8* genes in Purkinje neurons have been discovered—which is also consistent with our results for neuronal circuit regeneration and metabolic recovery (43, 44). Lange and Brand showed that most cell types in the adult zebrafish cerebellum can regenerate, a finding consistent with the general framework of our study (45). Furthermore, consistent with our study, the role of ventricular and neuroepithelial-like progenitors in regeneration of the adult zebrafish cerebellum was identified, with a gene profile somewhat related to our work. In contrast to one report, which focused primarily on progenitor populations, our study simultaneously monitored inflammatory and developmental gene expression over the entire regenerative period (20). Teleost models provide evidence for a multi-stage, conserved regeneration program. The gradual normalization of swimming depth and social behavior observed during cerebellar repair further supports the functional relevance of the histological and molecular changes reported in this study. Early impairments in locomotor coordination coincided with acute tissue disruption and inflammation, whereas improvements in swimming stability and group behavior paralleled structural reorganization and reactivation of neurogenic and synaptic markers.

Another noteworthy finding was the enhanced expression of *il-10* following metronidazole treatment during the acute phase of cerebellar regeneration. Although metronidazole is widely used in zebrafish research as part of the nitroreductase/metronidazole system for selective cell ablation, accumulating evidence suggests that this compound may also influence inflammatory responses and tissue repair processes indirectly by modulating immune cell activity (46, 47). Previous studies have demonstrated that depletion or functional alteration of microglia/macrophages profoundly affects regenerative neurogenesis in zebrafish by disrupting the balance between pro-inflammatory and anti-inflammatory signals (31, 48). In the present study, the stronger induction of *IL-10* in metronidazole-treated fish may

reflect a compensatory anti-inflammatory response aimed at restoring tissue homeostasis following altered immune cell dynamics. Because IL-10 is a key mediator of inflammation resolution and neuroprotection, its sustained up-regulation may facilitate the transition from the inflammatory phase to the regenerative phase by limiting excessive cytokine production and creating a permissive environment for progenitor activation and neuronal differentiation (35, 37). Nevertheless, because metronidazole can exert off-target effects independent of cell ablation, the precise mechanisms underlying its influence on cerebellar regeneration remain unclear and warrant further investigation using cell-specific genetic approaches.

Together, these observations suggest that cerebellar regeneration in adult zebrafish probably results in progressive restoration of motor function alongside tissue repair. For example, studies have shown that in *Apteronotus leptorhynchus*, radial glial fibers are markedly increased in the dorsal cerebellum 8 days after mechanical injury. In other words, newly generated cells are directed to the lesion site, and neuronal replacement occurs (49).

The present study not only contributes to a deeper understanding of the repair processes in zebrafish but also suggests avenues for future research in the field of neuroregenerative therapies. The findings of this study highlight pathways that could be considered as molecular targets to enhance cerebellar repair in higher vertebrates. However, several research gaps remain: first, the precise role of different stem and glial cell populations in the sequential stages of zebrafish cerebellar repair is still not fully understood. Second, it is unclear whether the temporal expression patterns of genes such as *shha*, *pax5*, *neurod*, and *slc17a7a* can also be reactivated in mammals. If these questions are investigated in the future, we may find ways to stimulate neuronal production and enhance cerebellar regeneration after injury in species such as humans. In addition, the use of new approaches such as cell tracking, live imaging, and gene-editing tools (e.g., CRISPR/Cas9) could significantly increase the power to elucidate the molecular mechanisms of regeneration.

The present study has several limitations. One limitation is that causal relationships between inflammatory signaling and developmental gene activation were not directly tested. Functional approaches, such as cytokine inhibition or genetic manipulation of the *shha* and *pax5* pathways, would further clarify their precise roles during cerebellar regeneration and should be addressed in future studies.

Although behavioral observations supported progressive functional recovery, behavioral assessment in the present study was qualitative. Quantitative behavioral analyses, such as tracking swimming speed and measuring bottom-dwelling, would provide additional functional evidence and should be considered in future studies.

We identified inflammatory cell populations based on morphology, but did not confirm them with immunohistochemical markers. Finally, we did not evaluate the functional integration of newly generated neurons electrophysiologically.

Conclusion

This study demonstrated that cerebellar regeneration in zebrafish follows a precise temporal cascade that begins with the activation of proinflammatory cytokines TNF- α

and IL-1 β , is then modulated by anti-inflammatory signals such as IL-10, and ultimately leads to the activation of developmental pathways (*shha* and *pax5*) and the expression of genes related to neuronal differentiation and maturation. This process suggests that the regulated interaction between inflammation and developmental programs is essential for successful cerebellar regeneration. Our findings not only provide a framework for understanding the molecular mechanisms of regeneration but also suggest potential targets for developing novel interventions to enhance cerebellar repair in species with limited regenerative capacity, such as mammals.

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Authors' Contributions

J RL conceived the study and handled data curation; T M developed the methodology and conducted the investigation; T M and T F supervised the process; all authors contributed to reviewing and editing the manuscript. All authors have read and approved the final version.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Declaration

The authors acknowledge using ChatGPT (OpenAI) to assist with drafting a small part of the introduction section. The authors reviewed and validated all content.

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