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Peer Review File

Single-cell analysis of innate spinal cord regeneration identifies intersecting modes of neuronal repair

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors used snRNA-seq to characterize and investigate the cellular basis of the innate ability of adult zebrafish to recovery from severe spinal cord injury. By analyzing over 50,000 nuclei across three different post-injury time points, the authors have generated an important adult zebrafish spinal cord database that will likely appeal to audience beyond spinal cord injury field. Using this dataset, the authors make important novel discoveries focusing on the role of neurons after SCI. They report that a wave of glutamatergic neurogenesis is followed by a slower wave of GABAergic neurons that contribute to a recovery of E/I balance in the chronic time point. A more detailed analysis of these neurons led to their discovery of a cluster of neurons that displayed a highly regenerative transcriptomic signature, which they termed iNeurons. Surprisingly, these iNeurons did not arise from neurogenesis, but rather were spared neurons that survived the injury and contributed to functional recovery through plasticity. This is significant because recovery after SCI in mammals is also not through neurogenesis, but rather plasticity of spared neurons and their axonal pathways. Thus, these iNeurons provide a novel model for utilizing the zebrafish to better understand recovery after SCI in mammalian models in future studies. I only have minor comments:

1) Although 90% of iNeurons come from those that survive the injury, it is interesting that 10% are generated via neurogenesis. It would be informative for the authors to comment on the nature of these minor INeurons and whether there are any interesting differences between the two populations.

2) The CellChat analyses showing that the strongest signals are between neurons (in particular iNeurons) and OPC are interesting, but is limited by the fact that many neurotransmitters, such as glutamate and GABA, are not expressed genetically, (i.e. there is no gene for glutamate). Thus, such neurotransmission would not be included in ligand-receptor analyses such as CellChat. The authors should discuss this limitation in their manuscript.

Reviewer #2 (Remarks to the Author):

The manuscript by Saraswathy et al. is a significant contribution to the spinal cord repair and regeneration field as it provides a single nuclei RNA atlas that was missing for the adult zebrafish. Together with the already generated atlases for mice, rats and humans, it has the potential to allow a more comprehensive understanding in future studies of the similarities and differences, at the cellular level, of regenerative versus fibrotic models. The authors generated a custom-assembled vertebrate CNS marker database to allow a more rigorous analysis of zebrafish marker genes that was valuable for this study and will certainly also be very useful for the zebrafish CNS community.

By performing a CellChat analysis, the authors did a comprehensive assessment of cell to cell interactions that suggest the potential of neurons as signaling hubs in homeostasis and in injury contexts. Their analysis identified a group of neurons (iNeurons) that are not generated de novo after injury but instead go through a process of plasticity acquiring a neuroblast-like transcriptional signature. They then go on to study the functional relevance of 2 of the markers expressed by this population - Syt11a/b (synaptotagmin XI a/b) and Vamp4 (vesicle-associate membrane protein 4). Using CRISPR/Cas9 they were able to downregulate these genes which led to an impairment of swimming recovery. These experiments show that these genes are important for spinal cord regeneration in zebrafish, but since these genes are expressed in other neuronal populations, it does not allow to conclude that iNeurons are important for spinal cord regeneration. The authors should

discuss this. In addition, there are a few points that need to be addressed.

Line 110-112, Fig 1C,E
1. Leukocytes B do not show the bone fide PTPRC/CD45 expression marker, but instead express zebrafish genes with unknown functions. Are there mammalian orthologues for these genes? Does their function make sense in an immunological context? What were the criteria to classify this cluster as Leukocytes?
2. What is the expression of other immune lineage genes in Leukocytes A and Leukocytes B (e.g. CD3 for T-cell, CD19/20 for B-cell, CD11b/c for myeloid, etc.)

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5. Would like to see, as an additional quality control, a nCounts vs nGenes scatter plot per cluster where each cell/nucleus is color codded with the percentage of counts coming from mitochondrial genes. For instance, low UMI count cells may have high mitochondrial RNA contribution and could then be flagged for removal from the cluster.
6. Fig 1E legend states that top 10 DE genes are shown for each major subpopulation. Yet, for Leucocyte B, OPC, Oigo B, Erythrocyte only has 8, 6, 2, 7 DE genes represented, respectively.

L213-220, Fig 3C,D,E,F
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8. Gad1b seems to be upregulated in the cells of the central canal with a morphology similar to CSF-contacting neurons, is these the case? And is it expected that these neurons are inhibitory?
9. In panels E and F it would be important to see not only the single channel for vglut2a and gad1b but also the single channel for HuC/D. It is very difficult to see colocalizations as it is.

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L390, Fig 7C, Fig S7C
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L412, Fig 7E
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20. Discussion could be improved by delving into how does iNeuron cluster compare to the pro-regenerative signature neuron cluster found in mice by Matson KJE et al, 2022 (doi: 10.1038/s41467-022-33184-1).

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Reviewer #1:

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Thank you for your time and suggestions!

Comment

1) Although 90% of iNeurons come from those that survive the injury, it is interesting that 10% are generated via neurogenesis. It would be informative for the authors to comment on the nature of these minor INeurons and whether there are any interesting differences between the two populations.

Response: We are equally intrigued by this observation and are currently building the genetic tools that will allow us to label and trace different iNeuron subtypes. The only observation we can make at this point is that EdU^+ iNeurons have distinctly smaller and rounder soma while the rest of iNeurons are morphologically more mature. To highlight this observation, we stated in **Lines 539-544**: "Although the majority of iNeurons are injury-surviving neurons that acquire an iNeuron signature at 1 wpi, 9% of iNeurons incorporated EdU in our proliferation assays. Supporting the premise that a minor fraction of *syt11b*$^+$ neurons are newborn, we noted that EdU^+ iNeurons have smaller soma that is characteristic of newborn neurons and different from the majority of iNeurons. The origin, type and function of EdU^+ iNeurons remain to be determined."

2) The CellChat analyses showing that the strongest signals are between neurons (in particular iNeurons) and OPC are interesting, but is limited by the fact that many neurotransmitters, such as glutamate and GABA, are not expressed genetically, (i.e. there is no gene for glutamate). Thus, such neurotransmission would not be included in ligand-receptor analyses such as CellChat. The authors should discuss this limitation in their manuscript.

Response: This is an important point, which we have now highlighted in the Results and Discussion section.

Lines 171-173 in Results state that: "This elevated neuronal signaling is not merely due to the inherent neurotransmission properties of neurons since major neurotransmitters are

non-protein-coding genes that are excluded by CellChat (Zhao et al., 2023, PMID: 36854676)".

Lines 499-506 in the Discussion state that: "As CellChat analysis is limited to short-range autocrine or paracrine signaling pathways and based on the expression of protein-coding transcripts, our analysis excludes long-range neuronal transmission and does not account for non-peptide neurotransmitters such as glutamate or GABA (Zhao et al., 2023, PMID: 36854676). Thus, our findings that neurons in general, and specifically iNeurons, are hubs of intercellular signaling compared to other spinal cell types is independent of neurotransmission. Further studies of injury-induced signaling pathways and neurotransmitter-mediated signaling will shed light into signaling pathways that direct regeneration- or plasticity-driven SC repair".

Reviewer #2:

The manuscript by Saraswathy et al. is a significant contribution to the spinal cord repair and regeneration field as it provides a single nuclei RNA atlas that was missing for the adult zebrafish. Together with the already generated atlases for mice, rats and humans, it has the potential to allow a more comprehensive understanding in future studies of the similarities and differences, at the cellular level, of regenerative versus fibrotic models. The authors generated a custom-assembled vertebrate CNS marker database to allow a more rigorous analysis of zebrafish marker genes that was valuable for this study and will certainly also be very useful for the zebrafish CNS community.

By performing a CellChat analysis, the authors did a comprehensive assessment of cell to cell interactions that suggest the potential of neurons as signaling hubs in homeostasis and in injury contexts. Their analysis identified a group of neurons (iNeurons) that are not generated de novo after injury but instead go through a process of plasticity acquiring a neuroblast-like transcriptional signature. They then go on to study the functional relevance of 2 of the markers expressed by this population - Syt11a/b (synaptotagmin XI a/b) and Vamp4 (vesicle-associate membrane protein 4). Using CRISPR/Cas9 they were able to downregulate these genes which led to an impairment of swimming recovery. These experiments show that these genes are important for spinal cord regeneration in zebrafish, but since these genes are expressed in other neuronal populations, it does not allow to conclude that iNeurons are important for spinal cord regeneration. The authors should discuss this. In addition, there are a few points that need to be addressed.

Thank you for the positive comments and valuable suggestions!

We completely agree and have highlighted this comment in the discussion **(Lines 559-565)**: "While our data reveals vesicular trafficking is an essential mechanism that underlies spontaneous plasticity and functional recovery after SCI, future cell-specific manipulations that enable iNeuron lineage tracing or ablation are needed to better understand their plasticity and requirement during SC regeneration. We propose that zebrafish provide a valuable model to investigate the origin and function of iNeurons during SC regeneration, elucidate the role of vesicular trafficking in spontaneous plasticity, and identify new manipulations to promote plasticity after SCI".

Comment *Line 110-112, Fig 1C,E*

1. Leukocytes B do not show the bone fide PTPRC/CD45 expression marker, but instead express zebrafish genes with unknown functions. Are there mammalian orthologues for these genes? Does their function make sense in an immunological context? What were the criteria to classify this cluster as Leukocytes?

Response: Leukocytes B is a coarse cluster comprised of 3 clusters (clusters 3, 20 and 23 in the complete dataset). To address this comment, we first identified the mouse orthologues for genes that are differentially expressed (DE) in these clusters. By evaluating the expression of these genes after mouse SCI, we found that most genes expressed in clusters 3, 20 and 23 were expressed in dividing myeloid cells (Milich et al, 2021, PMID:34132743). Therefore, we updated our VNM database to include zebrafish orthologous genes of the clusters "Div-Myeloid" and "Interferon Myeloid" from Milich et al, 2021 **(updated Table S1)**. Indeed, when we ran our marker scoring algorithm using the update VNM database, all three clusters of Leukocytes B showed significant enrichment for Dividing Myeloid genes **(Fig. S1E and Table S2)**. We have now included a new **Table S3** that details the specific DE genes that were used to call cell identities by our VNM database. We also updated the cluster ID sheets in **Table S4** to reflects this revised analysis.

Comment

2. What is the expression of other immune lineage genes in Leukocytes A and Leukocytes B (e.g. CD3 for T-cell, CD19/20 for B-cell, CD11b/c for myeloid, etc.)

Response: There are no zebrafish orthologues for CD3, CD19/20, CD11c according to either ensembl or zfin. *lck* (Nika et al, 2010, PMID: 20541955), *cd4-1* (Dee et al, 2016, PMID: 27694495) and *foxp3a* (Kasheta et al, 2017, PMID: 29066577) were shown to be markers of zebrafish T Cells. *mpx* (Renshaw et al, 2006, PMID: 16926288) and *lyz* (Meijer et al, 2008, PMID: 17553562) were shown to be markers of zebrafish neutrophils. *zbtb14* (Deng et al, 2022, PMID: 36205309) and *spi1a/b* (pu.1 0 Deng et al, 2022, PMID: 36205309) are markers of monocytes precursors and *itgam* (orthologue to *CD11b*) is a marker of myeloid cells in zebrafish.

We examined the expression of all these markers in **Lines 116-122**: "We noted that additional immune cell lineage genes were expressed in leukocytes A and B (Fig. S1G). The monocyte precursor marker *zbtb14* was enriched in less than 10% of leukocytes B, whereas *spi1a* and *spi1b* were enriched in 30-50% of leukocytes A. In contrast, T cell markers (*cd4-1, foxp3a*, and *lck*) and neutrophil markers (*mpx* and *lyz*) were enriched in less than 1% of leukocytes B, while 9% of leukocytes A expressed the myeloid marker *itgam* (orthologue of *CD11b*). Notably, we did not detect *bona fide* B cell markers in our dataset".

These findings are consistent with on our recent characterization of immune cell infiltration and activation after spinal cord injury (*manuscript in preparation*). In this study, we observed a transient and overwhelming activation of microglia and macrophages at the expense of other immune cell types.

Comment *Line 122, Fig 1E*

3. Leukocyte B and Erythrocytes have a lot of co-expressed genes, including common expression of mitochondrial COX3 and mt-co1. Single nuclei RNA-seq should not contain any, or at most, very low mitochondrial genes. Having them being selected as top DE genes may suggest not enough stringency in filtering out ambient RNA contaminated droplets.

Response: Our dataset is prefiltered using high-stringency parameters. Using decontx package, cells with contamination score greater than 0.75 were filtered out. This cut off removed ~15,000 cells and only cells with <5% mitochondrial content were analyzed **(Fig. S1A-B)**. Yet, despite these filtration steps, mitochondrial genes were still included in Leukocytes B and Erythrocyte. We believe Leukocytes B clusters are of hematopoietic origin, which may explain why many Leukocyte B markers were shared with Erythrocytes.

Comment
4. Additionally, are the selected genes expressed in a relevant minimum fraction of cells (30-40%) in those clusters?

Response: Throughout the manuscript, differential gene expression for individual cluster was identified using Wilcoxon rank sum test in the "FindAllMarkers" function. Marker genes detected in at least 25% of the clustered cells and with a logFC threshold of 0.25 were selected. Only positive markers were reported. We included these details in **Lines 675-678**.

Comment
5. Would like to see, as an additional quality control, a nCounts vs nGenes scatter plot per cluster where each cell/nucleus is color coded with the percentage of counts coming from mitochondrial genes. For instance, low UMI count cells may have high mitochondrial RNA contribution and could then be flagged for removal from the cluster.

Response: We plotted the suggested data **(Fig. S1H)**. As discussed in **Lines 122-128**: "Scatter plots representing the numbers of unique genes versus unique molecular identifiers (UMI) confirmed that the proportions of mitochondrial genes within each population does not exceed 5% (Fig. S1H). However, compared to all other cell populations, leukocyte B cells with the lowest UMIs showed higher mitochondrial gene content (Fig. S1H). (However), although neurons B and oligodendrocytes B did not show enrichment for classical markers (either), both clusters showed negligible mitochondrial gene expression (Fig. S1H)". Since multiple zebrafish clusters did not enrich for classical markers of their corresponding cell type despite having low mitochondrial gene content, we concluded in **Lines 128-131** that "our VNM database is likely still biased toward commonly used mammalian cell markers that are not necessarily the best markers for adult zebrafish, and that our single-cell atlas provides a platform to identify improved CNS markers for adult zebrafish cells".

Comment
6. Fig 1E legend states that top 10 DE genes are shown for each major subpopulation. Yet, for Leucocyte B, OPC, Oigo B, Erythrocyte only has 8, 6, 2, 7 DE genes represented, respectively.

Response: We have now corrected this glitch to include 10 DE genes for each cluster **(Fig. 1E)**.

Comment *L213-220, Fig 3C,D,E,F*
7. Some emphasis is put on these data, prompting it to be next topic studied, yet differences are not particularly striking (4% changes). Neither the pie charts or bar graphs. Is there statistical significance at least between control and 1wpi?

Response: We did not mean to emphasize the *in silico* calculations of E/I ratios, but rather to report them as the observations that prompted us to perform E/I calculations *in vivo*.

Two points to highlight in agreement with the Reviewer's comment: 1) We performed our snRNA-seq in duplicates. Thus, we were not able to calculate statistical significance of *in silico* E/I ratios. 2) Our snRNA-seq lacked spatial information and included tissue portions that are rostro-caudal and proximo-distal to the lesion. Thus, any E/I perturbations that occur proximal to the lesion were likely diluted when combined with spared/distal tissues.

To address the Reviewer's comment, we stated in the Results section **(Lines 236-240)**: "We note that our *in silico* E/I ratio estimates did not allow for statistical testing. Moreover, our snRNA-seq data lacks spatial information and includes tissue portions that are both proximal and distal to the lesion, ostensibly diluting E/I changes localized proximal to the lesion. Nonetheless, these studies prompted us to calculate E/I ratios *in vivo*". Our *in vivo* validation

showed that E/I ratios are significantly increased at 1 wpi (2-fold change) and recover to the baseline levels at 6 wpi **(Fig. 3I)**.

Comment
8. Gad1b seems to be upregulated in the cells of the central canal with a morphology similar to CSF-contacting neurons, is these the case? And is it expected that these neurons are inhibitory?

Response: Yes, CSF-contacting neurons have been shown to be GABAergic (Djenoune et al., 2014, PMID: 24834029).

Comment
9. In panels E and F it would be important to see not only the single channel for vglut2a and gad1b but also the single channel for HuC/D. It is very difficult to see colocalizations as it is.

Response: We reformatted the panels to include high magnification insets with dual and single channels for *vglut2a*, *gad1b* and HuC/D **(Fig 3E-F)**.

Comment *L240-246, Fig 4B*
10. Colocalization for vglut2a and gad1b with EdU are very difficult to see. Should improve these images. And also, it is a bit puzzling to see many more vglut2a.RFP at 1 wpi and almost no signal at 3 and 6 wpi. These neurons are not supposed to be disappearing as regeneration progresses.

Response: We reformatted the panels to include high magnification insets with dual channels for *vglut2a*, *gad1b* and EdU **(Fig 4B)**. By quantification, and although the absolute numbers of *vglut2a:RFP* cells showed a slight decrease at 6 wpi relative to 1 wpi **(Fig. S3F)**, the proportions of *vglut2a:RFP* cells did not decrease between 1 and 6 wpi **(Fig. 3G)**. This is likely due to the overall decrease in cell numbers between 1 and 6 wpi. We have edited Fig. 4B to be more representative of these quantifications.

Comment *L267-269, Fig 4G*
11. The same here, very difficult to see vglut2a and gad1b with EdU co-localizations. Most of the arrowheads are pointing to vglut2a or gad1b single positive cells.

Response: We reformatted the panels to include high magnification insets with dual channels for *vglut2a*, *gad1b* and EdU. This data is shown in **Fig 4F** of this revised submission.

Comment *L330-332, Fig 6D*
12. NRG1, NRG2, ERBB3/4 are mentioned in the text but not shown in Figure 6D.

Response: This typo was corrected to "(Fig. S5F)" in **Line 353-354** of the revised submission.

Comment *L343, Fig 6*
13. Figures could be improved and made clearer what is the marker analyzed in each graph B, C, E and F.

Response: We changed the Y axis labels in **Fig. 6B-C and E-F** to make the figure clearer. We also edited **Fig. 6G-H** in effort to improve the clarity of the data.

Comment *L349, Fig S6A,E-H*
14. Syt11a is expressed in ependymal cells, so not entirely neuron, particularly iNeuron, specific. Would like to see syt11a expression in S6A dotplot, to likewise discard expression in other neuron clusters.

15. Could syt11a expression in ependymal cells be a marker of neuron-biased precursors that could become syt11b upon maturation?

Response: To address comments related to *syt11a*:

We added a dopt plot for *syt11a* to **Fig. S6D** and clarified in the text **(Lines 371-377)** that *syt11a* expression is not specific to iNeurons. "The *syt11b* paralog, *syt11a,* was not identified as a unique iNeuron marker. However, as zebrafish paralogs have been shown to play redundant functions upon genetic perturbation (El-Brolosy, 2019, PMID: 30944477; Klatt Shaw and Mokalled, 2021, PMID: 33742663; Klatt Shaw et al, 2021, PMID: 33609461), we examined the expression of *syt11a* after SCI (Fig. S6D-H). Like *syt11b*, *syt11a* transcripts were upregulated in neurons after injury. However, unlike *syt11b*, *syt11a* expression was not restricted to neurons including expression in ependymal cells surrounding the central canal at 1 wpi (Fig. S6D-H)."

In general, two common themes have emerged from our studies of zebrafish paralogs during spinal cord regeneration, which seem to also apply to *syt11a/b*. a) Gene paralogs are differentially regulated and show distinct expression patterns after injury. b) Mutating a duplicated gene is likely to induce genetic compensation by its paralog even if said paralog has a distinct expression pattern in wild-type animals.

While we cannot exclude the possibility that *syt11a* is expressed in neurogenic progenitors, we think it is unlikely that *syt11a* labels neurogenic progenitors. In an independent study that we have in preparation, we mapped and validated 19 clusters of lineage-biased progenitors. This study mapped neurogenic progenitors to specific, localized niches around the ependyma. *syt11a* is expressed around the ependyma, but does not localize to these neurogenic niches.

Comment *L372, Fig S6F,I,J,K*

16. Figures could be improved and made clearer what is the marker analyzed in each graph.

Response: We changed the Y axis labels in **Fig. S6** to make the figure clearer.

Comment *L374, Fig S6K*

17. Should 2wpi be 3wpi? A typo?

Response: Thanks. We corrected the type to 3 wpi in **Line 403** of the revised submission.

Comment *L390, Fig 7C, Fig S7C*

18. Why no biocytin label at 150uM when applied at the rostral side (7C), yet observable when applied in the caudal side (S7C)? 7C and S7C refers to iNeurons specifically.

Response: Yes, Figures 7C and S7C refer to iNeurons.

We were equally intrigued by our initial observation that rostral biocytin did not label iNeurons at 150μM, but caudal biocytin did. However, our hypothesis was that absence of Biocytin-labeled iNeurons could be due to sample size for the following reasons:

- The numbers of HuC/D$^+$ neurons at 150 μm are marginally lower than in distal tissues (450 and 750 μm).
- Within HuC/D$^+$ neurons, the numbers of Biocytin-labeled neurons at an early time point of 1 wpi is even lower (<10% of neurons) (Fig. 7B and S7B).
- iNeurons account for only ~10-20% of total neurons.

To address this comment and possibility, we have now repeated this experiment using 14-15 independent animals **(Fig. 7A-C)**. Supporting our hypothesis, we were able to identify Biocytin$^+$ iNeurons in a small subset of animals **(Fig. 7C)**.

We have further addressed this comment in the Discussion **(Lines 529-531)** by stating: "While our dual-grade, biocytin-mediated tracing experiments established the integration of iNeurons in the spinal circuitry at 1 wpi, finer tracing tools are needed for detailed mapping of iNeuron connectivity".

Comment *L412, Fig 7E*

19. The only statistically significant mutants found are atf3 KO, syt11a/b double KO (dKO) and vamp4 KO. Incidentally, these are the conditions where more fish were analyzed. For instance, n=32 for aforementioned mutants versus n=22-23 in the single syt11a, sy11b mutants, respectively (>39% more fish). This may bias the claim that single mutants do not alter swim endurance. Would like to see the same n applied to all tested conditions, or at least single syt11a, syt01b single KO mutants.

Response: The first rounds of CRISPR screening were performed in duplicates with comparable numbers of animals for all the genes tested. These initial experiments yielded significant swim endurance defects in *atf3*, *syt11a/b* and vamp4. In order to perform subsequent swim behavior and histology, we also repeated swim endurance evaluation for *atf3*, *syt11a/b* and vamp4 and combined them to the initial dataset. To address the Reviewer's comment, we have now removed these additional replicates and are only showing the first two rounds of swim endurance that were performed on all targeted genes. To further address the Reviewer's comment, we also performed random sampling of the combined dataset and reached the same conclusions. The revised data is shown in **Fig. 7E**.

Comment

20. Discussion could be improved by delving into how does iNeuron cluster compare to the pro-regenerative signature neuron cluster found in mice by Matson KJE et al, 2022 (doi: 10.1038/s41467-022-33184-1).

Response: We revised the discussion **(Lines 535-539)** to state that: "Zebrafish iNeurons also share transcriptional similarities with a rare population of mouse spinal neurons that express regeneration associated genes including *Atf3* and *Sox11* after mouse SCI (Matson et al, 2022, PMID 36163250). Cross-species transcriptional comparisons and follow-up *in vivo* studies are required to determine the extents of anatomical and functional similarities between these neuronal populations". Our plan is to pursue these comparisons in a future follow-up study.