nature research

Peer Review File

Manuscript Title: Neonatal microglia-organized scar-free spinal cord repair

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Li et al from the Zhigang He lab have discovered a remarkable potential for long distance axon regeneration of serotonergic and cortico-spinal axons through and well beyond a lesion after complete transection crush injury of the spinal cord in neonatal mice. While spontaneous regeneration across such a devastating injury has been documented before in neonatal marsupials (that are born at a very early developmental stage), to my knowledge, this is the first demonstration of such robust, lengthy regeneration across a complete lesion in an immature rodent. However, the manuscript goes well beyond the observation of regeneration because the authors delve deeply into the cellular and molecular interactions that allow for such remarkable regeneration to occur. In general, the data are crisp, nicely presented, statistically sound and of high quality. The paper will likely become a landmark in the field because it is filled with a wealth of completely novel and exciting mechanistic findings. Firstly, the authors show convincingly using a variety of markers that the neonatal (P2) lesion environment unlike that which occurs in later stage postnates and the adult, heals without any scar. Indeed, the wound healing process in the neonate was so remarkably scar-free that there was no evidence of a lesion at all and with complete revascularization of the wound epicenter. The authors discovered that, unlike the adult, microglia in a homeostatic state persist in the core of the neonatal lesion throughout the period of wound resolution and surprisingly abundant axonal regeneration. They then proceeded with a thorough investigation of the possible role of immature versus mature microglia in the neonatal scar-free wound healing phenomenon. Elegant single cell RNAseq analyses revealed that immature microglia produce transient fibronectin and inflammation controlling proteinase inhibitors, so they examined the role of these immature cells themselves as well as their products. The transient microglial fibronectin production in the fashion of a bridge that seals the edges of the neonatal lesion was clearly shown via immunohistochemistry. They then went on to show the role of fibronectin in early lesion healing by deleting fibronectin specifically in microglia to show beautifully that bridge formation and sealing of the lesion edges did not occur. They deleted the young microglial population globally via drug as well as genetic strategies. All of these manipulations led to ectopic scar formation in the neonate and regeneration failure, even of young axons. Going even further they transplanted immature microglia as well as proteinase inhibited adult microglia into the adult lesion and both strategies lead to reduced scar formation and induced axonal regeneration in the adult, a remarkable and quite surprising result. Taken together, this beautiful work has opened a new appreciation of the role of immature microglia in scar free wound healing and regeneration after spinal cord injury. This elegant research is surely worthy of publication in Nature not only for its fundamental revelations about scar formation and axon regeneration as well as the intriguing time dependent changes in microglial biology but also for its potential translational significance.

I have but a few minor suggestions that could help clarify some issues that could help make this wonderful work even better.

In the extended data sections figs 5 and 11, the disc-shaped astrocytic walls that form in the lesion

epicenter after microglial depletion or microglial specific fibronectin depletion in the neonate are extremely interesting, especially since robustly growing, neonatal 5-HT axons stop abruptly just rostral and adjacent to them. This may be one of the most convincing demonstrations that astrocytes (perhaps largely by themselves) can form an axon regeneration blocking "scar" barrier. The question here is whether this barrier structure that develops postnatally is relatively free of the additional inflammatory cells as well as fibroblastic/basal lamina-like (see below) and additional ECM contributions to scar that form in the adult? Can the authors say anything about the presence or absence of various ECM molecules or fibroblasts or macrophages in this structure? Do you have higher magnification images to show more about its physical make-up (density, architecture of cells within the wall)?
At a minimum the authors could show higher mag images of this structure and its intimate relationship with the front of non-regenerating 5-HT axons. Also, I think this "astroglial scar" phenomena is certainly deserving of some discussion related to the issue, that has become somewhat controversial, of whether or not astroglial scar even exists and has the ability to impede, rather than simply aid, axonal regeneration. The authors might also mention the formation of an "astrocytic scar" in relation to the fibronectin depletion studies (there seems to be a far greater GFAP staining intensity in the lesion epicenter in fig S11 C, Cx3cr1,fn1 f/f).

The description of ECM in the lesion core after damage to P20 or adult mice is lovely but a bit incomplete. A major component of the scar matrix that develops in the adult after cord lesion is basal lamina which forms due to fibroblast or endothelial cell interactions with reactive astrocytes. The basal lamina is comprised, in part, of collagens 1 and 4, fibronectin, laminin and CSPGs among other proteins. It has been shown that swirls of basal lamina structures form after implant lesions in the mature cortex but not the neonatal cortex and that such scar matrix along with its associated cells when extracted and placed in vitro is non-permissive/inhibitory for axonal outgrowth unlike the highly axonal growth promoting scar/basal lamina-free wound tissue extracted from the neonate (Rudge and Silver, J Neurosci 1990). The authors should briefly discuss and cite this early work that is highly relevant to the present research because it showed that such scar tissues are overtly inhibitory to axonal outgrowth and which structures or molecules within scar inhibit regeneration. When such basal lamina swirls develop in a perpendicular orientation in relation to regenerating axons they can, in addition to their molecular inhibitory properties, provide a mechanical barrier as well. Can the authors verify whether basal lamina is present or not in the vicinity of the adult crush lesion 2 weeks after injury in their model? Basal lamina is likely to be most abundant where the collagen staining is most intense near the outer edges of the lesion core at the interface with astroglia (fig 1 e). Basal lamina could be revealed simply by staining with laminin antibodies or preferably via EM. Given the interest in CSPGs as inhibitory components of the scar/basal lamina ECM (See McKeon et al., 1991 among many other papers), the authors could also stain the mature lesion wound tissue with antibodies to CSPGs (compare P2 crush with adult crush as in fig 1).

Regarding the microglia transplant experiments, are there images of GFAP differences in the different groups?

Cite Jenny Zou paper about plexin B2 and microglia in scar formation.

Without going into great detail (realizing that functional recovery data may be the topic of a future publication) can the authors give a brief general description of the functional recovery of the animals with neonatal lesion and regeneration (these are the most interesting)? Also, older lesioned animals and especially those that had some induced regeneration after microglial transplants would be

interesting to know about their functional recovery.

For the sake of referencing the historical literature on the possibility of spinal cord regeneration in neonatal mammals, the authors might wish to cite some of the early work of Norman Saunders who showed regeneration of axons across complete transection lesions in the neonatal opossum. Although Marsupials are born at a very early stage of neural development, none-the-less, there is a body of previous work showing that regeneration in the neonatal mammal can occur albeit only in certain species that are born precociously.

There are a few grammatical errors scattered about. Here are a few that I caught.

Scar forming-based should just be scar-based

Pg 3 Interestingly these cells begun…. Should be .. these cells had begun….

…most microglia were ramified morphology should be most microglia displayed a ramified morphology…

Referee #2 (Remarks to the Author):

The manuscript by Li et al presents evidence for a critical role of microglia in scar-free tissue healing and axon growth after spinal cord injury in neonatal mice. They document extensive 5-HT and corticospinal axon growth following a spinal cord crush injury at P2 (postnatal day 2, or neonatal stage). This correlates with a dramatic difference in tissue bridging and healing between P2 and adult in fibronectin, microglia and macrophage dynamics as well as astrocyte scar and blood vessel repopulation at the injury site. Pharmacological and genetic depletion of microglia reduces tissue bridging and healing after neonatal injury. Single cell sequencing identified several types of neonatal microglia with MG3 being the type most enriched for fibronectin and peptidase inhibitor expression. Genetic depletion of microglia-derived fibronectin reduces tissue healing and 5-HT axon growth after neonatal injury. In contrast, transplanting neonatal microglia or peptidase inhibitor-treated adult microglia into the injury site improves tissue healing and 5-HT axon growth after injury in adult mice.

This is a highly significant study, including 1) the identification of microglia as a critical player in the tissue healing response after neonatal injury; 2) the identification of two molecular targets for this repair-promoting ability of microglia (fibronectin and peptidase inhibitors). The study was well designed with extensive genetic and pharmacological function-perturbation experiments. The results have important implications in both the fundamental understanding and potential clinical translation for spinal cord repair. The comments below are meant to improve the manuscript and can be addressed with discussion if data are not already available.

1. As the authors rightly pointed out, they "could not distinguish if these axons are regenerating or uninjured and late-arriving" after P2 injury (page 2). Thus, the authors may wish to clarify that "regeneration" here is broadly defined as axonal growth after injury, similar to that proposed by Sofroniew (2018). Alternatively, the authors may wish to use the term "axon growth" instead of "axon regeneration". Relevant to this, what is proportion of 5-HT and CST axons yet to pass T10 at P2?

2. Compared with adult, how consistent is a spinal cord crush injury at P2? How is this consistency determined?

3. How do the patterns of "regenerated" axons after P2 injury compare with the uninjured axons? Do P2 injured mice fully recover function such as locomotor behavior?

4. The data support the role of microglia-derived fibronectin in scar-free healing. Are fibroblasts present at 3 days after P2 injury?

5. The results from genetic and pharmacological depletion of microglia are convincing. However, is it possible that depletion of other cell types such as astrocytes after P2 injury may also compromise tissue repair at the injury site? If so, microglia is still an important cell type for this tissue repair, but may not be the only (or primary) cell type orchestrating tissue healing since other cell types may also be at play.

6. Ext data Fig. 11: what about CD68 and fibronectin at 14 dpi? This figure presents evidence for an important conclusion, and can be a main figure.

7. Fig. 4a, clarify the exact area for quantification (e.g. use dotted lines); Fig. 4c, no difference between control and vehicle adult microglia? Images suggest some difference, particularly at the perilesional area. Also, what happens to fibronectin here?

8. Neonatal microglia transplant is an important experiment. Suggest to include this result in Abstract. Also, did the authors assess CST regeneration with microglia transplants?

9. Is there any literature on the use of similar peptidases alone in spinal cord injury models? What is the potential of using such peptidases in the absence of microglia transplantation?

10. "This feature of neonatal microglia is different from the permanent activation of microglia in the adult spinal cord lesions." Could the results be interpreted as the absence of certain type of transiently activated microglia allows for the persistence of monocyte-derived macrophages at the adult injury core?

11. Have the authors tried another injury model other than crush?

Minor:
Page 3, "these cells begun" should be "these cells began".
"most microglia were ramified morphology". Check grammar.

Page 5, "MG1 cells in the spinal cord proximal to the lesion (Fig. 3h)". "Proximal" often refers to proximal vs distal. Do the authors mean just outside of the lesion core/epicenter, or around the lesion (as in figure legend)?

Page 7, "is required for bridge forming across the lesion site, but avoiding scar formation" is awkward. "CTCF" should be "CTGF".

Fig. 2c, e, Is the gap at the center of the lesion following microglia depletion negative for DAPI, meaning no cells?

Fig. 3f legend: "expression of Fn1 express in and around the lesion site". Remove "express".

Fig. 4c, gray scale coding is confusing and is probably not needed

Page 20, “as described previously”. Need reference.

Ext Data Fig. 11, 12: Scar bar (typo, three times)

Referee #3 (Remarks to the Author):

The paper by He and co authors reveals new insight into the remarkable regenerative capacity of the postnatal spinal cord. In an elegant series of experiments the authors identify microglia as key orchestrators of scar-free wound healing in mice with complete crush injuries, when the injury is performed at postnatal day 2 (P2). They reveal an orchestrated wound healing response over early time points after P2 crush injury. This is signified by rapid deposition and then clearance of fibronectin matrix in the lesion core, which is associated with transient activation of microglia. This response appears to enable the cut spinal cord stumps to efficiently knit together, effectively closing the wound. By 7 days post injury there is near complete healing - activated microglia (identified with CD68 and SPP1) have returned to a homeostatic state (identified with P2Y12), fibronectin is no longer present, there is no astrocytic scar and this remarkable wound repair is reflected by exuberant growth of serotonergic axons across the lesion. The phenotype and function of P2 microglia appears to be critical for this process. They use pharmacological (CSF1R inhibitor) and genetic (conditional knockout of CSFR1 in microglia) approaches to deplete microglia, and no longer see effective wound healing after P2 injuries, corroborating the key role of microglia in scar-free wound healing. Using single cell RNAseq they reveal that P2 microglia have a distinct gene signature. They identify transcriptionally distinct MG1 and MG3 injury-induced neonatal microglia as potential key mediators of scar-free wound healing. Finally, transplanted adult microglia, treated with peptidase inhibitors identified in P2 microglia, enabled improved wound healing in adult spinal cord lesions.

Although the concept of age-dependent differences in axon regeneration is not new, identifying the cell type, the phenotype, and the distinct molecular signature that underlies the regenerative and wound healing capacity of the neonatal spinal cord is novel and potentially transformative. The pivotal finding that P2 microglia have a distinct molecular profile that enables them to rapidly recover to a homeostatic state, and that this could be the key for scar-free wound healing, represents a step change in our understanding of wound healing and pathological scarring. This work has wide implications for nervous system disorders where regeneration failure, scarring and gliosis are common pathological hallmarks, potentially leading to new therapeutic avenues for treating traumatic injuries or diseases of the brain and spinal cord.

The work is exceptional. The findings are potentially ground-breaking. Below I list several comments and suggestions relating to the data presented.

1. Involvement of other extracellular matrix (ECM) components. Other than collagen-1 and fibronectin, have the authors examined other ECM expression changes in the different conditions? In particular, scar-associated ECM, particularly CSPGs, are known to play a role in restricting regeneration, amplifying the inflammatory response and propagating injury pathology and contributing to failure of effective wound healing. It would be interesting to know if there are CSPG expression changes in the different conditions: scar-free (P2) vs non-permissive (P7-adult) time points; after microglia depletion; after microglial transplantation (either P2 microglia or peptidase inhibitor-treated adult microglia)?

2. Did the authors ever assess whether there was full functional recovery after P2 lesion and effective scar-free wound healing e.g. were motor assessments performed in the mice kept for 10 weeks post

P2-injury, where extensive corticospinal tract axon growth was observed (Fig 1d)? Similarly, it would be useful to know if there was recovered locomotor function in the microglia transplanted and inhibitor treated adult lesioned mice – was motor recovery assessed over the 4 weeks post-injury?

3. P2 microglial activation and recovery to homeostatic conditions. An elegant data set is presented, which reveals a distinct injury-induced transcriptional signature of neonatal microglial cells. They show convincing evidence that initially activated microglia are rapidly transitioning back to the homeostatic stage at 5dpi, with the shift in microglia clusters from predominantly MGO (0 dpi) to MG1/MG3 (3 dpi) then MG2 (5dpi), with M0 increasing from 3 to 5dpi. However, why did the authors not also look at 7dpi (or 14dpi) - to show a return to homeostatic microglia at this time point, when scar-free wound healing has occurred (and ideally compared with adult also, where you would expect impaired recovery of homeostatic microglia)?

4. Dynamics of neonatal wound healing. The histological data are striking, and convincingly show effective and orchestrated wound healing. However, since the data is typically presented as low power images, it is difficult in some instances to see cellular morphology or co-localisation in any detail. For example, the histological images provided do not show high enough resolution to claim co-localisation (e.g. of CD68 and P2Y12, Extended data Fig 2), or morphological changes in microglia from ramified to amoeboid (Fig. 2b, Extended Data Fig. 2a) or fibronectin +ive cells. Where stated "By 3dpi fibronectin+ cells appeared between the gap", it is not clear from the images (Fig. 2) whether these are cells or matrix – it certainly looks more like matrix than fibronectin+ive cells. Some additional higher power images would add clarification. Also the P2Y12 3dpi data in fig 2b (where it fills the epicentre) seems different from P2Y12 3dpi data in extended fig 2 (where it is largely absent from the epicentre). For histological data, the authors should also avoid referring to inflammation resolution. While it is clear that homeostatic function and phenotype of microglia recovers, to convincingly conclude that there is resolution of inflammation would require analysis of phenotype, cytokine expression, enzymatic and gene expression changes in pathways linked with resolution.

5. Discussion points.
The injury model is a clean complete crush injury to the spinal cord. While this is an excellent model for looking at wound repair and regeneration, these types of injuries rarely occur clinically. Some discussion on whether they would expect similar scar-free wound healing orchestrated by neonatal microglia in contusion-type injuries would be appreciated.
The authors may want to discuss evidence that serotonin itself promotes regeneration e.g. evidence from zebrafish studies. The high levels of serotonin in the spinal cord at P2 (evident in images showing approximately 90% of the cord to be filled with serotonergic axons, Extended data Fig 1a) could potentially also influence the microenvironment. Also some inclusion of old literature on plasticity of the serotonergic system after neonatal spinal lesions (e.g. Bregman: https://www.ncbi.nlm.nih.gov/pubmed/3304541).

Author Rebuttals to Initial Comments:

Note: Author responses in black

Referee #1 (Remarks to the Author):

Li et al from the Zhigang He lab have discovered a remarkable potential for long distance axon regeneration of serotonergic and cortico-spinal axons through and well beyond a lesion after complete transection crush injury of the spinal cord in neonatal mice. While spontaneous regeneration across such a devastating injury has been

documented before in neonatal marsupials (that are born at a very early developmental stage), to my knowledge, this is the first demonstration of such robust, lengthy regeneration across a complete lesion in an immature rodent. However, the manuscript goes well beyond the observation of regeneration because the authors delve deeply into the cellular and molecular interactions that allow for such remarkable regeneration to occur. In general, the data are crisp, nicely presented, statistically sound and of high quality. The paper will likely become a landmark in the field because it is filled with a wealth of completely novel and exciting mechanistic findings. Firstly, the authors show convincingly using a variety of markers that the neonatal (P2) lesion environment unlike that which occurs in later stage postnates and the adult, heals without any scar. Indeed, the wound healing process in the neonate was so remarkably scar-free that there was no evidence of a lesion at all and with complete revascularization of the wound epicenter. The authors discovered that, unlike the adult, microglia in a homeostatic state persist in the core of the neonatal lesion throughout the period of wound resolution and surprisingly abundant axonal regeneration. They then proceeded with a thorough investigation of the possible role of immature versus mature microglia in the neonatal scar-free wound healing phenomenon. Elegant single cell RNAseq analyses revealed that immature microglia produce transient fibronectin and inflammation controlling proteinase inhibitors, so they examined the role of these immature cells themselves as well as their products. The transient microglial fibronectin production in the fashion of a bridge that seals the edges of the neonatal lesion was clearly shown via immunohistochemistry. They then went on to show the role of fibronectin in early lesion healing by deleting fibronectin specifically in microglia to show beautifully that bridge formation and sealing of the lesion edges did not occur. They deleted the young microglial population globally via drug as well as genetic strategies. All of these manipulations led to ectopic scar formation in the neonate and regeneration failure, even of young axons. Going even further they transplanted immature microglia as well as proteinase inhibited adult microglia into the adult lesion and both strategies lead to reduced scar formation and induced axonal regeneration in the adult, a remarkable and quite surprising result. Taken together, this beautiful work has opened a new appreciation of the role of immature microglia in scar free wound healing and regeneration after spinal cord injury. This elegant research is surely worthy of publication in Nature not only for its fundamental revelations about scar formation and axon regeneration as well as the intriguing time dependent changes in microglial biology but also for its potential translational significance.

We appreciate the positive comments of this reviewer.

I have but a few minor suggestions that could help clarify some issues that could help make this wonderful work even better.

1. In the extended data sections figs 5 and 11, the disc-shaped astrocytic walls that form in the lesion epicenter after microglial depletion or microglial specific fibronectin depletion in the neonate are extremely interesting, especially since robustly growing, neonatal 5-HT axons stop abruptly just rostral

and adjacent to them. This may be one of the most convincing demonstrations that astrocytes (perhaps largely by themselves) can form an axon regeneration blocking "scar" barrier. The question here is whether this barrier structure that develops postnatally is relatively free of the additional inflammatory cells as well as fibroblastic/basal lamina-like (see below) and additional ECM contributions to scar that form in the adult? Can the authors say anything about the presence or absence of various ECM molecules or fibroblasts or macrophages in this structure? Do you have higher magnification images to show more about its physical make-up (density, architecture of cells within the wall)?
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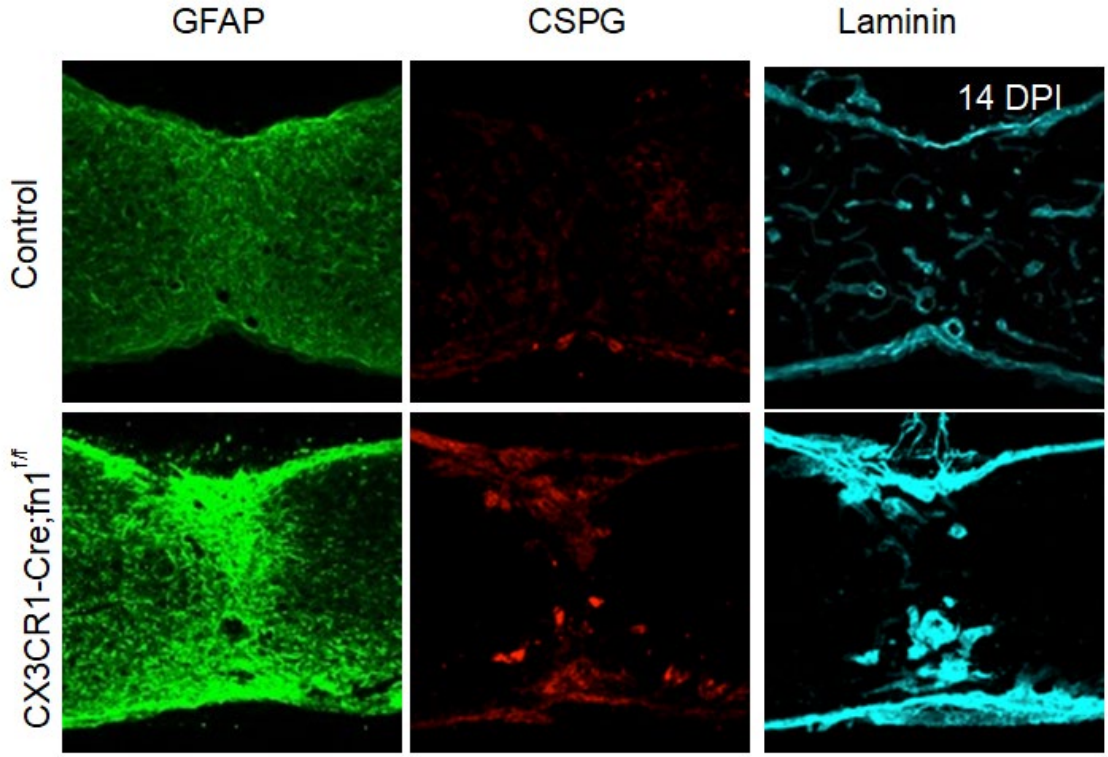
Following the advice of this reviewer, we performed additional IHC with the sections from neonatal injured mice with microglial depletion or fibronectin knockout. Interestingly, despite GFAP+ cell accumulation, very little basal lamina components, such as CSPG, laminin and collagen I were detected around the injury epicenter. High mag images showed that GFAP+-enriched area appears to be a barrier for axon crossing. A few axons still manage to cross it, perhaps due to the "loose" scar with limited CSPG accumulation. We have included these data in Fig. S5c&d (Microglia depletion), Fig. 4e and below (Cx3cr1-cre, $fn1^{f/f}$). Interestingly, as this reviewer pointed out, these data are consistent with what shown by Rudge and Silver, J Neurosci 1990 (please see our responses to the next question). We also emphasized this in the revision.



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lamina is comprised, in part, of collagens 1 and 4, fibronectin, laminin and CSPGs among other proteins. It has been shown that swirls of basal lamina structures form after implant lesions in the mature cortex but not the neonatal cortex and that such scar matrix along with its associated cells when extracted and placed in vitro is non-permissive/inhibitory for axonal outgrowth unlike the highly axonal growth promoting scar/basal lamina-free wound tissue extracted from the neonate (Rudge and Silver, J Neurosci 1990). The authors should briefly discuss and cite this early work that is highly relevant to the present research because it showed that such scar tissues are overtly inhibitory to axonal outgrowth and which structures or molecules within scar inhibit regeneration. When such basal lamina swirls develop in a perpendicular orientation in relation to regenerating axons they can, in addition to their molecular inhibitory properties, provide a mechanical barrier as well. Can the authors verify whether basal lamina is present or not in the vicinity of the adult crush lesion 2 weeks after injury in their model? Basal lamina is likely to be most abundant where the collagen staining is most intense near the outer edges of the lesion core at the interface with astroglia (fig 1 e). Basal lamina could be revealed simply by staining with laminin antibodies or preferably via EM. Given the interest in CSPGs as inhibitory components of the scar/basal lamina ECM (See McKeon et al., 1991 among many other papers), the authors could also stain the mature lesion wound tissue with antibodies to CSPGs (compare P2 crush with adult crush as in fig 1).

In this revision, we included the data from IHC with antibodies against additional basal lamina components on the sections from P20/adult injury. Our results support that basal lamina is a barrier for axon crossing (please also see the responses above), consistent with the results in Rudge and Silver, 1990 and McKeon et al., 1991, both of which are cited in this revision.

3. Regarding the microglia transplant experiments, are there images of GFAP differences in the different groups?

As shown in Fig. S10C of this revision, transplantation of neonatal microglia or adult microglia treated with proteinase inhibitors led to continuous GFAP+ cells and reduced CSPG accumulation (Fig S10c).

4. Cite Jenny Zou paper about plexin B2 and microglia in scar formation.

We cited this paper.

5. Without going into great detail (realizing that functional recovery data may be the topic of a future publication) can the authors give a brief general description of the functional recovery of the animals with neonatal lesion and regeneration (these are the most interesting)? Also, older lesioned animals and especially those that had some induced regeneration after microglial transplants would be interesting to know about their functional recovery.

We appreciate the kind consideration of this reviewer. The mice with neonatal injury do recover hindlimb locomotion, similar to what shown in rats by others (such as Miya et al., J. Neurosci 1997). However, it is unknown as to the contribution of lesion-crossing descending axons and/or intra-spinal re-organization after neonatal injury, which is a focus of our current work.

For the mice with adult lesion and grafting, we have not carefully analyzed their behavioral outcomes yet. Again, our future studies will address this.

6. For the sake of referencing the historical literature on the possibility of spinal cord regeneration in neonatal mammals, the authors might wish to cite some of the early work of Norman Saunders who

showed regeneration of axons across complete transection lesions in the neonatal opossum. Although Marsupials are born at a very early stage of neural development, none-the-less, there is a body of previous work showing that regeneration in the neonatal mammal can occur albeit only in certain species that are born precociously.

Thanks for this important point. We cited Fry et al., 2003 from Norman Saunders in this revision.

7. There are a few grammatical errors scattered about. Here are a few that I caught.

Scar forming-based should just be scar-based

Pg 3 Interestingly these cells begun…. Should be .. these cells had begun….

…most microglia were ramified morphology should be most microglia displayed a ramified morphology…

We fixed them in this revision.

Referee #2 (Remarks to the Author):

The manuscript by Li et al presents evidence for a critical role of microglia in scar-free tissue healing and axon growth after spinal cord injury in neonatal mice. They document extensive 5-HT and corticospinal axon growth following a spinal cord crush injury at P2 (postnatal day 2, or neonatal stage). This correlates with a dramatic difference in tissue bridging and healing between P2 and adult in fibronectin, microglia and macrophage dynamics as well as astrocyte scar and blood vessel repopulation at the injury site. Pharmacological and genetic depletion of microglia reduces tissue bridging and healing after neonatal injury. Single cell sequencing identified several types of neonatal microglia with MG3 being the type most enriched for fibronectin and peptidase inhibitor expression. Genetic depletion of microglia-derived fibronectin reduces tissue healing and 5-HT axon growth after neonatal injury. In contrast, transplanting neonatal microglia or peptidase inhibitor-treated adult microglia into the injury site improves tissue healing and 5-HT axon growth after injury in adult mice.

This is a highly significant study, including 1) the identification of microglia as a critical player in the tissue healing response after neonatal injury; 2) the identification of two molecular targets for this repair-promoting ability of microglia (fibronectin and peptidase inhibitors). The study was well designed with extensive genetic and pharmacological function-perturbation experiments. The results have important implications in both the fundamental understanding and potential clinical translation for spinal cord repair. The comments below are meant to improve the manuscript and can be addressed with discussion if data are not already available.

We appreciate the encouraging comments of this reviewer.

1. As the authors rightly pointed out, they “could not distinguish if these axons are regenerating or uninjured and late-arriving” after P2 injury (page 2). Thus, the authors may wish to clarify that “regeneration” here is broadly defined as axonal growth after injury, similar to that proposed by Sofroniew (2018). Alternatively, the authors may wish to use the term “axon growth” instead of “axon regeneration”. Relevant to this, what is proportion of 5-HT and CST axons yet to pass T10 at P2?

We appreciate this point. We emphasize that we cannot distinguish crossing axons are regenerating or

uninjured and late-arriving after P2 injury and also changed from regeneration to regrowth in the text. In addition, as shown in Fig. 1b/1d, about 80% and 32% of CST axons and serotonergic axons crossed the lesion after neonatal injury, respectively.

2. Compared with adult, how consistent is a spinal cord crush injury at P2? How is this consistency determined?

Compared to adult, the spinal cord of neonatal mice is soft and has robust growth ability, which makes the crush injury more consistent. We assessed the consistency by IHC with antibodies against 5-HT, GFAP and fibronectin at different time points post-injury. Based on these results, we eliminated a small number of animals showing spared axons. An important issue with neonatal injury is proper animal care during which close monitoring of feeding is needed, as well as avoiding cannibalism and sacrificing animals which lose too much weight. We added these details in the methods section of this paper.

3. How do the patterns of "regenerated" axons after P2 injury compare with the uninjured axons? Do P2 injured mice fully recover function such as locomotor behavior?

We included the images of CST projections from control and the mice with neonatal injury in Fig. S1c. It appears that after injury, axons showed delayed projection. In addition, unlike the tightly bundled CST axons seen in uninjured controls, more CST axons derailed from the main tract around the lesions.

As to the functional recovery, please see our responses to the question #5 of reviewer 1 above.

4. The data support the role of microglia-derived fibronectin in scar-free healing. Are fibroblasts present at 3 days after P2 injury?

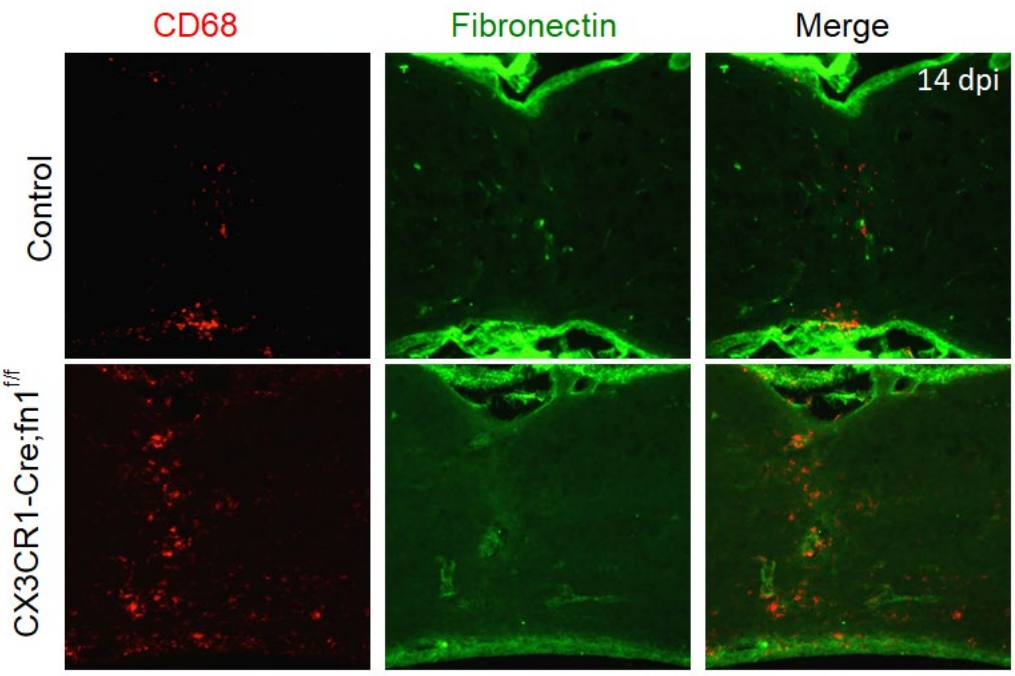
No. We added collagen I staining images in Fig. S3a. Only little collagen signals were detected in the blood vessels (likely expressed in pericytes).

5. The results from genetic and pharmacological depletion of microglia are convincing. However, is it possible that depletion of other cell types such as astrocytes after P2 injury may also compromise tissue repair at the injury site? If so, microglia is still an important cell type for this tissue repair, but may not be the only (or primary) cell type orchestrating tissue healing since other cell types may also be at play.

It is possible that other cell types also play important roles. However, astrocytes only start to accumulate in the lesion at a later time point, thus are less likely to be a primary organizer (Fig. S3).

6. Ext data Fig. 11: what about CD68 and fibronectin at 14 dpi? This figure presents evidence for an important conclusion, and can be a main figure.

Following the advice of this reviewer, we moved this to Figure 4 of the revision. At 14 dpi of Fn1 KO mice, some CD68+ cells remain, but only with very little fibronectin, possibly from other sources (like blood vessel, see below).

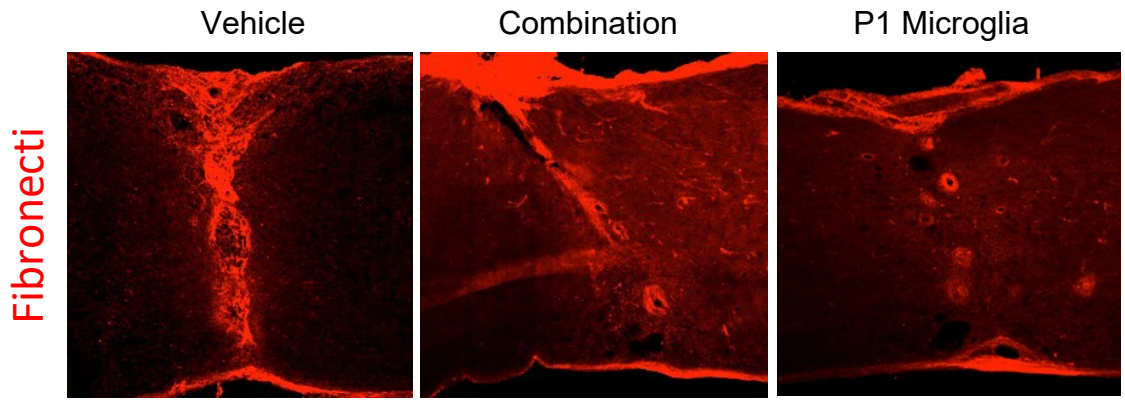


7. Fig. 4a, clarify the exact area for quantification (e.g. use dotted lines); Fig. 4c, no difference between control and vehicle adult microglia? Images suggest some difference, particularly at the perilesional area. Also, what happens to fibronectin here?

For panel a (now Fig 5), we only quantified the epicenter of lesion (100µm width region). We added dotted lines to the images.

For panel c (now Fig. 5), we agree that differences exist in the perilesional area, but not in the epicenter. An exception is GFAP as shown in Fig. 5c and S10c. We speculate that such differences in the perilesional area might be secondary to the reduced inflammation as indicated by profound reduction of Ly6G signal (for infiltrated immune cells). Thus, our quantification has focused on the epicenter of lesion.

The results of fibronectin are similar to that of collagen 1 (please see below).



8. Neonatal microglia transplant is an important experiment. Suggest to include this result in Abstract. Also, did the authors assess CST regeneration with microglia transplants?

We included this in the Abstract in the revision. Because of requiring additional surgical procedures, we hope that this reviewer agrees that we will leave analyzing CST and other descending axons for our future studies.

9. Is there any literature on the use of similar peptidases alone in spinal cord injury models? What is the potential of using such peptidases in the absence of microglia transplantation?

We are not aware of using such proteinase inhibitors in SCI models. However, in a recent study, Ou et al used the same proteinase inhibitors, together with a mitochondrial uncoupler, to convert a few types of cells to be cold resistant. We cited this paper in the revision. As our results suggested a transient requirement for these proteinase inhibitors, it is possible to test them in the absence of microglia in future studies.

10. "This feature of neonatal microglia is different from the permanent activation of microglia in the adult spinal cord lesions." Could the results be interpreted as the absence of certain type of transiently activated microglia allows for the persistence of monocyte-derived macrophages at the adult injury core?

It is possible. Future studies will test this.

11. Have the authors tried another injury model other than crush?

We have not done this yet but will consider it as an interesting future direction.

Minor:
Page 3, "these cells begun" should be "these cells began".
"most microglia were ramified morphology". Check grammar.

Page 5, "MG1 cells in the spinal cord proximal to the lesion (Fig. 3h)". "Proximal" often refers to proximal vs distal. Do the authors mean just outside of the lesion core/epicenter, or around the lesion (as in figure legend)?

Page 7, "is required for bridge forming across the lesion site, but avoiding scar formation" is awkward.
"CTCF" should be "CTGF".

Fig. 2c, e, Is the gap at the center of the lesion following microglia depletion negative for DAPI, meaning no cells?

No DAPI+ cells, but we cannot rule out the possibility that some red blood cells exist in the gap.

Fig. 3f legend: "expression of Fn1 express in and around the lesion site". Remove "express".
Fig. 4c, gray scale coding is confusing and is probably not needed.
Page 20, "as described previously". Need reference.
Ext Data Fig. 11, 12: Scar bar (typo, three times)

We appreciate the careful review of this reviewer and fixed these errors in the revision.

Referee #3 (Remarks to the Author):

The paper by He and co authors reveals new insight into the remarkable regenerative capacity of the postnatal spinal cord. In an elegant series of experiments the authors identify microglia as key orchestrators of scar-free wound healing in mice with complete crush injuries, when the injury is performed at postnatal day 2 (P2). They reveal an orchestrated wound healing response over early time points after P2 crush injury. This is signified by rapid deposition and then clearance of fibronectin matrix in the lesion core, which is associated with transient activation of microglia. This response appears to enable the cut spinal cord stumps to efficiently knit together, effectively closing the wound. By 7 days post injury there is near complete healing - activated microglia (identified with CD68 and SPP1) have returned to a homeostatic state (identified with P2Y12), fibronectin is no longer present, there is no astrocytic scar and this remarkable wound repair is reflected by exuberant growth of serotonergic axons across the lesion. The phenotype and function of P2 microglia appears to be critical for this process. They use pharmacological (CSF1R inhibitor) and genetic (conditional knockout of CSFR1 in microglia) approaches to deplete microglia, and no longer see effective wound healing after P2 injuries, corroborating the key role of microglia in scar-free wound healing. Using single cell RNAseq they reveal that P2 microglia have a distinct gene signature. They identify transcriptionally distinct MG1 and MG3 injury-induced neonatal microglia as potential key mediators of scar-free wound healing. Finally, transplanted adult microglia, treated with peptidase inhibitors identified in P2 microglia, enabled improved wound healing in adult spinal cord lesions.

Although the concept of age-dependent differences in axon regeneration is not new, identifying the cell type, the phenotype, and the distinct molecular signature that underlies the regenerative and wound healing capacity of the neonatal spinal cord is novel and potentially transformative. The pivotal finding that P2 microglia have a distinct molecular profile that enables them to rapidly recover to a homeostatic state, and that this could be the key for scar-free wound healing, represents a step change in our understanding of wound healing and pathological scarring. This work has wide implications for nervous system disorders where regeneration failure, scarring and gliosis are common pathological hallmarks, potentially leading to new therapeutic avenues for treating traumatic injuries or diseases of the brain and spinal cord.

The work is exceptional. The findings are potentially ground-breaking. Below I list several comments and suggestions relating to the data presented.

We appreciate the positive comments of this reviewer.

1. Involvement of other extracellular matrix (ECM) components. Other than collagen-1 and fibronectin, have the authors examined other ECM expression changes in the different conditions? In particular, scar-associated ECM, particularly CSPGs, are known to play a role in restricting regeneration, amplifying the inflammatory response and propagating injury pathology and contributing to failure of effective wound healing. It would be interesting to know if there are CSPG expression changes in the different conditions: scar-free (P2) vs non-permissive (P7-adult) time points; after microglia depletion; after microglial transplantation (either P2 microglia or peptidase inhibitor-treated adult microglia)?

We now included new data about IHC of CSPG in the revision in Fig. S1d-e (without manipulations), Fig. S5c (for microglia depletion) and transplantation (Fig. S10c). Please see our responses to the comments

#1 and #2 of reviewer 1.

2. Did the authors ever assess whether there was full functional recovery after P2 lesion and effective scar-free wound healing e.g. were motor assessments performed in the mice kept for 10 weeks post P2-injury, where extensive corticospinal tract axon growth was observed (Fig 1d)? Similarly, it would be useful to know if there was recovered locomotor function in the microglia transplanted and inhibitor treated adult lesioned mice – was motor recovery assessed over the 4 weeks post-injury?

Please see our responses to the question #5 of the reviewer 1.

3. P2 microglial activation and recovery to homeostatic conditions. An elegant data set is presented, which reveals a distinct injury-induced transcriptional signature of neonatal microglial cells. They show convincing evidence that initially activated microglia are rapidly transitioning back to the homeostatic stage at 5dpi, with the shift in microglia clusters from predominantly MGO (0 dpi) to MG1/MG3 (3 dpi) then MG2 (5dpi), with M0 increasing from 3 to 5dpi. However, why did the authors not also look at 7dpi (or 14dpi) - to show a return to homeostatic microglia at this time point, when scar-free wound healing has occurred (and ideally compared with adult also, where you would expect impaired recovery of homeostatic microglia)?

We appreciate the comment of this reviewer. When we designed the experiments, the primary objective was to analyze the dynamics of microglia at early post-injury points. Our future studies will compare these different time points from the mice with neonatal or adult injury.

4. Dynamics of neonatal wound healing. The histological data are striking, and convincingly show effective and orchestrated wound healing. However, since the data is typically presented as low power images, it is difficult in some instances to see cellular morphology or co-localisation in any detail. For example, the histological images provided do not show high enough resolution to claim co-localisation (e.g. of CD68 and P2Y12, Extended data Fig 2), or morphological changes in microglia from ramified to amoeboid (Fig. 2b, Extended Data Fig. 2a) or fibronectin +ive cells. Where stated “By 3dpi fibronectin+ cells appeared between the gap”, it is not clear from the images (Fig. 2) whether these are cells or matrix – it certainly looks more like matrix than fibronectin+ive cells. Some additional higher power images would add clarification. Also the P2Y12 3dpi data in fig 2b (where it fills the epicentre) seems different from P2Y12 3dpi data in extended fig 2 (where it is largely absent from the epicentre). For histological data, the authors should also avoid referring to inflammation resolution. While it is clear that homeostatic function and phenotype of microglia recovers, to convincingly conclude that there is resolution of inflammation would require analysis of phenotype, cytokine expression, enzymatic and gene expression changes in pathways linked with resolution.

Following the advice of this reviewer, we included high-resolution images of CD68/P2Y12 (Fig.S2a right panels), CD68/Fibronectin/DAPI (Fig. S2b) as well as the morphological changes after injury in Fig.S2a.

We also removed inflammation resolution accordingly.

5. Discussion points.
The injury model is a clean complete crush injury to the spinal cord. While this is an excellent model for looking at wound repair and regeneration, these types of injuries rarely occur clinically. Some discussion on whether they would expect similar scar-free wound healing orchestrated by neonatal microglia in contusion-type injuries would be appreciated.

The authors may want to discuss evidence that serotonin itself promotes regeneration e.g. evidence from zebrafish studies. The high levels of serotonin in the spinal cord at P2 (evident in images showing approximately 90% of the cord to be filled with serotonergic axons, Extended data Fig 1a) could potentially also influence the microenvironment. Also some inclusion of old literature on plasticity of the serotonergic system after neonatal spinal lesions (e.g. Bregman: https://www.ncbi.nlm.nih.gov/pubmed/3304541).

We included these to the revision.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have done an admirable job of responding to my previous critiques as well as those of the other reviewers. Indeed, the addition of new data has made this already incredibly interesting paper even better. The authors are to be heartily congratulated on this lovely and potentially landmark work. I have a couple of suggestions and a comment.

On Page 4 the sentence…. "The majority of axons stalled at the lesion epicenter, with only few penetrating into GFAP+ cells,"…. describes a wonderful new finding but the wording of the sentence is a bit awkward. I would suggest breaking the sentence into 2 parts. You might say something like….. The great majority of axons stalled at the lesion epicenter directly abutting the dense wall of GFAP+ cells. However, a few axons did penetrate into and through the condensed GFAP+ region, perhaps due to the lack of additional inhibitory basal lamina components24.

I'm not sure why the authors have a need to put quotation marks around "astroglial scar". It is what it is.

The authors have shown really strong and incredibly beautiful evidence suggesting that the physical nature of the astroglial scar, even when lacking basal lamina constituents is likely to be a barrier to axonal growth, albeit not absolute, at least when formed in the neonate. The authors have an opportunity to set the record straight about the inhibitory nature of the astroglial scar. The data are clear, the images are remarkable and I encourage, but leave it to them, if they wish to make a stronger statement about the axon and apparently blood vessel growth inhibitory role of the astroglial scar in the results or discussion.

Jerry Silver

Referee #2 (Remarks to the Author):

The revised manuscript from He and colleagues has addressed all my suggestions for revision. The new Figure S1c comparing P2-injured and uninjured control CST axon trajectory 4 week after injury is especially informative (However, please check figure legend; may need to remove the reference on "adult crush").

Overall, this paper is beautifully done, will be highly cited, and may prove to be a classic.

One point for clarification regarding the authors' response to my original comment #1: "In addition, as

shown in Fig. 1b/1d, about 80% and 32% of CST axons and serotonergic axons crossed the lesion after neonatal injury, respectively." Although this is also useful information, this was not what I was asking for. What I meant was to get a sense of how many axons (number and percentage) have crossed T10 at P2 in UNINJURED mice. This will give a sense on the proportions of axons that have been cut and thus would need to regenerate through the P2 injury site versus those have not yet reached T10 by P2 so in essence they were still developing at the time and would cross T10 sometime after injury. I understand that these numbers may not be readily available from this study but wonder if they exist in the literature (for mice or rats).

Referee #3 (Remarks to the Author):

I would have liked to see inclusion of the functional data (as supplementary data, or at least a more full description), rather than simply stating "these mice were able to achieve some degree of hindlimb locomotor function". Otherwise satisfied with the revised version. This is an excellent paper.

Author Rebuttals to First Revision:

Note: Author responses in black

Responses to reviewers' comments:

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However, a few axons did penetrate into and through the condensed GFAP+ region, perhaps due to the lack of additional inhibitory basal lamina components24.

We modified this accordingly in the revision.

I'm not sure why the authors have a need to put quotation marks around "astroglial scar". It is what it is.

We removed it in the revision.

The authors have shown really strong and incredibly beautiful evidence suggesting that the physical nature of the astroglial scar, even when lacking basal lamina constituents is likely to be a barrier to axonal growth, albeit not absolute, at least when formed in the neonate. The authors have an opportunity to set the record straight about the inhibitory nature of the astroglial scar. The data are clear, the images are remarkable and I encourage, but leave it to them, if they wish to make a stronger statement about the axon and apparently blood vessel growth inhibitory role of the astroglial scar in the results or discussion.

We will include more discussion in the text. Referee

#2 (Remarks to the Author):

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especially informative (However, please check figure legend; may need to remove the reference on “adult crush”).

Overall, this paper is beautifully done, will be highly cited, and may prove to be a classic.

One point for clarification regarding the authors’ response to my original comment #1: “In addition, as shown in Fig. 1b/1d, about 80% and 32% of CST axons and serotonergic axons crossed the lesion after neonatal injury, respectively.” Although this is also useful information, this was not what I was asking for. What I meant was to get a sense of how many axons (number and percentage) have crossed T10 at P2 in UNINJURED mice. This will give a sense on the proportions of axons that have been cut and thus would need to regenerate through the P2 injury site versus those have not yet reached T10 by P2 so in essence they were still developing at the time and would cross T10 sometime after injury. I understand that these numbers may not be readily available from this study but wonder if they exist in the literature (for mice or rats).

We thank this reviewer for this interesting question. We have not examined this thoroughly , but according to Bareyre et al (Nature Medicine 11, 1355, 2005), CST axons in the main tract reached T3 by P1, and L5 by P6. Thus, it is likely that significant numbers of crossing axons after P2 injury might be late-growing and un-injured axons, a point we have emphasized in the text.

Referee #3 (Remarks to the Author):

I would have liked to see inclusion of the functional data (as supplementary data, or at least a more full description), rather than simply stating “these mice were able to achieve some degree of hindlimb locomotor function”. Otherwise satisfied with the revised version. This is an excellent paper.

We added more precise indications of locomotor function, stepping and inter-limb coordination, in the revision. As multiple mechanisms might be involved in such behavioral outcomes, our current studies are towards dissecting their detailed mechanisms.