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FGF22 signaling regulates synapse formation during post-injury remodeling of the spinal cord

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Transaction Report:

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1st Editorial Decision   11 December 2014

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see, both referees find the analysis interesting and support publication here. They raise a number of different issues with the analysis, but none of them should involve too much work to resolve. I would therefore like to invite you to submit a suitably revised version for our consideration. Both referees also suggest extending the result section and the description of how the work was done. I think that would be very good and space is not a problem. Should you have any further questions please don't hesitate to contact me.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORT

Referee #1:

Plasticity and the creation of bypass circuits is the main mechanism of functional recovery after spinal cord injury. The paper demonstrates a role for FGF22 signalling in this process. The experiments are thorough and well-performed. If space permits, more detail of the results would improve the readability of the paper.

1. The effects shown in the paper require that there be FGF receptors on the corticospinal axons. The authors show that the relevant neurons express the mRNA for these receptors. However many growth factor receptors are restricted to the dendrites of CNS neurons. If the authors can find a suitable antibody, it would be good to see that the receptors are actually present on the CST axons.

2. Figure 1b. What spinal level is this section from?

3. The authors show pictures of a few of the swellings on the sprouted axons co-stained with a presynaptic marker. Do any of them correspond with a postsynaptic marker? If so, what percent?

4. In the assays of sprouting the authors counted the number of sprouts exiting the dorsal columns. Were there any obvious differences in the length of the sprouts?

5. Why do the authors think that there is no apparent developmental effect of the FGF22 knockout, where the references cited in the paper are all about developmental defects?

6.. Do the authors have an explanation for the compensatory increase in sprouting of CST axons in the single FGF receptor knockdowns?

Referee #2:

This manuscript reports that FGF22 signaling through FGFR1 and FGFR2 regulates and is critical for synaptic sprouting that underlies spontaneous functional recovery after hemisection lesions of the spinal cord. The experiments seem rigorously conducted and well controlled. The data figures are of good quality and the data presented are convincing. The conclusions seem reasonable and not overstated. The findings are interesting and advance information regarding factors that regulate synaptic reorganization associated with spontaneous recovery after partial SCI and that might be targeted for therapeutic interventions. I have only some minor concerns.
The description of what the authors mean by a "dorsal hemisection" should be more detailed and should not simply refer to previous papers. Does "dorsal hemisection" means that the spinal cord was cut on both sides (bilaterally) but only part way down? If so, how far down, and how reproducible or consistent is the cut to the same depth. It will only take a few words to make this clear. Also, does this cut interfere with at least some of the propriospinal projections? This should be discussed more clearly. Lastly, the text on page 4 and 5 should make it more clear that the sprouting of CST is being studied in the cervical region after the transection of the CST at T8. At least this is my interpretation of what is shown. The main text is too short and vague in this regard. The authors may think that it is obvious, but it is not.

Point-to-point response

Reviewer #1

(1.0) Plasticity and the creation of bypass circuits is the main mechanism of functional recovery after spinal cord injury. The paper demonstrates a role for FGF22 signalling in this process. The experiments are thorough and well-performed. If space permits, more detail would improve the readability of the paper.

We thank the reviewer for his/her positive assessment of our work and, as suggested, we have added experimental details to the **results** section and expanded the **discussion** section in the revised manuscript.

(1.1) The effects in the paper require that there be FGF receptors on corticospinal axons. The authors show that the relevant neurons express the mRNA for these receptors. However many growth factor receptors are restricted to the dendrites of CNS neurons. If the authors find a suitable antibody, it would be good to see that the receptors are actually present on the CST axons.

As suggested by the reviewer we have searched for appropriate antibodies that allow immuostaining for FGFR1 and FGFR2 on PFA-fixed cryosections. While we did not find suitable FGFR1 antibodies, we could perform immunostainings for FGFR2. We first confirmed the specificity of the FGFR2 antibody by comparing the staining pattern on tissue from FGFR2 competent and forebrain FGFR2 deficient mice (**Figure E6A**) and then analysed FGFR2 expression in the corticospinal tract of spinal cord-injured mice. Our results show that the receptor is indeed present not only in cortical projection neurons but also along their axons and on their putative synaptic boutons (**Figure E6B-C**). We present these new results in a new **expanded view figure** (**Figure E6**), describe the FGFR2 immunostaining in the revised **methods** (p. 17/18) and discuss the results in the revised discussion section (p.8).

(1.2) Figure 1b. What spinal level is this section from?

The section shown in **Figure 1b** is from cervical spinal level C4/C5 – we apologize for not providing this information in our original submission and have now included it in the revised **figure legend** of **Figure 1**.

(1.3) The authors show pictures of a few swellings on the sprouted axons co-stained with a pre-synaptic marker. Do any of them correspond with a postsynaptic marker? IF so, what percent?

We agree with the reviewer that it would be helpful to know whether the newly formed CST boutons co-localize with a post-synatic marker. To address this question, we have now performed immunostainings for PSD95 (Synaptic Systems PSD95 124 011), an established marker of post-synaptic densities. Our results show that indeed CST boutons commonly co-localize with PSD95-positive densities (see **Reviewer Figure 1**). However the very high density of PSD95-positive structures in the cervical grey matter makes it very difficult to specifically assign post-synaptic densities to individual CST boutons and to our mind unfortunately preclude a reliable quantitative analysis in our system.
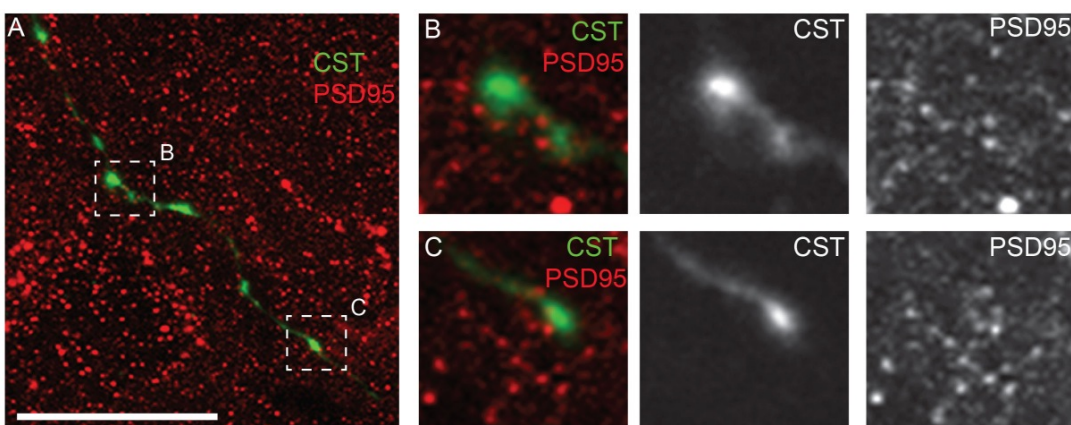
in the grey matter of the cervical spinal cord after counterstaining with an anti-PSD95 antibody (PSD95: red; CST: green). (**B**,**C**) Single confocal image planes showing CST boutons immediately adjacent to PSD95-positive post-synaptic structures (magnified 3 times from the boxes in (**A**); PSD95: red; CST: green). Images on the right are single channels. Scale bar in (**A**) equals 25mm.

(1.4) In the assays of sprouting the authors counted the number of sprouts exiting the dorsal columns. Were there any obvious differences in the length of the sprouts?

Following the reviewer's suggestion we have gone back to our data and compared the length of the collaterals that exited the CST in the different FGF and FGFR deficient mouse lines and their respective control groups. Our analysis shows that there are no significant differences in the length

of individual CST collaterals between these groups. We now present these results in a new **expanded view figure** (**Figure E2**) and include them in the revised **results** section of the manuscript (p. 6)

(1.5) Why do the authors think that there is no apparent developmental effect of the FGF22 knockout, where the references cited are all about developmental defects.

The reviewers raises an interesting point, namely why we did not detect differences in CST development in FGF 22 deficient mice despite the fact that developmental alterations in other parts of the nervous system have been previously described in these mice[1-3]. There are a number of possible explanation for our finding: i) It might be that the developmental defects in FG22 deficient mice are cell-type specific and thus present in some neurons e.g. in the cerebellum[1], the dorsal lateral geniculate nucleus[2] and the hippocampus[3] but not in others like the corticospinal projection neurons. ii) It is possible that corticospinal neurons show initial developmental defects that are however compensated over time and would thus not be detected by our analysis that was performed in adult mice (at 6 weeks of age). The finding that newly formed CST synapses in FGF22 deficient mice still show some maturation between 3 and 12 weeks after lesion (**Figure 5**) would be compatible with such as delayed compensation process. iii) Finally it is conceivable that the CST shows developmental defects that are however not detected by our anatomical analysis. Subtle synaptic deficits for example of the composition of the pre-synaptic vesicle pool that affect neither overall locomotor function nor collateral morphology or synapse number would not be detected by our analysis. We now discuss this important aspect in the revised **discussion** and explicitly mention the limitations of our analysis of normal CST development (p. 9/10).

(1.6) Do the authors have an explanation for the compensatory increase in the sprouting of CST axons in the single FGF receptor knockdowns?

This is another very good question by the reviewer. As this compensatory response is only observed in single FGF receptor deficient mice (both after viral and genetic deletion) but neither in FGF22 deficient nor in the hindlimb motor cortex FGFR1 and FGFR2 double-deficient mice there are to our mind two possible explanations: (i) FGF22 itself has a pro-sprouting effect that can be mediated by either FGFR1 or FGFR2 and is thus present in both single receptor deficient mice but absent when the ligand or both receptors are missing. (ii) The induction of the compensatory response requires the presence of at least some "mature" and thus likely functional synapses on the corresponding CST collateral. As synapse maturation deficits are more pronounced in the FGF22 deficient and the hindlimb motor cortex FGFR1 and FGFR2 double-deficient mice (**Figure 5**) this could prevent the induction of such compensatory response. While it is difficult to definitively resolve this question in our system we now discuss the possible explanations in the revised **discussion** (p. 8/9) of the manuscript.

Reviewer #2

(2.0) The manuscript reports that FGF22 signaling through FGFR1 and FGFR2 regulates and is critical for synaptic sprouting that underlies spontanous functional recovery after hemisection lesions of the spinal cord. The experiments seem rigorously conducted and well controlled. The data figures are of good quality and the data presented are convincing. The conclusions seem reasonable and not overstated. The findings are interesting and advance information regarding factors that regulate synaptic reorganization associated with spontaneous recovery after partial SCI and that might be targeted for therapeutic interventions.

We thank the reviewer for his/her positive assessment of our work.

(2.1) The description of what the authors mean by a "dorsal hemisection" should be more detailed and should not simply refer to previous papers. Does "dorsal hemisection" mean that the spinal cord was cut on both sides (bilaterally) but only part the way down? If so, how far down, and how reproducible or consistent is the cut to the same depth. It will only take some words to make this clear.

(2.2) Also, does this cut interfere with at least some of the propriospinal connections? This should be discussed more clearly.

The reviewer raises important points and we apologize for not defining our lesion model (a thoracic dorsal bilateral hemisection[4]) better. We have now performed additional analyses to more

comprehensively characterize the lesion extent as well as the possible interference with propriospinal connections (see **Reviewer Figure 2**). The results of these analyses show: (i) The maximal lesion depth (measured on longitudinal sections through the lesion) was relatively precisely around 50 % of the spinal cord thickness and did not differ significantly between the different experimental groups (**Reviewer Figure 2A**,**B**; here analysed for FGF22 deficient and forebrain FGFR1 and FGFR2 double-deficient mice and there respective controls as these were the only groups showing significant differences in locomotor recovery). (ii) The 3-dimensional lesion volume calculated on consective longitudinal sections through the spinal cord did not differ between FGF22 deficient and forebrain FGFR1 and FGFR2 double-deficient mice and their respective control groups (**Reviewer Figure 2C**).
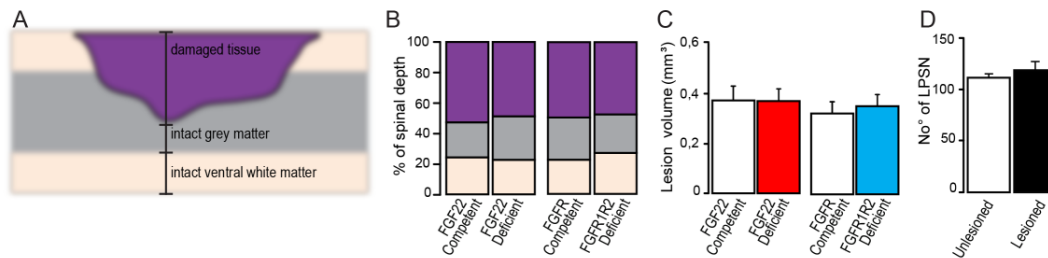
Reviewer Figure 2:(**A**) Schematic drawing of a thoracic dorsal bilateral hemisection of the spinal cord. The maximal depth of the lesion was calculated as follows: a line was drawn from the dorsal surface of the spinal cord to the lowest point of the lesion to measure the damaged tissue (purple). Then the thickness of the intact grey (grey) and white (beige) matter was measured. All values were related to the entire dorso-ventral extent of the spinal cord and then used to create the quantification in (**B**, n=7 mice per group). (**C**) Lesion volumes were also calculated as described in the manuscript and average 0.37 ± 0.047 mm^3 in FGF22 deficient vs 0.37 ± 0.055 mm^3 in FGF22 competent control mice and 0.35 ± 0.03 mm^3 in FGFR1/FGFR2 hindlimb motor cortex deficient mice vs 0.32 ± 0.054 mm^3 in the FGFR competent controls; n=5-7 mice per group. (**D**) Quantification of the number of retrogradely labeled long propriospinal neurons in the cervical spinal cord in lesioned and unlesioned mice. The number of labeled neurons is not significantly different in both groups indicating that the thoracic dorsal bilateral hemisection spares the long propriospinal connections.

This indicates that FGF22 signaling does not interfere with lesion formation and thus the observed differences in locomotor recovery are likely related to an altered axonal remodeling response to the lesion and not to a primary difference in the number of lesioned axons. (iii) Finally, two lines of arguments support the view that propriospinal connections are not affected by the lesion. First, as described above we have examined the dorso-lateral lesion extent and confirmed that none of the lesions reaches the ventral white matter that contains the propriospinal axons (**Reviewer Figure 2A**,**B**). Additionaly we counted the number of retrogradely traced propriospinal neurons in the cervical spinal cord of lesioned mice and unlesioned control mice and found similar number of labelled neurons again indicating that propriospinal axons that retrogradely transport the dye from the lumbar injection site are not transected by the thoracic dorsal bilateral hemisection (**Reviewer Figure 2D**). In the revised **manuscript** we now use the more precise term "thoracic dorsal bilateral hemisection" whenever we refer to the lesion model and better explain the lesion extension in this model in the revised **methods** section (p. 14). We also present the quantification of the lesion volumes in the different experimental groups in the **results** section (p. 7) and of the labeled propriospinal neurons in lesioned and unlesioned mice in the revised **methods** section (p. 15/16).

(2.3) Lastly the text on page 4 and 5 should make it more clear that the sprouting of CST is being studied in the cervical region after transection of the CST a T7. At least this is my interpretation of what is shown. The main text is too short and vague in this regard. The authors might think that it is obvious but it is not.

Indeed CST sprouting was always analysed in the cervical spinal cord after a dorsal bilateral hemisection of the CST at T8. As also suggested by the editor and reviewer 1 we have now extended the **results** section of the manuscript and provide more details on the way experiments were performed including the information where the CST was lesioned and where sprouting was analysed.

References

1.   Umemori H, Linhoff MW, Ornitz DM, Sanes JR (2004) FGF22 and its close relatives are presynaptic organizing molecules in the mammalian brain. *Cell* 118: 257-270.

2.   Singh R, Su J, Brooks J, Terauchi A, Umemori H, Fox MA (2012) Fibroblast growth factor 22 contributes to the development of retinal nerve terminals in the dorsal lateral geniculate nucleus. *Front Mol Neurosci* 4: 61.

3.   Terauchi A, Johnson-Venkatesh EM, Toth AB, Javed D, Sutton MA, Umemori H (2010) Distinct FGFs promote differentiation of excitatory and inhibitory synapses. *Nature* 465: 783-7.

4.   Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. (2004) The injured spinal cord spontanously forms a new intraspinal circuit in adult rats. *Nat Neurosci* 7: 269-277.