EMBO reports

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STAT3 promotes corticospinal remodeling and functional recovery after spinal cord injury

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 26 February 2013

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, the referees agree that the data are mostly convincing and well presented. Referee 1 is surprised by the high infection efficiency and asks for additional data on the proportion of neurons expressing exogenous pSTAT3. The remaining referee comments mainly concern re-writing of the manuscript text.

Given these positive comments, we would like to invite you to revise your manuscript with the

understanding that the referee concerns must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Also, the revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 figures plus 5 supplementary figures, which should directly relate to their corresponding main figure. The current character count slightly exceeds our limits, and the manuscript text could therefore be shortened a little. Please note that the materials and methods essential for the understanding of the experiments described in the main manuscript file must remain in the main text.

Please remember to specify the number (n) of experiments, the error bars (standard deviation, standard error, etc), the 1, 2 or 3 stars and the statistical tests used to calculate p-values for all quantifications in the corresponding figure and supplementary figure legends. This information is currently incomplete.

When submitting your revised manuscript, please include:

A Microsoft Word file of the manuscript text, editable high resolution TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format), a letter detailing your responses to the referee comments, and a two sentence summary of your findings and their significance.

We also recently decided to offer the authors the possibility to submit "source data" with their revised manuscript that will be published in a separate supplemental file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) of your key experiments together with the revised manuscript.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready.

REFEREE REPORTS:

Referee #1:

This manuscript from Florence Bareyre's group indicates that overexpression of STAT3 can promote sprouting of corticospinal neurones after spinal cord injury, and that there are functional benefits to this finding. The paper is clearly written, the data are mostly convincing (except the amount of cortical transgene infection), and the findings represent an important conceptual advance. However, some specific points need to be addressed.

In Fig 2 the authors show that overexpression of pSTAT3 in the corticospinal neurones enhances axonal sprouting after hemisection. The authors show increases in pSTAT3 labelling in the cortex, and in Fig 2c and d indicate that 30 percent of cortical neurones are pSTAT3 positive. This is a high proportion of cells; one would assume that only neurones infected with the pSTAT3 trangene are showing enhanced pSTAT3. This is surprisingly high, and diverges from previous studies; I would like to see more evidence that the vector is indeed this efficient. GFP labeling should be shown to provide corroboration. Many cortical neurones do not project to the spinal cord, thus what proportion of corticospinal neurones are infected by pSTAT3? Please provide this information.

There are some important limitations to the experimental design that should be noted in the text and discussed at the end of the paper. First, the animals underwent injections of pSTAT3 before the spinal cord injury, and this represents a pre-treatment paradigm. This approach might not work as a post-treatment design, and this should be pointed out. This does not alter the finding that STAT3 influences corticospinal growth, and that finding of the paper remains noteable. The authors might consider emphasizing the mechanistic importance of their findings, rather than the practical findings, since it is not known in fact whether this will be practical (if targeted for treatment after injury). It would be nice to see some re-writing of the paper around this point. Second, the case for regeneration of axons in this study is weaker than the evidence to support sprouting. The increased density of axons that are present around the injury site could be a result of growth of unlesioned axons originating adjacent to the injury site (that normally terminate in gray matter just above the lesion), or growth of cut axons (there is a nice summary of this possibility in the review by Tuszynski and Steward in Neuron 2012). It would be better to refer to the observation of more axons around the lesion site as "growth" rather than regeneration, since a complete transection is the only way to clearly claim that there is enhanced regeneration.

Minor:
Fig 1a-d the authors show pSTAT3 expression in intact, lesioned and STAT3 deficient mice. I think they should point out in the figure legend that panel B shows 24hr and panel C shows 3 weeks; this is easily overlooked and might confuse the reader. But the data appear convincing.

Referee #2:

The manuscript by Lang et al. reports that overexpression of STAT3 in cortical motor neurons promotes axon sprouting and functional recovery after spinal cord injuries. In contrast, the authors showed that deletion of STAT3 had no effect on injury-induced axonal sprouting. Overall, the experiments were well done and the data are solid and well presented. Many studies, including authors' previous study, have shown the important role of STAT3 signaling in axon regeneration. The current study provides another evidence that activation of this pathway in cortical motor neurons can promote spinal cord regeneration via enhanced CST axonal sprouting. However, the study falls short of providing mechanistic insight into how STAT3 enhances CST sprouting. Some experiments that reveal the molecular mechanisms by which STAT3 regulates axonal remodeling will greatly improve the manuscript. For instance, how do overexpression of STAT3 induce axonal sprouting and promote axonal midline crossing?

By conditionally knocking out STAT3 in cortical motor neurons, the authors conclude that transient expression of endogenous STAT3 does not contribute to CST injury-induced endogenous axonal remodeling. However, under normal condition, only about 5% of cortical motor neurons showed elevated STAT3 expression upon CST injury. Even if they contributed to endogenous axonal remodeling, it may not be detected.

In Fig. 4, for EMG recording experiments, the authors should also provide the amplitude and the duration of EMG responses.

Referee #3:

Plasticity mechanisms (growth of new collaterals and lesion-circumventing circuitry) can lead to measurable recovery of function after incomplete spinal cord injuries. It would be of considerable clinical interest if we could enhance such post-injury plasticity. The transcription factor STAT3 is important for regeneration of injured sensory axons in the PNS, and deletion of a STAT3 antagonist improves regeneration in the optic nerve. The authors here test for possible effects of STAT3 in two mouse models of incomplete SCI, thoracic dorsal hemisection and unilateral pyramidotomy.
The results are quite interesting: while STAT3 was activated in motor cortex after dorsal hemisection, plasticity appeared normal in Emx-Cre conditional STAT3 nulls. Conversely, overexpression of STAT3 in motor cortex resulted in increased sprouting of collaterals from cervical CST - weakly in injured mice (because the injury alone already induces collateral growth) and strongly in non-injured mice! Only in the non-injured mice (and then in the pyramidotomy model) did the authors observe enhanced formation of contacts between CST collaterals and propriospinal neurons. STAT3 overexpression measurably improved behavioral recovery after unilateral pyramidotomy.
As a bottom line, STAT3 emerges as a general inducer of collateral sprouting in the spinal cord, with no specific link to SCI and the post-injury responses.
This is a fine concise study that in my opinion advances the field and is appropriate for publication in EMBO Reports. The authors may want to attempt a better explanation of their one baffling observation, that STAT3 overexpression increases the number of new contacts onto proprioceptive interneurons (and motoneurons) in the pyramidotomy paradigm, but not in the thoracic hemisection paradigm.

1st Revision - authors' response 29 June 2013

Point to Point response – EMBOR-2013-37152V1

Reviewer 1

The manuscript by Florence Bareyre`s group indicates that the overexpression of STAT3 can promote sprouting of corticalspinal neurons after spinal cord injury, and that there are functional benefits to this finding. The paper is clearly written, the data are mostly convincing (..), and the findings represent an important conceptual advance.

We would like to thank the reviewer for his/her overall encouraging and balanced assessment of our work. We have addressed the remaining concerns as outlined below.

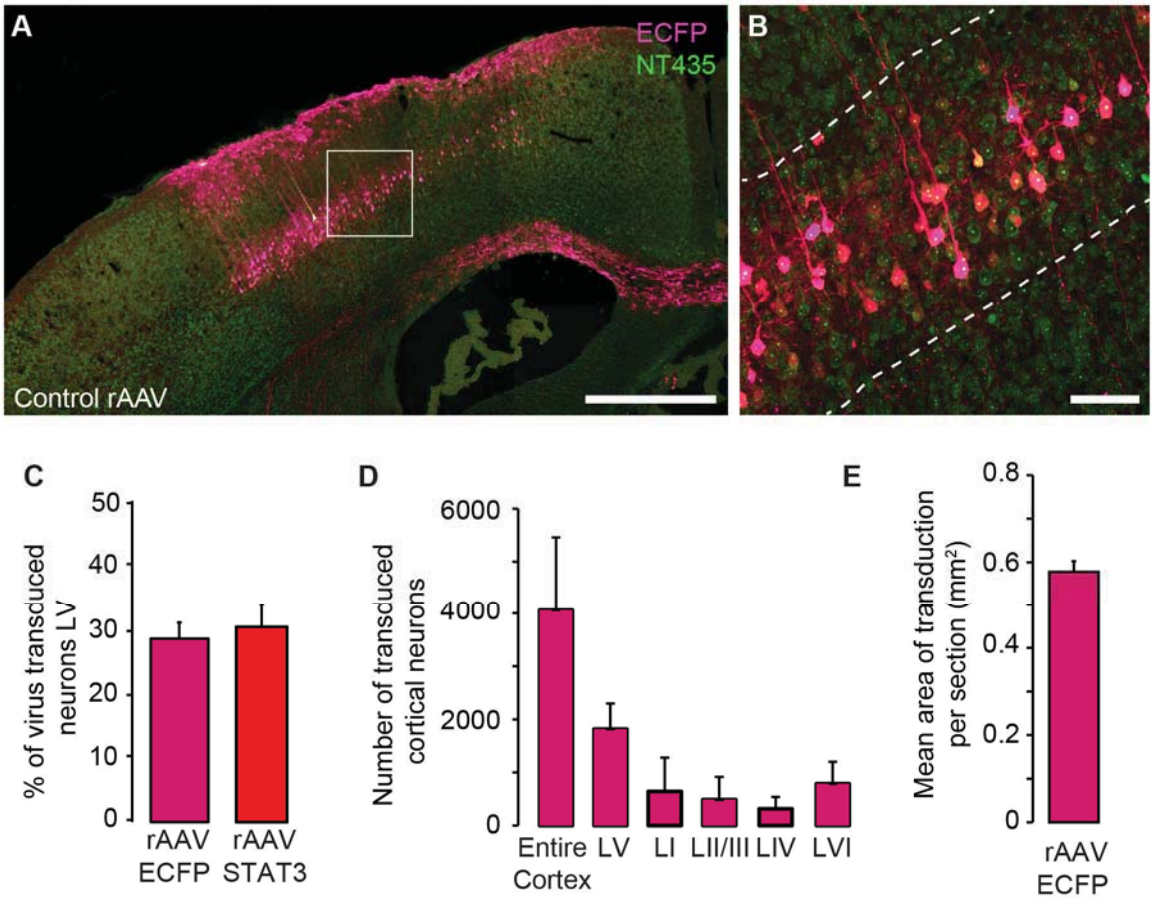
1) The authors show increases in pSTAT3 labelling in the cortex, and in Fig 2c and d indicate that 30 percent of cortical neurons are pSTAT3 positive. This is a high proportion of cells; one would assume that only neurons infected with the transgene are showing enhanced pSTAT3. This is surprisingly high, and diverges from previous studies, I would like to see more evidence that the vector is indeed this efficient. GFP labelling should be shown to provide corroboration. Many cortical neurons do not project to the spinal cord, thus what proportion of corticospinal neurons are infected by pSTAT?

Following the reviewers suggestion we have performed two new sets of experiments to confirm the efficiency of the viral gene transfer to cortical projection neurons:

First, we injected an rAAV expressing enhanced cyan fluorescent protein (ECFP) stereotactically to the hindlimb motor cortex and then quantified the number of layer V neurons (identified by NeuroTrace labeling) that express CFP (see **Reviewer Figure 1**). We found that - similarly to the rAAV-STAT3 experiment presented in the manuscript - about 30% of layer V neurons in the hindlimb motor cortex are transduced by the rAAV-ECFP. With regard to the high rate of transfection, it is important to consider that (as stated in the **Supplementary Information**) we determined the percentage of pSTAT3 positive neurons only in layer V neurons and only in the transduced area around the injection site (imaging fields of 423 µm x 423 µm were counted on five sections before and five sections after the injection site; 50 µm thick sections covering around 500 µm). This area should cover at least a substantial part of the hindlimb motor cortex (Neafsey *et al*, 1986). The percentage of STAT3-positive neurons in the entire motor cortex is however likely to be smaller (see **Reviewer Figure 1**). Indeed, when we calculated the total number of transduced neurons in the cortex (on average 4059 $\pm$ 1399, n = 3 mice) as well as the mean area of transduction per section (0.578 $\pm$ 0.129 mm^2, n = 3 mice) after injection of rAAV-ECFP, we obtained values that are in good agreement with previously published work on rAAV-mediated gene transfer in the cortex (Hutson *et al*, 2012). We apologize for not stating this clearer and now better specify where the quantification was performed in the **revised Figure Legend** and the **revised Supplementary Information (p. 4/5)**.

Second, to address directly which percentage of lesioned CST projection neurons were transduced to express STAT 3, we retrogradely labeled the transected hindlimb projection neurons from the thoracic lesion site using dextran tetramethylrhodamine 3000 MW and then quantified the percentage of the retrogradely labeled neurons in the cortex that expressed p-STAT3 at 4 weeks after virus injection (3

weeks after lesion). This experiment showed that about 30 % of retrogradely-labeled neurons expressed p-STAT3. These results validate our quantification approach and confirm that a substantial fraction of the hindlimb CST projections neurons can be targeted by rAAV-based viral gene transfer. We have included these new results in the **revised Figure 2 (Panel C, E)**.



Reviewer Figure 1. Following injection of rAAV-ECFP at -1.3/1/0.6 mm from bregma, rAAV infected cells were detected in the motor cortex with a rostral-caudal spread of 0.96 ± 0.05 mm (n = 3 mice). (**A**) Confocal images of the mouse motor cortex -1.22mm caudal from bregma (ECFP, red; Neurotrace, green). (**B**) Higher-magnification image of area boxed in (**A**) shows ECFP-positive neurons in layer V (outlined by dashed lines). (**C**) Quantification of the percentage of transduced cortical neurons in layer V (LV) of the hindlimb motor cortex (evaluated as described above in 10 sections between 1.05 - 1.55 mm from bregma, n = 3 mice) after injection of rAAV- STAT3 (red bar, as presented in the manuscript in **Figure 2D**) and after injection of rAAV-ECFP (purple bar). (**D**) Quantification of the number of transduced cortical neurons in the entire mouse brain as well as the different cortical layers after injection of rAAV-ECFP. Note that the highest number of transduced neurons is located in layer V to which the injection was targeted. (**E**) Quantification of the mean transduced area per cortical section after injection of rAAV-ECFP (10 sections per mouse were evaluated for 3 mice). All values represent mean + SEM. Scale bar in (**A**) 500 µm and in (**B**) 50 µm.

2) First, the animals underwent injections of pSTAT3 before the spinal cord injury, and this represents a pre-treatment paradigm. This approach might not work as a post-treatment design, and this should be pointed out. This does not alter the finding that STAT3 influences corticospinal growth and that finding of the paper remains notable.

As suggested by the reviewer we have revised the corresponding section of the discussion to better explain the limitations of the pre-treatment paradigm used in our study (see **revised Manuscript**, **p. 10**). While we agree with the point raised by the reviewer, it is worth noting that rAAV-STAT3 injection was also able to induce collateral sprouting of unlesioned fibers in control animals as well as in the pyramidotomy paradigm. This might suggest that the temporal relation of STAT3 induction to the lesion is less critical.

3) Second, the case for regeneration of axons in this study is weaker than the evidence to support sprouting. The increased density of axons that are present around the injury site could be the result of unlesioned axons originating adjacent to the injury site (that normally terminate in the gray matter just above the lesion); or growth of cut axons (..). It would be better to refer to the observation of more axons around the lesion as "growth" rather than regeneration…

We tried to minimize a contribution of spared fibers by (i) only counting fibers that were emerging from the dorsal main CST and extending in the dorsal funiculus and (ii) verifying the absence of labeled dorsal fibers distal from the lesion site (at thoracic level 11) in all animals used for the analysis (see **Supplementary Information**). We however agree with the reviewer that it is difficult to completely rule out a contribution of other sprouts e.g. those emerging from unlesioned fibers that enter the dorsal funiculus above the level of the lesion and we have therefore replaced the term "regeneration" with the less charged term "growth" throughout the manuscript.

4) Minor: Fig 1a-d the authors show pSTAT3 expression in intact, lesioned and STAT3 deficient mice. I think they should point out in the figure legend that panel B shows 24hr and panel C shows 3 weeks; this is easily overlooked and might confuse the reader.

We thank the reviewer for his suggestion and have ameliorated the figure legend accordingly.

Reviewer 2

The manuscript by Lang et al. reports that overexpression of STAT3 in cortical motor neurons promotes axon sprouting and functional recovery after spinal cord injuries. In contrast, the authors showed that deletion of STAT3 had no effects on injury-induced axonal sprouting. Overall the experiments were well done and the data are solid and well presented.

We would like to thank the reviewer for his/her overall encouraging and balanced assessment of our work. We have addressed the remaining concerns as outlined below.

1) However the study falls short of providing mechanistic insight into how STAT3 enhances CST sprouting. Some experiments that reveal the molecular mechanisms by which STAT3 regulates axonal remodelling will greatly improve the manuscript…

We agree with the reviewer that the molecular mechanisms that mediate the effects STAT3 on neuronal growth and remodeling is a fascinating and important topic. Indeed in recent years a number of

STAT3 regulated genes have been identified (Coqueret and Gascan, 2003; Pradervand *et al*, 2004; Smith *et al*, 2011). A conclusive experimental analysis of these candidates however would to our mind require the generation of novel tools (in particular floxed mouse lines for the candidate genes) and very substantial additional experimental analysis. After discussing this aspect with the editor, we feel that the downstream mechanisms of STAT3 are clearly important but not within the scope of this particular manuscript that primarily focuses on analyzing the anatomical and functional consequences of STAT3-induced axonal remodeling after spinal cord injury. We however now discuss this important point in the **revised Manuscript** (**p.7**) and cite reports that have identified potential downstream mediators of STAT3.

2) By conditionally knocking out STAT3 in cortical motor neurons, the authors conclude that transient expression of endogenous STAT3 does not contribute to CST injury-induced endogenous axonal remodelling. However, under normal conditions only about 5 % of cortical motor neurons showed elevated STAT3 expression upon CST injury. Even if they contributed to the endogenous axonal remodelling, it may not be detected.

The reviewer correctly points out that only a relatively small proportion of cortical projection neurons (about 5-10 %) transiently increase STAT3 expression after injury and as such, it might be difficult to detect changes on neuronal remodeling caused by the conditional deletion of this expression. While this is clearly a possible argument, we would have assumed that - if STAT3 is indeed a key element of endogenous growth induction – the neurons that actually express STAT3 should be those that attempt to remodel endogenously. While these neurons would only be a small proportion of all neurons in the cortex they should represent a much more substantial proportion of those neurons that initiate growth. While we thus still think that our results make a substantial contribution of STAT3 expression to endogenous remodeling at least unlikely, we agree that the more conservative interpretation of our data is that we can only rule out that those cells that transiently express STAT3 are primarily responsible for endogenous remodeling. We have now revised the discussion of these experiments to take the reviewers concern into account (see **revised Manuscript**, **p. 5/6**).

3) In Fig. 4, for EMG recording experiments, the authors should also provide the amplitude and the duration of EMG responses

As suggested by the reviewer we have now included the amplitude and duration of the EMG responses in the **revised Supplementary Information** (**p. 10**).

Reviewer 3

This is a fine concise study that in my opinion advances the field and is appropriate for publication in EMBO Reports.

We would like to thank the reviewer for his/her overall encouraging and balanced assessment of our work. We have addressed the remaining concerns as outlined below.

The authors may want to attempt a better explanation of their one baffling observation, that STAT3 overexpression increases the number of new contacts onto propriospinal interneurons (and motoneurons) in the pyramidotomy paradigm, but not in the thoracic hemisection paradigm.

We thank the reviewer for raising this interesting point. We believe that this difference - that is indeed surprising - is likely explained by the different size of the STAT3 effects on the remodeling of lesioned and unlesioned fibers. Indeed one of the key findings of our study is that while STAT3 leads only to moderate changes in the growth response of lesioned CST fibers, it can induce substantial de novo remodeling of unlesioned fibers. For example, the collateral sprouting of lesioned CST axons in the thoracic hemisection paradigm is only increased by about 25 % (compared to mice injected with Control-rAAV) - an increase that may be too small to lead to significant (and measurable) changes of the formation of detour circuits and more specifically of the percentage of long propriospinal neurons that are contacted by these collaterals. In contrast, in the pyramidotomy paradigm the number of unlesioned fibers that sprout and cross the spinal midline is increased more than 3-fold (compared to controls) and as a result about 4-times more short propriospinal neurons and more than 10-times more motoneurons are contacted by these fibers. This argument is supported by our experiments in which we investigate detour circuit formation in the absence of a lesion. In this case injection of rAAV STAT3 into the cortex leads to more than 4-fold increase in the sprouting of cervical collaterals and as a result to a significant increase of the percentage of long propriospinal neurons that are contacted (**Fig. 2 O**). We have included this explanation in the **revised Manuscript (p. 7)**.

References

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Neafsey EJ *et al* (1986) The organization of the rat motor cortex: a microstimulation mapping study. Brain Res **396**:77–96

Pradervand S *et al* (2004) Small proline-rich protein 1A is a gp130 pathway- and stress-inducible cardioprotective protein. *EMBO J* **23**: 4517-25.

Smith RP, Lerch-Haner JK, Pardinas JR, Buchser WJ, Bixby JL, Lemmon VP (2011) Transcriptional profiling of intrinsic PNS factors in the postnatal mouse. *Mol Cell Neurosci* **46**: 32-44.

2nd Editorial Decision 09 July 2013

Thank you for the submission of your revised manuscript. Referee 1 is happy with the revised version and we can therefore in principle accept your manuscript now.

However, the character count largely exceeds our limit (currently 34.809), and the manuscript text therefore needs to be shortened by at least 4000 characters. Regarding the Methods section, I think the generation and production of AAV vectors, the surgical procedures, the tissue processing and histological analysis, image processing, statistical evaluation and may be behavioral analysis and electrophysiology can be deleted, especially if the same information provided in the method section now is already given in the main manuscript text (and figure legends). Regarding the statistics, do the explanations of the values and error bars at the end of each figure legend always apply to the entire figure? If yes, can you please explain this in the figure legend by saying, for example, "all bars and error bars in this figure represent mean+/-SEM". In order to reach our limit of 30.000 characters, the main manuscript text will most likely need to be shortened. Please let me know if you have any further questions.

I look forward to seeing a new revised version of your manuscript as soon as possible.

REFEREE REPORT:

Referee #1:

The authors have addressed my concerns nicely.

2nd Revision - authors' response 15 July 2013

Thanks for your mail - please find attached:

A) a shortened version of the manuscript (now at 29 900 characters) - we have removed the methods parts as suggested, shortened some discussion of our data and removed non -essential references. Further we have added the statement " All bars and error bars are mean +/- SEM" in all Figure Legends.

B) a new version of the Supplementary Information - here we have added the methods describing the statistical analysis and added the statement " All bars and error bars are mean +/- SEM" in all Supplementary Figure Legends

Please let me know if we should also upload these files through the electronic submission system and/or if you have any additional suggestions.

Thanks again for your support and advice and best regards.

3rd Editorial Decision 16 July 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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