Positional differences of axon growth rates between sensory neurons encoded by RUNX3


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(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 02 May 2012

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.

Both referees appreciate the analysis, but they also find that the analysis needs to be extended in order to consider publication here. Referee #1 finds that some further insight into either the upstream regulator of Runx3 or into the downstream effectors of Runx3 is needed. Looking into the latter aspect would also tie in the transcriptome analysis better, which at the moment is a bit disconnected from the rest of the analysis. Performing these experiments in cultured neurons is sufficient. I don't know if you have data on hand to address either of these issues, but anything that you can add along those lines will clearly strengthen the analysis. We can discuss this issue further should that be helpful. Referee #2 suggests a few experiments, but also finds that some of the statements regarding the novelty of the findings needs to be toned down a bit.

Should you be able to extend the findings along the lines as indicated by the referees then I would like to invite you to submit a revised version of the manuscript. I should add that it is EMBO Journal policy to allow only a single major round of revision only and that it is therefore important to address the concerns raised at this stage.

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://www.nature.com/embr/about/process.html
Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Lallemend and coworkers present a new function for the transcription factor Runx3 in sensory axon growth. Increasing the levels of Runx3 by in ovo transfection increased axon growth of cultured sensory neurons; interfering with Runx3 expression by RNAi knockdown or gene targeting in mouse impaired axon growth in vivo and in vitro. The abundance of endogenous Runx3 along the rostro-caudal axis correlated with differential sensory axon growth rates: the higher Runx3 abundance, the faster axons grew. The authors also performed a transcriptome analysis of brachial DRGs of WT and Runx3 KO embryos for RNA transcripts which were under control of Runx3 expression. However, none of the candidates were followed up in this study.

The data in general are of high technical quality and convincing. The correlation between Runx3 expression along the rostro-caudal axis and axon growth rate is novel and interesting. The story remains somewhat descriptive, as the upstream cues which regulate Runx3 expression and the downstream effectors of Runx3 were not investigated.

1. The authors convincingly show that extrinsic cues from the target tissue (limbs) do not regulate Runx3 expression, but do not explore the more locally operating extrinsic cues that might directly regulate Runx3 expression. Instead they argue that DRG sensory neurons display intrinsic differences in growth rate which are encoded by Runx3. I find that confusing. The authors should only use the term 'intrinsic', when they can be sure that Runx3 expression is regulated by upstream transcriptional programs and not simply by a local extrinsic cue.

2. For the readers of the EMBO Journal, the story would gain considerable interest, if the authors could validate one of the Runx3-regulated genes at least in cultured neurons. In the present manuscript, the transcriptome analysis remains disconnected from the rest of the story.

3. The observation that Runx3 controls axon growth of sensory neurons is not entirely novel and was previously proposed by Jessell and coworkers. The present work contains convincing data which indicates that the previously proposed direct role of Runx3 in axon growth in the spinal cord is likely to be indirectly due to a failure of peripheral growth. This observation, however, would be more appropriate for a specialized neuroscience journal.

In conclusion, the present story seems better placed in a neuroscience journal, but it could potentially be suited for the EMBO Journal, if the authors added some (in vitro) data on either the upstream regulation of Runx3 in the relevant sensory neuron subpopulation, or on a potential downstream effector of Runx3.

Referee #2 (Remarks to the Author):

Lallemend and colleagues describe intrinsic differences in axon growth rate from early spinal sensory neurons of different axial levels that are correlated with the distance these axons have to grow to reach their peripheral targets. This is not a new concept, contrary to the authors' assertion. This has already been extensively documented for different populations of cranial sensory neurons over two decades ago. Nonetheless, the contribution of this paper is that it begins to provide
evidence for aspects of the molecular mechanism underlying intrinsic differences in early sensory neuron axon growth rate, and as such is a valuable addition to the literature.

Using very similar methodology to that previously employed for demonstrating intrinsic differences in initial axonal growth rate correlated with target distance for different populations of cranial sensory neurons, the authors demonstrate intrinsic differences in initial axonal growth rate correlated with target distance for different populations of spinal sensory neurons (those at lumbar and brachial levels extend axons faster than those at thoracic levels). The authors then show that the spatial and temporal patterns of expression of the transcription factor Runx3 in early DRG neurons of different axial levels in vivo are correlated with intrinsic differences in axonal growth rate. Furthermore, Runx3 expression levels in early DRG neurons of different axial levels were unaffected by early target field ablation, indicated the level of Runx3 expression is determined independently of the peripheral target. To test the role of Runx3 in governing intrinsic differences in axon growth rate, the authors manipulated the expression level or function of Runx3 in early chick DRG neurons by electroporating these neurons with siRNA or plasmids that overexpress Runx3 or express a mutant Runx3 that interferes with the function of the endogenous protein. They show that overexpression enhances axon growth rate whereas reducing expression or interfering with function reduces axon growth rate. Furthermore, they show that the extent of neurite growth from E11.5 brachial DRG established from Runx3-/- mice is significantly reduced compared with explants established from wild type mice. Finally, whole transcriptome analysis of E11.5 brachial DRGs of WT and Runx3-/- mice revealed that many of the positively regulated Runx3 genes are involved in microtubule dynamics, although the involvement of these and other Runx3-regulated genes in intrinsic differences in axon growth rate has not been evaluated.

This manuscript makes an important start to elucidating the molecular mechanisms that are responsible for intrinsic differences in initial axon growth rate. The data seem robust and reliable and the study is competently executed and well illustrated. The study raises import further questions such as what controls the neuronal differences in Runx3 expression, which Runx3 regulated genes are necessary and sufficient for regulating intrinsic differences in axonal growth and whether Runx3 plays a role in regulating axon growth rate in other populations of neurons in which intrinsic differences in early axon growth have been described.

I have the following points:

The in vitro mouse axon growth data is based on the analysis of neurite growth from explants. Although the authors have obtained results (in as far as they go) that are consistent with those of the rest of the study, quantifying axonal growth from explants is notoriously difficult. It would be far better and much more convincing to obtain axonal growth rate data from neurons grown in low density dissociated cultures.

Although the authors have shown that the extent of neurite outgrowth from brachial DRG explants of E11.5 Runx3-/- mice was reduced and comparable to that from wild type thoracic DRG explants, is there any effect of Runx3 deletion on the growth of thoracic DRG axons? This needs to be carefully addressed in dissociated cultures. This is an important and interesting question in that it addresses the issue of whether endogenous Runx3 is required for axon growth per se and to what extent it enhances axon growth above a certain basal level, Is growth from brachial DRG neurons close to a hypothetical basal level, for example?

The really glaring omission and distortion in this manuscript is the claim that a novel developmental principal in the nervous system is being described for the first time, namely, that there are intrinsic differences in axonal growth rate among different populations of a particular neuron subtype that are correlated with the distance axons have to growth to reach their targets. Indeed, the Abstract begins with a contrary assertion "..... implying a similarity between neuronal subtypes in terms of nerve extension", as if all that is known about this issue is misleading and inaccurate supposition. Furthermore, in the Introduction the authors predict "intrinsic differences in the propensity of axonal growth between different neurons" from the from the fact that some neurons establish very long projections while others form contacts locally. There's no prediction about it. It has already been clearly and convincingly demonstrated that there are marked intrinsic differences in axonal growth rate from different populations of cranial sensory neurons that are correlated with the length of their projections in the developing chicken embryo (Intrinsic differences in the growth rate of early nerve
fibres related to target distance. Nature 1989, 337:553-555). While the authors make a valuable start to understanding the molecular mechanism underlying intrinsic differences in axonal growth rate from developing sensory neurons, they must make it abundantly clear in their manuscript that this is all they do and do not claim to have described a novel developmental principle for the first time. They need to cite the original discovery of this principle and concede that they neither predicted nor discovered it. I'm really surprised by this omission as it seems inconceivable that the authors are not familiar with the relevant literature.

Response to reviewers:

Reviewer 1

1. We agree with the reviewer that our results does not exclude that Runx3 is regulated by local patterning molecules or other extrinsic cues. Our rational for claiming an intrinsic mechanism in the manuscript was based on that the propensity of growth once encoded within the cell is independent on extrinsic factors. Hence, if neurons are taken out of the tissue and cultured at very low density, they display different axon growth properties. Such differences must be cell intrinsic, since the neurons are grown on exactly the same substrate, yet display different growth rates. The experimental basis for this conclusion is paralleled by previous work (Davies AM, Nature 1989, 337:553-555). However, we realize by the reviewerís comment that this concept can easily be misinterpreted and have therefore followed the advice of the reviewer and removed all claims to an intrinsic mechanism of growth rate in the revised manuscript.

2. We have performed new experiments to fully respond to the suggestion to validate Runx3-regulated genes. Pathway analysis revealed up as well as down-regulated genes in the non-muscle myosin II pathway. These results shows that Rock1 and Rock2 are increased while protein phosphatase 1 regulatory subunit 12C (PP1) is decreased. Rock activity activates and PP1 inhibits NMII. In new functional experiments, we have identified the presence of an ongoing Rock activity which restricts axon growth in early sensory neurons. We furthermore show that this activity is regulated by Runx3 because when we block Rock, the axon growth deficits of Runx3-/- neurons is rescued. This shows that a Runx3 suppressed Rock activity confers some of its effects on increasing the rate of axon growth. This new data has been added to the revised Figure 6.

3. Jessell group showed a deficit in target-innervation in the spinal cord. This deficit could be caused by any of several different mechanisms including for instance changes in cell fate or changes in expression of receptors for guidance molecules. Hence, this study did not directly address the role of Runx3 for axon growth. Consistently, we show in the present study that the deficit of spinal cord innervation in Runx3-/- mice is caused by its requirement for peripheral target innervation that is necessary for ER81 expression, which in turn, determines central termination patterns. We therefore believe that our study provides new knowledge on the function of Runx3 and specifically show that Runx3 levels molecularly define a transcriptional program underlying growth differences between positionally different neurons during development.

The reviewer states that our study could be suited for the EMBO Journal if we add some in vitro data on potential downstream effectors of Runx3. As outlined above, we have identified the Rock-NMII pathway as critical for axial differences in axon growth and that Runx3 activity suppresses this pathway resulting in increased axon growth of brachial DRG neurons. We have also revised the manuscript in accordance with the other concerns of the reviewer also outlined above. We hope that the reviewer finds our revised manuscript suitable for EMBO J.
Reviewer 2

1. The mouse explants culture system we use is an established model system used by many established researchers in the field (see for example Graef et al., Cell, Vol. 113, 657-670, May 30, 2003 and Wickramasinghe et al., Neuron 58, 532-545, May 22, 2008). We nevertheless agree with the reviewer that quantification of axon growth from explants is more difficult that single cells. However, the effect of Runx3 is large and robust (nearly digital) in the explants model (see Figure 5) and several biological replicating experiments conducted at different times has led to similar results, as can be seen by the significance of the experimental data. Furthermore, these in vitro experiments using mouse as model system is confirmed by in vivo experiments (Figure 6). We have also conducted large numbers of experiments using low density dissociated cultures of chick neurons, in accordance with the proposal of the reviewer that corroborates these results (Figure 4 and "Results " section under the title "Axial differences in growth functionally determined by Runx3”).

2. The reviewer asks us to carefully follow up our results showing that DRG explants from E11.5 Runx3-/- mice is similar to thoracic DRG explants. The reviewer asks us to examine if this effect is specific to the brachial neuron by examining if thoracic Runx3-/- neurons also display deficits of axon growth. This would answer if Runx3 enhances growth above a certain basal level and thereby specifically determines the positional differences in axon growth. We have conducted new experiments to address this question. Axon growth was measured from brachial and thoracic neurons from wild-type and Runx3-/- mice. While axon growth of brachial neurons is reduced nearly half to that of wild-type mice, the growth from thoracic levels is unaffected by Runx3-deletion (Figure 5F). Similarly, transfecting HHst25 thoracic neurons with pCARunx1d construct, which impedes Runx activity, does not affect axon growth compared to controls in dissociated cultures. This data is included now in the main manuscript, in "Results " section under the title "Axial differences in axonal growth functionally determined by Runx3”. This shows that Runx3 is critical for encoding axial differences in axon growth and does not affect growth of sensory neurons having short axons and low levels of endogenous Runx3. To maintain a consistent experimental approach in the manuscript, experiments performed in mouse were conducted using the explants culture system. However, the effects are very large, robust and reproducible (see Figure 5F) and are similar to those observed in dissociated cultures using chicken DRG and we hope that altogether these experiments are acceptable to the reviewer.

3. The reviewer points out that we have a glaring omission of citation in the introduction of previous work on the correlation of rate of axon growth with distance to target for those neurons which show an intrinsic difference in axon growth between neurons (Davies AM, Nature 1989, 337:553-555). Although not an adequate excuse but rather an explanation - this reference and its results must have been mistakenly removed during one of the rounds of preparation of the manuscript. We sincerely apologize for this omission and have corrected the introduction to fully include and cite this work. These corrections clarify the sentence “that axon length of pioneering neurons invading peripheral tissue at different axial levels is determined by differences in axon growth rate during development and that such a process is transcriptionally encoded” is based on previous state-of-the-art refers to a prediction in our model system. We have also rephrased the introduction to clarify that we have studied the “molecular mechanism underlying differences in axon growth” in accordance with the reviewer's suggestion. Again, we apologize for these omissions in the first version of the manuscript and hope that our revised manuscript fully has included previous work in this area of research.

Acceptance letter

20 July 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I asked both referees to take a look at the revision and have now received their comments. As you can see below, both referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore very pleased to proceed with the acceptance of the paper for publication here. You will
receive the formal acceptance letter shortly.

Thank you for submitting your interesting study to the EMBO Journal!

Yours sincerely
Editor
The EMBO Journal

Referee #1

The authors have adequately addressed my concerns and have added novel data on a potential mechanism operating downstream of Runx3. I therefore feel that this manuscript is suitable for publication in the EMBO Journal.

Referee #2

I am satisfied that the authors have adequately addressed my concerns.