

LTK and ALK promote neuronal polarity and cortical migration by inhibiting IGF1R activity

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This manuscript describes studies that indicate roles for the ALK and LTK receptors in neuronal polarity, cortical patterning and behavior in mice. I really liked the study and overall think that it deserves publication in a high-ranking journal. It reports important and novel results and benefits from a comprehensive analysis at multiple levels, including cell biological, biochemical and behavior. The points raised below are suggestions for consideration at the discretion of the authors.

1. The term "DKO" appears in the Introduction without explanation. I assume this means double KO mice lacking both receptors from birth. It should be indicated here, just in case.
2. The last paragraph of the Introduction is redundant with the Abstract. This is a stylistic question, which is up to the authors. Nevertheless, as a suggestion, they could take the opportunity here to explain the rationale of the study and why they did what they did.
3. Is "single cell in situ mRNA analysis" standard in situ hybridization or something else? Why is it called "single-cell"? It could be misleading.
4. In Fig. S1B, could the authors please include expression patterns of LTK in adult brain? It'd seem that is the most relevant place to look given the analysis that follows in the paper.
5. I have an issue in general in the first part of the manuscript with regards to the labeling of cortical layers. How were CP, IZ and SVZ/VZ defined? Specific markers should be used to identify their actual boundaries. Guesswork from the DAPI pattern (if that is what was used) is not really appropriate.
6. Comparing Fig. 1 and Fig. S2, there would seem to be little or no additive nor synergistic effects of the double mutation, as the phenotype in the DKO appears to be completely attributable to the Ltk KO. What does this mean? Providing the expression patterns of the two receptors at the ages used here (i.e., P2 and P7) would also be helpful.

7. In Fig. 1F, again, how were the boundaries between the cortical areas (dotted lines) determined? This is particularly important for the mutant sections, as apparent cortical thickness would be easily be affected by the plane of the section. Simply assuming that the CP is of equal thickness than the one in the WT may be incorrect. I feel the authors cannot just place dotted lines in the figure without explaining the criteria that was used to determine their location. Also, there is a significant (many fold) increase in Ctip2 cells in the IZb of the mutant (1F) that it's not explained in the text. The quantification of Ctip2 cells in the CP and IZa of the mutant is missing in the histogram. It should be indicated, even if very low. Again, the key point here is the criteria used for the boundaries between areas. May be what it's marked as IZa in the mutant is still part of the CP, in which case the number of Ctip2 cells would be increased there, not decreased, as claimed in the text.

8. In Fig. S3C-F, the all-critical quantification of Ctip2 cells at P2 seems to be missing in this figure. It would important to provide this in light of the comments above. Again, the same problem with the layer boundaries is clear here. The Ltk KO would have normal levels of Ctip2 cells if the CP thickness were to be larger (due to e.g., the plane of the section not being perfectly perpendicular to the brain surface).

9. In Figure 2A and B, % positive cells is plotted but we are not told what is the reference (100%) level. Was it the total number of cells in the entire cortex (including SVZ and VZ)? That cannot be the case, since CP+IZ in WT alone reaches almost 100%. What is 100% here please? Also, the idea of drawing a little rectangle in the IZ and CP and counting only there is flawed. The values would change drastically depending on where the rectangle is placed. They need to count the whole field of view, as it was done in the previous figures. Finally, again, we are not told how the boundaries of the different cortex areas were established. As explained earlier, distance from the surface (or from the bottom) of the cortex would be greatly affected by the plane of the section. This problem will need a more satisfying solution for the data to be interpreted in the way it has been done.

10. At the end of page 8, it is concluded that Alk/Ltk promote neuronal migration. Is this a cell-autonomous effect? Given the very sparse expression of these receptors (Fig S1), cell-autonomy (which is being implied by the authors) is not at all clear. Is the migration of Alk⁺ cells affected in the Ltk mutant? Vice-versa?

11. In Fig. S4A, as every cell in these panels bears probe signal, it'd be important to present a negative control, perhaps from KO cultures or wild type cells lacking receptor expression in the same field as expressing cells. At a 75%, 1 in 4 cells in any

field should be receptor-negative.

12. Figure S4B is difficult to interpret in the absence of Tau and MAP2 markers, as GFP does not discriminate between axons and dendrites. In general, the authors are recommended to show more than one cell per condition in their figures. Readers need to be convinced that these are robust phenotypes easily observed on many cells in the same field.

13. In Fig. S4C and D, do the KO neurons become bipolar? I don't see examples of multipolar neurons in the images provided.

14. Is there a way to quantify the effects shown in Fig. 3E?

15. The DKO display a dramatically different behavior phenotype compared to single Kos. How can this result be explained given that DKOs are indistinguishable from single KOs in all other parameters studied?

16. At the end of the behavior section, the authors attribute the phenotypes observed to defects in neuronal polarization. Given that polarization was only studied in vitro, it may be a premature to conclude that neurons fail to polarize in vivo in the absence of direct evidence showing this.

17. Regarding P-AKT studies, it would be interesting to assess the effects of the ALK7LTK ligands (e.g., from conditioned medium) on the levels of P-AKT in WT neurons.

18. In the mid part of page 14, the sentence "Treatment of WT cortical neurons with AG1024 at a dose (1 μ M) at which only IGF-1R but not InsR was inhibited restored the single axon phenotype in DKO neurons" is confusing. Treatment performed in WT neurons but assessed in DKO neurons? This must be a typo.

19. For completion, it would be informative to test whether IGF-1 antagonizes the effects of ALK and LTK ligands in axon formation.

20. The quality of the blot provided to illustrate levels of activated Igf-1r in Fig. 7A is clearly suboptimal. It is not apparent from that blot that phosphorylation of Igf1r is increased in the mutant neurons as the band intensities are indistinguishable. Was this performed in cortex extracts or cultured neurons? Is it affected by treatment with ALK/LTK ligands?

21. Given the physical interaction between ALK/LTK and IGF-R1, these receptors are

presumably co-internalized upon ligand treatment, or? Does treatment with IGF1 induces internalization of ALK or LTK?

22. The last paragraph in the Results section may be more appropriate for Discussion to avoid repetition. But it is of course up to the authors to decide on stylistic issues.

23. There is a discussion of possible redundancies between ALK and LTK in the Discussion section which appears to contradict itself. It is first stated (end of p. 18) that the two receptors are not redundant but both required for function. But in p. 19, the significant behavioral phenotypes observed in DKO mice, but not in single KO mice, are attributed to redundancy and compensation between the receptors. This needs some clarification. It's difficult to understand how there can be redundancy for behavior but not for structure or function.

2. Significance:

Significance (Required)

see above

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

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Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Christova et al. analyzed single and double knockout mice for Alk and Ltk to investigate their function in the nervous system and describe defects in cortical development and behavioral deficits. The defects in the formation of cortical layers suggest a delay in radial migration. In culture, 40% of cortical neurons from knockout embryos extend multiple axons. The mechanism responsible for this phenotype is explored in some detail. The authors conclude that Alk and Ltk function non-redundantly to regulate the Igf-1 receptor (Igf-1r). Inactivation of Alk or Ltk increases surface expression and activity of Igf-1r, which induces the formation of multiple axons. The authors propose that Alk and Ltk interact with Igf-1r and promote its endocytosis after activation by their ligand Alkal2, thereby preventing the formation of additional axons. However, the defects in neurogenesis, migration and behavior may have a different cause and should not be attributed only to Igf-1r.

****Major comments****

1) The role of Alk/Ltk in suppressing the formation of multiple axons is demonstrated by culturing neurons from knockout mice, suppression with siRNAs and treatment with inhibitors. These experiments consistently show that about 40% of cultured neurons extend more than one axon when Alk, Ltk or both are inactivated. Single and double knockout mice are largely normal with the exception of a delay in the formation of distinct cortical layers. The phenotypes of the knockout lines indicate a function in cortical development but Alk and Ltk are not "indispensable" as suggested (p. 18). The morphology of cortical neurons was analyzed by Golgi staining. A few potential axons (Fig. 3E) were identified only by an absence of dendritic spines and their aberrant trajectory. These results indicate that there are ectopic extensions in the cortex but do not demonstrate that neurons extend multiple axons also in vivo. It has to be confirmed that these extensions are positive for axon-specific markers and that several axons originate from one soma to demonstrate a multiple axon phenotype in vivo. A quantification of the number of neurons with multiple axons would be required to conclude that this phenotype occurs at a similar frequency in vivo.

2) According to the model presented in Fig. 7, Alkal2 activates Alk and Ltk, which stimulate the endocytosis of Igf-1r and thereby prevents the formation of additional axons. A quantification of Igf-1r surface levels by the biotinylation of surface proteins and Western blot shows an increase in knockout neurons. The authors suggest that Alk/Ltk activation stimulates Igf-1r endocytosis but do not demonstrate this directly. An increase in surface expression could also result from a stimulation of exocytosis or recycling.

3) The localization of Alk, Ltk and Alkal2 was determined by in situ hybridization. The signals are weak and it is not clear if they are specific because a negative control is missing. An analysis by immunofluorescence staining would be more informative. Alk appears to be expressed mainly in the ventricular zone (VZ) while Ltk shows a low expression in the SVZ and the cortical plate (CP). This expression pattern is not consistent with a function in regulating axon formation in multipolar neurons, which extend axons in the lower intermediate zone (IZ) (Namba et al., Neuron 2014) and not in the VZ or SVZ (p. 18).

It is also essential to analyze the subcellular localization of Alk and Ltk at least in cultured neurons. Ltk has been reported as an ER-resident protein that regulates the export from the ER (Centonze et al., 2019), which would not be consistent with the model.

4) The results convincingly show that an increased activity of Igf-1r is responsible for the formation of additional axons by cultured knockout neurons. The model in Fig. 7 explains how Alk/Ltk suppress the formation of multiple axons in culture but a key question remains to be addressed: why does Igf-1r remain active in the future axon? Are Alk/Ltk restricted to or selectively activated in dendrites? Which cells produce Alkal2 in neuronal cultures and in vivo? These points can be easily addressed and should be investigated. It is important to determine if Alk and Ltk are absent from the future axon before or after neuronal polarity is established. Why does an increase of Igf-1r surface expression in knockout neurons result in a stimulation of Igf-1r autophosphorylation? Neurons are cultured in a defined medium without Igf-1 and increased surface levels by themselves should not lead to an increased activity. The results presented in this manuscript are consistent with a role of Igf-1r in the formation of multiple axons in the absence of Alk/Ltk. However, inhibition of Igf-1r by various means does not prevent axon formation in controls. Igf-1 has been implicated in axon formation (Sosa et al., 2006) but a knockout of Igf-1r does not result in a loss of axons but a reduction of axon length in cultured neurons (Jin et al., PLoS One 2019). Axon-specific markers are used only for some experiments but not in Figs. 3D, 5B-D and 6 where the neuronal marker Tuj1 does not allow the unambiguous identification of axons. Staining with an axonal marker and a quantification of axon length are required to distinguish between a block in axon

formation and a reduction in axon growth in Figs. 3A, 5 and 6.

5) The analysis with layer specific markers and BrdU labeling reveals defects in the formation of cortical layers that suggest a delay in neuronal migration. The number of Sox2⁺ and Tbr2⁺ cells is lower in knockout neurons indicating a possible reduction in the number of proliferating progenitors and a defect in neurogenesis (Fig. 1). The number of neurons positive for layer-specific markers or BrdU was quantified as the percent of DAPI-positive cells. This does not allow distinguishing between a change in the distribution and a reduction in the number of neurons due to defects in neurogenesis. It would be more informative to quantify the total number Ctip⁺, Satb2⁺ or BrdU⁺ cells in the VZ, SVZ, IZ and CP.

6) The deficits observed in behavioral tests do not correlate with the defects in neuronal development. While the single knockouts show defects in cortical development only the double knockout displays behavioral deficits. The behavioral phenotype could be completely independent of Igf-1r. Alk has been implicated in regulating retrograde transport (Fellows et al., EMBO Rep. 2020) and synaptic scaling (Zhou et al., Cell Rep. 2021). Since there is no clear correlation between structural and behavioral changes these data are not obviously linked to the other results.

****Minor comments****

1) Fig. 3 shows defects in the corpus callosum where axons are restricted to the upper half in the wild type but not the knockout. These results could indicate a guidance defect but do not show a "failure in axon migration through the corpus callosum" (p. 17). It is also not demonstrated "that the aberrant axon tracts may be the result of effects on neuronal morphology" (p. 19). Without additional experiments to trace axonal projections e.g. by DiI labeling it is not possible to determine the actual cause for the observation shown in Fig. 3F.

2) Active kinases from SignalChem are used for the in vitro kinase assays. The increased phosphorylation of Igf-1r could also result from a stimulation of auto-phosphorylation and not a direct phosphorylation by Ltk. Previous results indicate that phosphorylation of Y1250/1251 leads to increased internalization and degradation (Rieger et al., Sci. Signal. 2020), which would be an alternative explanation how Alk/Ltk regulate surface expression. Antibodies that are specific for Igf-1r phosphorylation at Y1135/1136 or Y1250/1251 could address this possibility (Rieger et al., Sci. Signal. 2020).

3) The specificity of the siRNAs has to be verified in neurons by rescue experiments

and the suppression of the targeted proteins confirmed by immunofluorescence staining.

4) The position of molecular weight markers is missing for most Western blots.

5) It is not indicated which conditions show a significant difference in Fig. 6.

6) Why does the Western blot in Fig. 7A show a double band with the anti-phospho-Igf-1r antibody in the knockout? Which of the bands was used for the quantification?

7) Details of the plasmids used and information (catalog number) for recombinant GST-Ltk and His-Igf-1r should be included in Materials and Methods.

2. Significance:

Significance (Required)

The receptor tyrosine kinase Alk has been studied mainly for its involvement in several types of cancer but the physiological functions of Alk and its close relative Ltk remain poorly understood. The regulation of Igf-1r is an interesting and important result to understand the physiological function of Alk and Ltk. However, several points have to be addressed before the manuscript would be suitable for publication.

3. How much time do you estimate the authors will need to complete the suggested revisions:

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Response to Reviewers

Reviewer #1:

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We thank the reviewer for the positive and enthusiastic comments on our study and especially for noting that it is appropriate for publication in a high-ranking journal. We greatly appreciate the valuable suggestions, the majority of which we have incorporated into the revised manuscript.

1. The term "DKO" appears in the Introduction without explanation. I assume this means double KO mice lacking both receptors from birth. It should be indicated here, just in case.

We have added text at the first appearance of DKO (ie results section) to indicate that this refers to double knockout mice that lack both Ltk and Alk from birth.

2. The last paragraph of the Introduction is redundant with the Abstract. This is a stylistic question, which is up to the authors. Nevertheless, as a suggestion, they could take the opportunity here to explain the rationale of the study and why they did what they did.

We have made some modifications to provide an indication of the rationale for the studies.

3. Is "single cell in situ mRNA analysis" standard in situ hybridization or something else? Why is it called "single-cell"? It could be misleading.

This was a typographical error and has been corrected to single molecule in situ.

4. In Fig. S1B, could the authors please include expression patterns of LTK in adult brain? It'd seem that is the most relevant place to look given the analysis that follows in the paper.

We have replaced the previous panels with new plots (now Fig. S1G) showing the relative expression of Ltk, Alk and their ligand, Alkal2 in embryos (E15.5), newborn (P0) and post-natal Day 2 (P2) and Day 7 (P7) and in adults both in the cortex and whole brain. The results confirm that Alk and Ltk are both expressed in the cortex and brain but in varying patterns with Alk expression decreasing with age and Ltk increasing, particularly in the cortex. In contrast, Alkal2 expression is relatively constant throughout.

Related comments #5, #7, #8 and #9.

5. I have an issue in general in the first part of the manuscript with regards to the labeling of cortical layers. How were CP, IZ and SVZ/VZ defined? Specific markers should be used to identify

their actual boundaries. Guesswork from the DAPI pattern (if that is what was used) is not really appropriate.

7. In Fig. 1F, again, how were the boundaries between the cortical areas (dotted lines) determined? This is particularly important for the mutant sections....

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9. In Figure 2A and B, % positive cells is plotted but we are not told what is the reference (100%) level. ... Also, the idea of drawing a little rectangle in the IZ and CP and counting only there is flawed. ...Finally, again, we are not told how the boundaries of the different cortex areas were established. ...

Response to related comments #5, #7, #8 and #9.

As exemplified in the related comments above, the reviewer indicated that they “*have an issue in general in the first part of the manuscript with regards to the labeling of cortical layers.*”

We thank the reviewer for this insightful comment. Development of the mouse cortex follows a stereotypical pattern, thus we used a combination of DAPI (ie nuclear density is characteristic of some layers), and layer specific markers (Satb2, Ctip2, Pax6, Sox2, Tbr2) to label the cortical layers. While this is generally acceptable for wild type mice, we agree with the reviewer’s comment that this may not be appropriate in mutant mice. Accordingly, we have now taken a more unbiased approach and repeated all of the quantitation after creating equally sized bins that span the entire cortical length and have plotted the quantitation by bin location. The general location of layers in WT mice has been marked on the images for reference. Our conclusions that there are defects in early patterning that are resolving by ~P7 is unchanged.

With this re-quantitation, some of the previous reviewer comments within #5, 7, 8 and 9 no longer apply (ie a missing plot, box placement being subjective, etc) and so have not been responded to. With regards to the question of what is the reference (ie 100%) for the plots showing the y-axis as % positive; this was determined based on the total number of DAPI+ cells counted in each region. This information has been added to the legends and methods along with details of the new quantitation method.

6. Comparing Fig. 1 and Fig. S2, there would seem to be little or no additive nor synergistic effects of the double mutation, as the phenotype in the DKO appears to be completely attributable to the Ltk KO. What does this mean? Providing the expression patterns of the two receptors at the ages used here (i.e., P2 and P7) would also be helpful.

The relative contribution of Alk or Ltk in comparison to the DKO, varies as a function of age (E15.5, P2, P7) that generally correlates with their level of expression, as per the Reviewer’s suggestion. For example, at E15.5, a reduction in the number of Sox2+ or Tbr2+ cells is

observed for either Alk or Ltk knockouts alone, with a more prominent reduction in the case of Alk alone, and with the DKO showing the greatest reduction. In contrast, when examining Ctip2 levels at P2, the loss of Ltk alone yields a stronger effect. In agreement with these observations, analysis of mRNA expression levels show that Alk levels are highest in the embryonic cortex and brain and steadily decline until adulthood, while Ltk expression increases with maximal levels occurring post-natally. As indicated for our reply to comment #4, we have now added plots showing the relative level of expression of Alk and Ltk at various ages from embryos to adults (Fig S1G).

10. At the end of page 8, it is concluded that Alk/Ltk promote neuronal migration. Is this a cell-autonomous effect? Given the very sparse expression of these receptors (Fig S1), cell-autonomy (which is being implied by the authors) is not at all clear. Is the migration of Alk+ cells affected in the Ltk mutant? Vice-versa?

In our analysis of mRNA expression using RNAscope we originally included a widefield image that depicts the entire cortex where it is difficult to see expression at the cell level. We now also provide a magnified image of the E15.5 SVZ/VZ that shows that most cells do express the receptors (Fig. S1B). Thus, the results are consistent with the idea that the defect in migration is a cell autonomous effect.

11. In Fig. S4A, as every cell in these panels bears probe signal, it'd be important to present a negative control, perhaps from KO cultures or wild type cells lacking receptor expression in the same field as expressing cells. At a 75%, 1 in 4 cells in any field should be receptor-negative.

As requested, we now provide images with a wider field of view that includes negative cells.

12. Figure S4B is difficult to interpret in the absence of Tau and MAP2 markers, as GFP does not discriminate between axons and dendrites.

In the original submission we quantitated Tau-1 and MAP2 co-stained neurons in many experiments to demonstrate that Ltk/Alk act on axons, but in some cases, we used Tuj1 to more easily visualize and quantitate neurites. Nevertheless, as requested by the reviewers, in the revised manuscript we have repeated and replaced most of the results with Tuj1 or phalloidin staining with experiments using Tau-1 and MAP2 antibodies, including Fig. 5B-D and Fig. 6A-D and G as well as for Fig. S4B. The new data is consistent with our results using Tuj1 staining and further support our conclusions that Ltk/Alk act via Igf1-r to regulate neuronal polarity.

In general, the authors are recommended to show more than one cell per condition in their figures. Readers need to be convinced that these are robust phenotypes easily observed on many cells in the same field.

Due to space constraints, we included only a single representative image for each condition and then provided quantitation to support our conclusions. We have numerous images for all of the presented data and could provide a collage for all panels if considered appropriate. In the

meantime, we have added additional images for several experiments in the Main Figures (Fig. 5A-D, Fig. 6A, C) and in Suppl. Figure S4A, B, C where sufficient space was readily available.

13. In Fig. S4C and D, do the KO neurons become bipolar? I don't see examples of multipolar neurons in the images provided.

Upon siRNA mediated knockdown of Ltk and/or Alk, we observe about 50% of the neurons are bipolar (ie display the typical wild type single axon phenotype) while roughly 40% display the multiple axon phenotype. With the exception of the control (siCTL), the images provided were selected to show neurons with multiple axons. However, in some of the images, the arrowheads pointing to the axons were inadvertently omitted. These have now been added.

14. Is there a way to quantify the effects shown in Fig. 3E?

We attempted to quantitate the number and direction of neurites in the brain sections but because this is a dense tissue, even with Golgi staining, we found it impossible to trace individual neurites back to the cell body and thus were unable to quantitate the effects. As an alternative, we have provided additional images (Fig. S3B) from distinct mice to support our observations of aberrant horizontal neurites in the adult cortex.

15. The DKO display a dramatically different behavior phenotype compared to single Kos. How can this result be explained given that DKOs are indistinguishable from single KOs in all other parameters studied?

The reviewer is correct, that the single KO mice do not manifest noticeable behavioural defects except when older and challenged with the most demanding task, the Puzzle box, which measures complex executive functions. We speculate that alternative cortical re-wiring in the single knockouts is sufficient to maintain normal circuitry that cannot be compensated when both Ltk and Alk receptors are deleted. It is also possible that Ltk/Alk regulated signalling events, besides Igf-1r/PI3K could contribute to the behavioural defects observed in the DKO mice, such as the ALK-LIMK-cofilin pathway which regulates synaptic scaling mentioned by the reviewer (Zhou et al., Cell Rep. 2021). Nevertheless, the strong phenotype of the DKOs confirms that Ltk/Alk are important for proper brain function, thus our preference is to retain the behavioural data in the manuscript but to discuss that alternative Ltk/Alk pathways could contribute to the phenotype (which we have now incorporated into the text).

16. At the end of the behavior section, the authors attribute the phenotypes observed to defects in neuronal polarization. Given that polarization was only studied in vitro, it may be a premature to conclude that neurons fail to polarize in vivo in the absence of direct evidence showing this.

We agree and have modified the text to remove this inaccurate assertion.

17. Regarding P-AKT studies, it would be interesting to assess the effects of the ALK7LTK ligands (e.g., from conditioned medium) on the levels of P-AKT in WT neurons.

We agree that this would be interesting and we had attempted this experiment, but found that treatment of WT cortical neurons with medium conditioned with the ALKAL2 ligand did not change the levels of pAKT under our experimental conditions (namely 20-30 min treatment with ACM). Because the data is negative, it makes it difficult to make a firm conclusion, but if true, it is possible that other pathways might be involved when WT cortical neurons are stimulated with ligand.

18. In the mid part of page 14, the sentence "Treatment of WT cortical neurons with AG1024 at a dose (1 μ M) at which only IGF-1R but not InsR was inhibited restored the single axon phenotype in DKO neurons" is confusing. Treatment performed in WT neurons but assessed in DKO neurons? This must be a typo.

Thank you for pointing out this typo. It has been corrected.

19. For completion, it would be informative to test whether IGF-1 antagonizes the effects of ALK and LTK ligands in axon formation.

As suggested, we performed the requested experiment (with 3 independent repeats). In brief, four hours post-plating neurons were treated with control or ALKAL2-conditioned media and Igf-1 was added after 1 hour. Neurons were fixed at 36 hours, stained for MAP2 and Tau-1 and axons (Tau-1+) quantitated. Consistent with our previous findings, Igf-1 promotes the formation of multiple axons while ligand inhibits axon formation. In the ligand-treated neurons, addition of Igf-1 did not result in a statistically-significant change in the number of axons. These findings are consistent with our model that activation of Ltk/Alk promotes a decrease in cell-surface Igf1-r. This data has been added to the manuscript (Fig. 7J).

20. The quality of the blot provided to illustrate levels of activated Igf-1r in Fig. 7A is clearly suboptimal. It is not apparent from that blot that phosphorylation of Igf1r is increased in the mutant neurons as the band intensities are indistinguishable. Was this performed in cortex extracts or cultured neurons? Is it affected by treatment with ALK/LTK ligands?

We apologize for a labelling error that has caused confusion for both reviewers. We have replaced the blots and corrected the labels. We have noted in the legend that the experiments were performed using cultured cortical neurons.

21. Given the physical interaction between ALK/LTK and IGF-R1, these receptors are presumably co-internalized upon ligand treatment, or? Does treatment with IGF1 induces internalization of ALK or LTK?

This is a very interesting question. Unfortunately, due to the lack of suitable antibodies for the mouse versions of Alk or Ltk, we are not able to perform these experiments in cortical neurons with endogenous receptor expression. However, our co-immunoprecipitation experiments and in vitro kinase assays, indicate that only versions of LTK and/or ALK with active kinase domains

can interact with IGF-1R and that the activated LTK/ALK receptors then phosphorylate IGF-1R and trigger IGF-1R internalization (Fig. 7 and Fig. 8 model). Thus, we would expect that treatment with IGF-1 in the absence of LTK/ALK activation will not affect LTK/ALK internalization but will trigger IGF-1R endocytosis.

22. The last paragraph in the Results section may be more appropriate for Discussion to avoid repetition. But it is of course up to the authors to decide on stylistic issues.

We prefer to include a summary of the experimental findings and the model figure at the end of the results.

23. There is a discussion of possible redundancies between ALK and LTK in the Discussion section which appears to contradict itself. It is first stated (end of p. 18) that the two receptors are not redundant but both required for function. But in p. 19, the significant behavioral phenotypes observed in DKO mice, but not in single KO mice, are attributed to redundancy and compensation between the receptors. This needs some clarification. It's difficult to understand how there can be redundancy for behavior but not for structure or function.

We have clarified in the discussion, that both receptors are required in the context of neuronal polarity and migration whereas in the case of behaviour, compensatory mechanisms in neural circuitry or perhaps non-redundant Igf-1r independent pathways result in a strong phenotype only in DKO and can compensate for single but not double knockouts.

Reviewer #1 (Significance (Required)): see above

Reviewer #2:

Christova et al. analyzed single and double knockout mice for Alk and Ltk to investigate their function in the nervous system and describe defects in cortical development and behavioral deficits. The defects in the formation of cortical layers suggest a delay in radial migration. In culture, 40% of cortical neurons from knockout embryos extend multiple axons. The mechanism responsible for this phenotype is explored in some detail. The authors conclude that Alk and Ltk function non-redundantly to regulate the Igf-1 receptor (Igf-1r). Inactivation of Alk or Ltk increases surface expression and activity of Igf-1r, which induces the formation of multiple axons. The authors propose that Alk and Ltk interact with Igf-1r and promote its endocytosis after activation by their ligand Alkal2, thereby preventing the formation of additional axons. However, the defects in neurogenesis, migration and behavior may have a different cause and should not be attributed only to Igf-1r.

We would like to thank the reviewer for all the insightful comments and suggestions which we feel have strengthened our study.

We appreciate the reviewer's acknowledgement that we have shown that Igf-1r is involved in Alk/Ltk-mediated regulation of axon outgrowth. To provide evidence that Igf-1r is also important for Ltk/Alk regulated migration *in vivo*, we explored the effect of the Igf-1r inhibitor, PPP on the migration of neurons in WT and DKO mice by BrdU labelling. Excitingly, this analysis revealed that PPP administration resulted in a partial rescue of the migration defect in Ltk/Alk DKO mice, with BrdU+ neurons being localized to the most superficial layers in P2 mice (Fig. 6F). Thus, these data are consistent with our model that loss of Ltk/Alk can disrupt both neuronal polarity and migration via IGF-1r. We do agree with the reviewer that we have not directly shown that the behavioural defects can be attributed to Igf-1r and it is certainly possible that other pathways or mechanisms may be involved in the complex phenotype. We have updated the manuscript and discuss the potential involvement of other pathways in the discussion.

Major comments

1) The role of Alk/Ltk in suppressing the formation of multiple axons is demonstrated by culturing neurons from knockout mice, suppression with siRNAs and treatment with inhibitors. These experiments consistently show that about 40% of cultured neurons extend more than one axon when Alk, Ltk or both are inactivated. Single and double knockout mice are largely normal with the exception of a delay in the formation of distinct cortical layers. The phenotypes of the knockout lines indicate a function in cortical development but Alk and Ltk are not "indispensable" as suggested (p. 18).

We will modify the wording to remove the statement that Alk and Ltk are "indispensable" for cortical patterning and rather will indicate that the receptors 'contribute' to the timing of cortical patterning.

The morphology of cortical neurons was analyzed by Golgi staining. A few potential axons (Fig. 3E) were identified only by an absence of dendritic spines and their aberrant trajectory. These results indicate that there are ectopic extensions in the cortex but do not demonstrate that neurons extend multiple axons also in vivo. It has to be confirmed that these extensions are positive for axon-specific markers and that several axons originate from one soma to demonstrate a multiple axon phenotype in vivo. A quantification of the number of neurons with multiple axons would be required to conclude that this phenotype occurs at a similar frequency in vivo.

As indicated in response to reviewer #1, we attempted to quantitate the Golgi stained images but found it impossible to trace individual neurites to the cell body and thus could not unambiguously identify and quantitate axons. Accordingly, and as suggested by the reviewer, we have modified our conclusion to simply state there are aberrant extensions in the cortex in vivo. Although we were unable to do quantitation, to further support our conclusions, we have provided additional Golgi stained images of WT and DKO mice from an independent experiment (Fig. S3B).

2) According to the model presented in Fig. 7, Alkal2 activates Alk and Ltk, which stimulate the endocytosis of Igf-1r and thereby prevents the formation of additional axons. A quantification of

Igf-1r surface levels by the biotinylation of surface proteins and Western blot shows an increase in knockout neurons. The authors suggest that Alk/Ltk activation stimulates Igf-1r endocytosis but do not demonstrate this directly. An increase in surface expression could also result from a stimulation of exocytosis or recycling.

We showed that ligand-induced activation of Ltk/Alk in WT neurons resulted in a loss of biotin-labelled cell-surface Igf-1r, which is strongly indicative of increased internalization and cannot be explained by exocytosis. However, the reviewer is correct, that we cannot exclude the possibility that changes in exocytosis or recycling might also occur and that in the unstimulated DKO neurons, the increase in surface expression of Igf-1r could also result from a stimulation of exocytosis or recycling. Indeed, several papers (Laurino et al, 2005, PMID: 16046480; Oksdath et al, 2017, PMID: 27699600; Quiroga et al, 2018, PMID: 29090510) have reported that exocytosis mediated transport of IGF-1R and activation of IGF-1R/PI3K pathway is essential for the regulation of membrane expansion during axon formation. Accordingly, we have modified the discussion text to incorporate this possibility.

3) The localization of Alk, Ltk and Alkal2 was determined by in situ hybridization. The signals are weak and it is not clear if they are specific because a negative control is missing. An analysis by immunofluorescence staining would be more informative.

RNAscope is designed so that a single molecule of RNA is visualized as a punctuate signal dot with high specificity. In lower magnification images, such as those we showed to provide an overall view of expression in the cortex, it is difficult to discern the individual 'dots', particularly for genes with low expression, giving the impression that the signal is weak. However, at high magnification (63X) the signals are readily visible as seen in a new panel in Fig. S1B). We also neglected to mention that positive probes with all 3 labels (POLR2A: Channel C1, PPIB: Channel C2, UBC:Channel C3) as well as a negative probe (Bacterial dap gene) supplied by the manufacturer were used on our samples to validate specificity. We have corrected the oversight and have now added this information to the methods section.

Regarding immunofluorescence, we have rigorously tested numerous commercially-available antibodies and have undertaken repeated attempts to produce our own antibodies that recognize mouse Ltk or Alk, and are appropriate for immunofluorescence, but have had no success. The high specificity enabled by the RNAscope technology is thus currently the most reliable way we can examine expression, with the added advantage that we can simultaneously assess expression of both receptors and the ligand in an individual cell within a section.

Alk appears to be expressed mainly in the ventricular zone (VZ) while Ltk shows a low expression in the SVZ and the cortical plate (CP). This expression pattern is not consistent with a function in regulating axon formation in multipolar neurons, which extend axons in the lower intermediate zone (IZ) (Namba et al., Neuron 2014) and not in the VZ or SVZ (p. 18).

It is well described that multipolar neurons can be found in the SVZ, while bipolar neurons are preferentially in the IZ. Neurons expressing Ltk, Alk and their ligand, Alkal2 can be found in

both compartments (albeit levels appear higher in the SVZ), thus we feel our results are consistent with a role for the receptors in regulating neuronal polarization.

It is also essential to analyze the subcellular localization of Alk and Ltk at least in cultured neurons. Ltk has been reported as an ER-resident protein that regulates the export from the ER (Centonze et al., 2019), which would not be consistent with the model.

Unfortunately, the lack of antibodies with mouse reactivity prevents us from analyzing the subcellular localization of Alk and Ltk in cultured neurons. As mentioned by the reviewer, LTK has been reported as an ER-resident protein (in cancer cells) and similarly, many other tyrosine kinase receptors including IGF1R, have been reported to be localized to diverse intracellular compartments like Golgi, nucleus or mitochondria (reviewed in Rieger and O'Connor, 2021, Front Endocrinol:PMID: 33584548). However, since extracellular ligands for LTK and ALK are known, we feel it is a reasonable expectation that they will have a role as cell-surface receptors. Understanding the functions of RTK receptors and the interplay between the various compartments would nevertheless be an interesting area for future research.

4) The results convincingly show that an increased activity of Igf-1r is responsible for the formation of additional axons by cultured knockout neurons. The model in Fig. 7 explains how Alk/Ltk suppress the formation of multiple axons in culture but a key question remains to be addressed: why does Igf-1r remain active in the future axon? Are Alk/Ltk restricted to or selectively activated in dendrites? It is important to determine if Alk and Ltk are absent from the future axon before or after neuronal polarity is established.

We thank the reviewer for acknowledging that we have provided convincing data that increased activity of Igf-1r is responsible for the formation of multiple axons. Addressing why Igf-1r remains active in the future axon and if and how Ltk/Alk are selectively activated in dendrites and axons are all excellent questions, which we plan to pursue in future work, particularly when antibodies for Alk and Ltk become available.

Which cells produce Alkal2 in neuronal cultures and in vivo? These points can be easily addressed and should be investigated.

We have confirmed that Alkal2 is expressed in the isolated cortical neurons, consistent with our demonstration that siRNA-mediated abrogation of Alkal2 expression in cultured neurons regulates polarity and that ligand levels do not change in Ltk/Alk double knock out mice (Fig. S1G and S6A). Whether other non-neuronal cell types also express Alkal2 would be an interesting future direction.

Why does an increase of Igf-1r surface expression in knockout neurons result in a stimulation of Igf-1r autophosphorylation? Neurons are cultured in a defined medium without Igf-1 and increased surface levels by themselves should not lead to an increased activity.

We have not mechanistically determined why/how Igf-1r displays enhanced autophosphorylation in DKO neurons. Thus, we can only speculate about possibilities. Perhaps

there are low levels of Igf-1 in the cortical cell extracts, or is produced by the cortical neurons; there may be compensatory mechanisms engaged when Ltk/Alk are lost to ensure neuronal survival, or perhaps the increase in cell-surface Igf-1r promotes ligand-independent activation of receptors in the absence of ligand.

The results presented in this manuscript are consistent with a role of Igf-1r in the formation of multiple axons in the absence of Alk/Ltk. However, inhibition of Igf-1r by various means does not prevent axon formation in controls. Igf-1 has been implicated in axon formation (Sosa et al., 2006) but a knockout of Igf-1r does not result in a loss of axons but a reduction of axon length in cultured neurons (Jin et al., PLoS One 2019).

Axon-specific markers are used only for some experiments but not in Figs. 3D, 5B-D and 6 where the neuronal marker Tuj1 does not allow the unambiguous identification of axons. Staining with an axonal marker and a quantification of axon length are required to distinguish between a block in axon formation and a reduction in axon growth in Figs. 3A, 5 and 6.

In the original submission we quantitated Tau-1 and MAP2 co-stained neurons in many experiments to demonstrate that Ltk/Alk act on axons, but in some cases we used Tuj1 to more easily visualize and quantitate neurites. Nevertheless, as requested by the reviewers, in the revised manuscript we have repeated and replaced most of the results with Tuj1 or phalloidin staining with experiments using Tau-1 and MAP2 antibodies, including Fig. 5B-D and Fig. 6A-D and G, as well as for Fig. S4B requested by reviewer #1). The new data is consistent with our results using Tuj1 staining and further support our conclusions that Ltk/Alk act via Igf1-r to regulate neuronal polarity. With regards to Fig. 3D, we have been experiencing ongoing technical issues in generating human stem cell derived cortical neurons and have been unable to undertake Tau1/MAP2 staining of the human cortical neurons. Given that the point being made is minor, we have removed this panel from the paper.

With regards to the comment on that inhibition of Igf1-r did not prevent basal axon formation: in our prior quantitation of WT neurons in which Igf1-r was inhibited using either silgf1-r or PPP, we noticed a trend towards an increase in the number of neurons with no axons, but this was not statistically significant. Upon the repeat of experiments and re-quantitation with Tau-1/MAP2 co-staining, we do see a statistically-significant increase in the number of WT neurons without axons. This is in agreement with several prior studies (including one cited by the reviewer) indicating Igf1-r is important for neuronal polarity (Sosa, 2006; PMID: 16845384, Neito Guil 2017 PMID: 28794445). The text has been modified accordingly.

5) The analysis with layer specific markers and BrdU labeling reveals defects in the formation of cortical layers that suggest a delay in neuronal migration. The number of Sox2+ and Tbr2+ cells is lower in knockout neurons indicating a possible reduction in the number of proliferating progenitors and a defect in neurogenesis (Fig. 1). The number of neurons positive for layer-specific markers or BrdU was quantified as the percent of DAPI-positive cells. This does not allow distinguishing between a change in the distribution and a reduction in the number of neurons due to defects in neurogenesis. It would be more informative to quantify the total number Ctip+, Satb2+ or BrdU+ cells in the VZ, SVZ, IZ and CP.

In the in vivo BrdU labelling experiment, we did not co-stain sections with DAPI. However, in the immunofluorescence analysis in mice of the same ages, we did determine the total number of cells (ie by DAPI) that is shown in the plots in Fig. 1A and Fig. S2A/B. These results show that there are a similar number of cells in WT and mutant SVZ/VZ, consistent with the notion that there is a change in distribution rather than in reduction in the number of neurons due to defective neurogenesis. We neglected to mention this important point in the results and have now modified the text accordingly.

6) The deficits observed in behavioral tests do not correlate with the defects in neuronal development. While the single knockouts show defects in cortical development only the double knockout displays behavioral deficits. The behavioral phenotype could be completely independent of Igf-1r. Alk has been implicated in regulating retrograde transport (Fellows et al., EMBO Rep. 2020) and synaptic scaling (Zhou et al., Cell Rep. 2021). Since there is no clear correlation between structural and behavioral changes these data are not obviously linked to the other results.

The reviewer is correct, that the single KO mice do not manifest noticeable behavioural defects except when older and challenged with the most demanding task, the Puzzle box, which measures complex executive functions. We speculate that alternative cortical re-wiring in the single knockouts is sufficient to maintain normal circuitry that cannot be compensated when both Ltk and Alk receptors are deleted. However, we do agree that Ltk/Alk regulated signalling events, besides Igf-1r/PI3K could contribute to the behavioural defects observed in the DKO mice, such as the ALK-LIMK-cofilin pathway which regulates synaptic scaling as cited by the reviewer (Zhou et al., Cell Rep. 2021). Nevertheless, the strong phenotype of the DKOs confirms that Ltk/Alk are important for proper brain function, thus our preference is to retain the behavioural data in the manuscript but to discuss that alternative Ltk/Alk pathways could contribute to the phenotype (which we have now incorporated into the text). It should be noted that the study by Fellows et al in EMBO Rep 2020 shows Igf1-r, not ALK regulates retrograde transport so we have not included this study in the updated text.

Minor comments

1) Fig. 3 shows defects in the corpus callosum where axons are restricted to the upper half in the wild type but not the knockout. These results could indicate a guidance defect but do not show a "failure in axon migration through the corpus callosum" (p. 17). It is also not demonstrated "that the aberrant axon tracts may be the result of effects on neuronal morphology" (p. 19). Without additional experiments to trace axonal projections e.g. by Dil labeling it is not possible to determine the actual cause for the observation shown in Fig. 3F.

We agree with the reviewer and have modified the concluding sentence so that the defects are described without attributing the cause to the defects on neuronal morphology.

2) Active kinases from SignalChem are used for the in vitro kinase assays. The increased phosphorylation of Igf-1r could also result from a stimulation of auto-phosphorylation and not a direct phosphorylation by Ltk. Previous results indicate that phosphorylation of Y1250/1251 leads

to increased internalization and degradation (Rieger et al., Sci. Signal. 2020), which would be an alternative explanation how Alk/Ltk regulate surface expression. Antibodies that are specific for Igf-1r phosphorylation at Y1135/1136 or Y1250/1251 could address this possibility (Rieger et al., Sci. Signal. 2020).

It is rather surprising that for the Igf-1r, which is such a well-studied receptor, the mechanisms that regulate trafficking, exocytosis recycling, etc are so poorly understood and that this topic is currently an active area of investigation. The focus of our study was on understanding the role of Ltk/Alk in the brain and as part of this effort we demonstrated that Ltk/Alk can control neuronal polarity through Igf-1r phosphorylation. We believe that shedding light on the detailed mechanism of how enhanced Igf-1r phosphorylation induced by Ltk/Alk activation regulates Igf-1r trafficking is an exciting project for future work, but we feel that to thoroughly investigate this question is beyond the scope of the current study. We have, nevertheless, highlighted these points with additional references in the discussion.

3) The specificity of the siRNAs has to be verified in neurons by rescue experiments and the suppression of the targeted proteins confirmed by immunofluorescence staining.

We agree that rescue experiments are the gold standard, and we attempted to do this. However, we found that nucleofection of both siRNAs and cDNAs encoding either EGFP alone or Ltk/Alk was highly toxic to neurons with few surviving the treatment. As an alternative we used a pool of siRNAs, to minimize off-target effects and used genetic KOs or chemical inhibitors to verify the observations.

4) The position of molecular weight markers is missing for most Western blots.

We added the position of molecular weight markers for all the western blots in the revised manuscript.

5) It is not indicated which conditions show a significant difference in Fig. 6.

We thank the reviewer for pointing this out. We added the significant differences to all figures, including Fig. 6.

6) Why does the Western blot in Fig. 7A show a double band with the anti-phospho-Igf-1r antibody in the knockout? Which of the bands was used for the quantification?

We apologize for a labelling error that has caused confusion for both reviewers. We have replaced the blots and corrected the labels.

7) Details of the plasmids used and information (catalog number) for recombinant GST-Ltk and His-Igf-1r should be included in Materials and Methods.

The additional information and catalog numbers have been added to the Materials and Methods.

Reviewer #2 (Significance (Required)):

The receptor tyrosine kinase Alk has been studied mainly for its involvement in several types of cancer but the physiological functions of Alk and its close relative Ltk remain poorly understood. The regulation of Igf-1r is an interesting and important result to understand the physiological function of Alk and Ltk. However, several points have to be addressed before the manuscript would be suitable for publication.

We thank the reviewer for indicating that this is interesting and important study. We trust that the additional data and clarifications provided, have addressed the reviewers concerns.

Dear Liliana,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees that were asked to assess it. Former referee 1 was unfortunately not available to re-review your study for us, so I contacted a novel referee, now referee 2. Referee 1 below is former referee 2.

As you will see, referee 1 still has several concerns with the revised manuscript. However, upon cross-commenting (see below) referee 2 does not agree with these and we agree that the cross-comments are reasonable. I would therefore like to ask you to revise your manuscript along the lines referee 2 suggests. Please co-submit a point-by-point response to all (main) referee comments to ease the evaluation of your final manuscript version.

A few editorial requests will also need to be addressed before we can proceed with the official acceptance of your manuscript:

1. During a standard image analysis we detected potential aberrations in the figure set, and we would like to clarify these issues. We kindly invite you to check the composition of:

- Figure 2C and Figure 6F
- Figure 3E and Figure S3B
- Figure S3A yourself, and to send us the related source data. If you make changes to the figures, please include a point-by-point describing what you have changed.

Image source data should be provided as one file per figure that contains the original, uncropped and unprocessed scans of all or key gels/microscopy images used in the figure. The file(s) should be labeled with the appropriate figure/panel number, and should display molecular weight markers; further annotation may be useful but is not essential. Source data files will be published online with the article as supplementary "Source Data."

2. Please add up to 5 keywords to the manuscript file.

3. Please add a Data Availability Section (DAS) that lists links to publicly deposited data and accession numbers. If no data were deposited in public databases please mention this fact in the DAS.

4. Please correct the conflict of interest subheading to "Disclosure and Competing Interest Statement"

5. Please enter all author credits in our online manuscript submission system.

6. The REFERENCE FORMAT is not correct. Not more than 10 authors may be listed. Please correct to EMBO reports (Harvard) style.

7. The "DATA NOT SHOWN" statement on page 6 must be deleted, as per journal policy. Please either show the data or re-write.

8. The author checklist is missing. Please send us a completed author checklist, which you can download from our author guidelines. The completed checklist will also be part of our transparent peer-review file.

9. Please upload all main and all EV figures as separate files.

10. The FIGURE CALLOUTS for Appendix Fig S2B, S3A&B are missing. Please add.

11. The ms has 7 supplementary figures and 3 supplementary tables. The nomenclature needs correcting to either Appendix or EV figures and tables. You can find more info in our guide to authors at

12. I attach to this email a related ms file with comments by our data editors. Please address all comments in the final ms file.

I would like to suggest a small change to the title. Do you agree with:

LTK and ALK promote neuronal polarity and cortical migration by modulating IGF1R activity

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the final manuscript.

I am looking forward to receiving the final ms files as soon as possible. Please let me know if you have any questions.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In the revised manuscript, Christova et al. re-investigated the effect of Igf-1R suppression with siRNAs and the inhibitor PPP using axon-specific markers and now observe significant effects. However, several questions remain to be addressed and the analysis has to go further to offer a novel mechanistic insight into neuronal polarization. Are Alk/Ltk involved in initiating neuronal polarization or act later to prevent excessive Igf-1R receptor activity?

Major points:

1) The authors included new results with the Igf-1R inhibitor PPP and report that it inhibits axon formation in culture and neuronal migration in the embryonic cortex. The effects of PPP are more severe than an Igf-1R knockout in the cortex (Jin et al. (2019), doi: 10.1371/journal.pone.0219362). The knockout phenotype indicates that Igf-1R is not required for axon formation and neuronal migration. In culture, PPP is used at concentrations that are far above the IC₅₀ (IC₅₀ = 1 nM in cell-free kinase assay and {less than or equal to} 60 nM for cell lines). Off-target effects are not uncommon for inhibitors, especially at higher concentrations. This also applies to the Alk inhibitors TAE684 (Hellwig et al. (2012); doi: 10.1016/j.chembiol.2012.01.020) and Crizatinib (inhibits also c-Met, TrkA/B/C) but their effect is corroborated by the knockout phenotype, in contrast to PPP. An Igf-1R-independent effect on microtubules has been described for PPP that is a stereoisomer of podophyllotoxin, an inhibitor of microtubule polymerization (Wu et al., 2013; doi: 10.1158/1535-7163.MCT-13-0091). It cannot be excluded that targets other than Igf1-R are responsible for at least some of the PPP effects.

2) The authors could not verify the specificity of the siRNAs in neurons by rescue experiments because "nucleofection of both siRNAs and cDNAs encoding either EGFP alone or Ltk/Alk was highly toxic to neurons". These toxic effects are highly concerning. Rescue experiments are essential controls to confirm the specificity of a knockdown. A pool of siRNAs is not sufficient to exclude off-target effects.

3) The model in Fig. 8 contains several errors and has to be corrected. It should also be described in more detail in the figure legend. Alk/Ltk and Igf-1R are depicted to function in neurons that are apparently localized in the SVZ (the schematic representation of the cortex is not labeled). The orange cells could be migrating bipolar neurons or radial glia. Axon formation and the transition to a bipolar morphology happen in the lower IZ, axons extend tangentially in the IZ and not along migrating bipolar neurons (or radial glia) as depicted.

Wild type neurons are shown to contain phosphorylated Alk/Ltk and inactive Igf-1R while Igf-1 bound, activated Igf-1R is present in knockout neurons. Do the authors suggest that active Alk/Ltk interacts specifically with ligand-free Igf-1R or that it blocks the binding of Igf-1 to its receptor? The latter would be consistent with the increased activation of Igf-1R in the knockout but was not shown experimentally. It remains unclear why Igf-1R autophosphorylation is increased in knockout neurons. The authors speculate that "there are low levels of Igf-1 in the cortical cell extracts, or is produced by the cortical neurons; there may be compensatory mechanisms engaged when Ltk/Alk are lost to ensure neuronal survival, or perhaps the increase in cell-surface Igf-1r promotes ligand-independent activation of receptors in the absence of ligand." These are all not very convincing explanations.

Additional points:

1) The expression pattern of Alk/Ltk is important to understand their role in neuronal development. The authors state "It is well described that multipolar neurons can be found in the SVZ, while bipolar neurons are preferentially in the IZ. Neurons expressing Ltk, Alk and their ligand, Alkal2 can be found in both compartments (albeit levels appear higher in the SVZ), thus we feel our results are consistent with a role for the receptors in regulating neuronal polarization." Alk/Ltk display the lowest (or no) expression in the SVZ/IZ (Fig. S1A), i.e. the region where multipolar neurons transit to the bipolar morphology and form an axon. This expression pattern is more consistent with a function after axon formation to prevent the extension of additional axons and not in initiating axon formation.

2) The Alk/Ltk knockout results in a defect in neurogenesis, visible by a severe reduction in the number of Sox2- and Tbr2-positive progenitors. A reduction in the number of progenitors is expected to decrease the number of neurons in the Alk/Ltk knockout, in addition to the defects in migration. The authors state "there are a similar number of cells in WT and mutant SVZ/VZ, consistent with the notion that there is a change in distribution rather than in reduction in the number of neurons due to defective neurogenesis". A premature onset of neurogenesis can deplete the pool of progenitors without a severe reduction in the number of cells in the SVZ/VZ at this stage. The analysis of this phenotype remains incomplete although most of the data

should already be available. To address this point the total number of Ctip2- or Satb2-positive neurons has to be determined together with the number of deeper layer (e.g. Tbr1-positive) neurons.

3) The authors favor the idea that defects in neuronal polarity are responsible for the behavioral deficits in the double knockout. They attribute the absence of behavioral deficits in the single knockouts to unspecified compensatory mechanisms. The loss of Alk and Ltk results in a delay of neuronal migration but does not abolish it. Neuronal migration appears to catch up at later stages since the distribution of Satb2- and Ctip-2-positive cells does not show a significant difference between wild type and knockout at P7 (Fig. 1,B-E). Do the adult mice still display structural defects when they were evaluated for their performance in behavioral tests? Overall, the results do not support a strong link between the defects observed at early stages of neuronal development and behavioral deficits. A more parsimonious conclusion is that the behavioral deficits are largely independent of the developmental phenotype. Other mechanisms have to be considered since Igf-1 also serves as a trophic factor for cortical neurons (Ueno et al., 2013, doi: 10.1038/nn.3358) and regulates vesicle transport (Fellows et al. (2020)). The authors did not discuss the regulation of transport because the study by "Fellows et al in EMBO Rep 2020 shows Igf1-r, not ALK regulates retrograde transport". The Alk/Ltk knockout affects Igf1-R signaling and, therefore, is expected to also affect transport, which may be relevant especially in the context of the behavioral deficits.

4) The authors mention that they used a negative control but did not include the images for it. Alk and Ltk display the strongest expression in the choroid plexus (Fig. S1E) that is considerably higher than in the cortex. The choroid plexus produces cerebrospinal fluid that regulates progenitor proliferation.

5) The number of embryos analyzed in Fig. 3F and G should be included.

6) The Alkal2 knockdown induces the formation of additional axons. Is the efficiency of the knockdown sufficient to reduce the amount of Alkal2 in the medium or does Alkal2 act in an autocrine manner?

7) The total number of positive cells in the SVZ/VZ is shown in Figure 1 and S2 but the area or size of the bin is not indicated.

Referee #2:

The manuscript by Christova et al. provides novel insight into the functions of Alk and Ltk in cortical patterning, neuronal polarization and migration, and behavior. Their studies using Alk/Ltk double knockout mice in vivo, complemented with morphological and biochemical studies in cultured cortical neurons, provide physiological and functional evidence for these genes/proteins in these processes, as well as potential cellular and molecular mechanisms that might contribute to the phenotypes observed. In addition, the involvement of Alk and Ltk in neurodevelopment and cognition, and the elucidation of a potential ALK/LTK/IgF-1R mechanism, will not only appeal to the neuroscience community, but also to cancer biologists, given the association of ALK and LTK dysregulation with cancers, and clinicians utilizing ALK/LTK small molecular inhibitors as part of their treatment regimen. Overall, the manuscript is improved by the changes made, and it is now suitable for publication. However, there are a few minor points that need to be addressed.

1.) Do Alk/Ltk double knockout mice have defects in the radial glial scaffold that could contribute to the delayed migration phenotype? Defects in the radial glia fibers could result from primary effects in the glia or secondary effects from neurons or other cells types and could impact neuronal migration. The authors should provide an image of the radial glial fibers at appropriate time points, and if they are aberrant, they should comment on this in the discussion.

In general, since these are not conditional KOs/DKO, the authors should discuss the potential impact of other cell types with potential loss of Alk/Ltk on the neuronal phenotypes observed.

2.) Page 5, "ittle" should be "little" in the following: "ALK and LTK have been extensively studied in cancer, but ittle is ..."

3.) In Figure 3E, the outlined ROI in the left DKO panel does not correspond to the Layers IV-V images for the DKO in the center panels.

4.) In the higher magnification images of WT and DKO neurites, right panels for Figure 3E, one arrow points to a dendrite in the WT, and the other arrow points to a potential axon in the DKO image, but the legend says "higher magnification images of WT and DKO neurites". Why point to a dendrite in the WT image, if you are trying to highlight potentially aberrant axons?

From Figure 3E golgi images and others, do you think there is a difference in spines between your WT and DKO mice that could contribute to the behavioral phenotypes observed? While quantitating spine differences is beyond the scope of this manuscript at this point, it could provide a potential explanation for behavioral differences that manifest in adults. Perhaps you can comment on this in the discussion.

5.) Define red arrows versus red arrowheads for Figure 3E in the text and the figure legend. For Figure 3E, you state: "Examples of disrupted directional growth and extension of projections in pyramidal neurons in knockout mice are marked (red arrowheads)." In the text, you state: "In contrast, pyramidal neurons in single and double knockout mice exhibited an abnormal pattern of neurites that included aberrant horizontal projections across the cortical layers (red arrows) that were more prominent in DKO." Why are there arrowheads pointing to aberrant projections in most panels of Figure 3E, but then arrows in the higher magnification panels for WT and DKO, with one pointing to a dendrite with spines in the WT panel?

6.) For Figure 5E and S6G, the pAKT should be normalized to total AKT. Also, for Figure S7A, plgF-1R should be normalized to total IgF-1R.

Cross-comments from referee 2:

I think this reviewer is being unfair and is requesting things that were not asked for in the original review. The reviewer who could not do read the revision was more positive and would, I think agree with me. In my view, they addressed most of the concerns. I agree that some of the issues, like using an inhibitor versus a genetic experiment are slight concerns, but there are off target effects in genetic models as well. The authors use a combination of in vivo, culture, and behavioral assays. The cellular and molecular mechanisms identified in culture may or may not contribute to the in vivo effects, but they report what they see and cleaned up their language to say that other mechanisms could be involved in vivo they are not able to address at this point. I think most studies would have this issue. EMBO's guide for reviewers says that physiological and functional significance is required, but not detailed mechanism. I thought it was a nice contribution and don't agree with the other reviewer.

More detailed comments:

Referee #1

In the revised manuscript, Christova et al. re-investigated the effect of Igf-1R suppression with siRNAs and the inhibitor PPP using axon-specific markers and now observe significant effects. However, several question remain to be addressed and the analysis has to go further to offer a novel mechanistic insight into neuronal polarization. Are Alk/Ltk involved in initiating neuronal polarization or act later to prevent excessive Igf-1R receptor activity?

Major points:

1) The authors included new results with the Igf-1R inhibitor PPP and report that it inhibits axon formation in culture and neuronal migration in the embryonic cortex. The effects of PPP are more severe than an Igf-1R knockout in the cortex (Jin et al. (2019), doi: 10.1371/journal.pone.0219362). The knockout phenotype indicates that Igf-1R is not required for axon formation and neuronal migration. In culture, PPP is used at concentrations that are far above the IC50 (IC50 = 1 nM in cell-free kinase assay and {less than or equal to} 60 nM for cell lines). Off-target effects are not uncommon for inhibitors, especially at higher concentrations. This also applies to the Alk inhibitors TAE684 (Hellwig et al. (2012); doi: 10.1016/j.chembiol.2012.01.020) and Crizatinib (inhibits also c-Met, TrkA/B/C) but their effect is corroborated by the knockout phenotype, in contrast to PPP. An Igf-1R-independent effect on microtubules has been described for PPP that is a stereoisomer of podophyllotoxin, an inhibitor of microtubule polymerization (Wu et al., 2013; doi: 10.1158/1535-7163.MCT-13-0091). It cannot be excluded that targets other than Igf1-R are responsible for at least some of the PPP effects.

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"A cell-permeable cis-cyclolignan compound that acts as a substrate competitive, reversible, potent, and specific inhibitor of IGF-1R both in vitro (IC50 = 1 nM in cell-free kinase assay; {less than or equal to} 60 nM for cell viability and receptor autophosphorylation in melanoma cell lines) and in vivo (complete inhibition of IGF-1R-dependent tumor cell growth at 20 mg/kg/12 hr, i.p., in SCID mice) with minimum toxic effect (LD50 >500 mg/kg). Exhibits little effect towards IR, FGFR, PDGFR and EGFR, and exerts no effects on microtubules and DNA topoisomerase II. Targets the phosphorylation of Y1136 in the activation loop and is the first inhibitor reported to discriminate between IGF-1R and IR."

2) The authors could not verify the specificity of the siRNAs in neurons by rescue experiments because "nucleofection of both siRNAs and cDNAs encoding either EGFP alone or Ltk/Alk was highly toxic to neurons". These toxic effects are highly concerning. Rescue experiments are essential controls to confirm the specificity of a knockdown. A pool of siRNAs is not sufficient to exclude off-target effects.

>> While rescue experiments are the gold standard, most of the siRNA experiments were corroborated by knockout and inhibitor experiments in the manuscript, and or they used "validated" siRNAs. The authors could just add a sentence to explain "validated".

The toxic effects are not highly concerning. The co-electroporations for rescue experiments can be quite tricky to get just the right amount of plasmid to effectively rescue the phenotype and not be toxic. Likely, the authors would need to titrate the plasmid to find just the right amount. This would be time consuming. If the siRNAs were the only method to produce a loss of function, then the rescue experiments would be critical. However, for most of the siRNA experiments, alternative methods corroborate the phenotype(s) observed.

3) The model in Fig. 8 contains several errors and has to be corrected. It should also be described in more detail in the figure legend. Alk/Ltk and Igf-1R are depicted to function in neurons that are apparently localized in the SVZ (the schematic representation of the cortex is not labeled). The orange cells could be migrating bipolar neurons or radial glia. Axon formation and the transition to a bipolar morphology happen in the lower IZ, axons extend tangentially in the IZ and not along migrating bipolar neurons (or radial glia) as depicted.

Wild type neurons are shown to contain phosphorylated Alk/Ltk and inactive Igf-1R while Igf-1 bound, activated Igf-1R is present in knockout neurons. Do the authors suggest that active Alk/Ltk interacts specifically with ligand-free Igf-1R or that it blocks the binding of Igf-1 to its receptor? The latter would be consistent with the increased activation of Igf-1R in the knockout but was not shown experimentally. It remains unclear why Igf-1R autophosphorylation is increased in knockout neurons. The authors speculate that "there are low levels of Igf-1 in the cortical cell extracts, or is produced by the cortical neurons; there may be compensatory mechanisms engaged when Ltk/Alk are lost to ensure neuronal survival, or perhaps the increase in cell-surface Igf-1r promotes ligand-independent activation of receptors in the absence of ligand." These are all not very convincing explanations.

>> Easy enough to fix the figure. The reviewer is still concerned about elucidating mechanisms that are beyond the scope of this paper.

Additional points:

1) The expression pattern of Alk/Ltk is important to understand their role in neuronal development. The authors state "It is well described that multipolar neurons can be found in the SVZ, while bipolar neurons are preferentially in the IZ. Neurons expressing Ltk, Alk and their ligand, Alkal2 can be found in both compartments (albeit levels appear higher in the SVZ), thus we feel our results are consistent with a role for the receptors in regulating neuronal polarization." Alk/Ltk display the lowest (or no) expression in the SVZ/IZ (Fig. S1A), i.e. the region where multipolar neurons transit to the bipolar morphology and form an axon. This expression pattern is more consistent with a function after axon formation to prevent the extension of additional axons and not in initiating axon formation.

>> Looking at the images and the graphs, there is expression of both Alk and Ltk at all time points and in all areas, although to different degrees. Just because cells might have low expression of a protein does not mean that it is not important or functional. Loss of Alk/Ltk also clearly results in the presence of multiple axons in cultures, whether the mechanism by which ALK/LTK prevents the formation of multiple axons during neuronal development involves preventing the extension of additional axons or is involved in the initiation of axon formation is something that can be discussed. Zooms of the cells electroporated with a fluorophore expressing construct in vivo would reveal the morphologies of the cells in the different layers of the cortex to see exactly where the multipolar cells are located and live imaging over time would tell whether they recovered or had excessive processes/axons at later stages of development, but these experiments were not requested during the first round of reviews and are a big undertaking.

2) The Alk/Ltk knockout results in a defect in neurogenesis, visible by a severe reduction in the number of Sox2- and Tbr2-positive progenitors. A reduction in the number of progenitors is expected to decrease the number of neurons in the Alk/Ltk knockout, in addition to the defects in migration. The authors state "there are a similar number of cells in WT and mutant SVZ/VZ, consistent with the notion that there is a change in distribution rather than in reduction in the number of neurons due to defective neurogenesis". A premature onset of neurogenesis can deplete the pool of progenitors without a severe reduction in the number of cells in the SVZ/VZ at this stage. The analysis of this phenotype remains incomplete although most of the data should already be available. To address this point the total number of Ctip2- or Satb2-positive neurons has to be determined together with the number of deeper layer (e.g. Tbr1-positive) neurons.

>> This additional analysis would be informative.

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discuss the regulation of transport because the study by "Fellows et al in EMBO Rep 2020 shows Igf1-r, not ALK regulates retrograde transport". The Alk/Ltk knockout affects Igf1-R signaling and, therefore, is expected to also affect transport, which may be relevant especially in the context of the behavioral deficits.

>> The authors conceded that other mechanisms might be responsible and added that to the discussion. It is beyond the scope of this manuscript to identify the exact mechanisms responsible for the behavior deficits, but the behavior deficits provide further support for the physiological and functional relevance/significance of these genes/proteins to development. The reviewer seems particularly upset that the authors decided not to include the references they suggested.

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>> Figure 3G is has Golgi images from adult cortices, and there is no Fig. 3G.

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>> Easy enough for the authors to indicate.

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Corr_author: Attisano

Title: LTK and ALK regulate neuronal polarity and cortical migration by modulating IGF1R activity

Response to Reviewer Comments (Full Version).

We thank the referees for their helpful comments and especially appreciate referee #2's *cross-comments* and for considering our manuscript a nice contribution.

Referee #1.

Our response to the main outstanding comments from this referee, taking into account cross-comments from referee 2 (in italics) are indicated below.

In the revised manuscript, Christova et al. re-investigated the effect of Igf-1R suppression with siRNAs and the inhibitor PPP using axon-specific markers and now observe significant effects. However, several questions remain to be addressed and the analysis has to go further to offer a novel mechanistic insight into neuronal polarization. Are Alk/Ltk involved in initiating neuronal polarization or act later to prevent excessive Igf-1R receptor activity?

Cross-comments from referee 2:

I think this reviewer is being unfair and is requesting things that were not asked for in the original review. The reviewer who could not do read the revision was more positive and would, I think agree with me. In my view, they addressed most of the concerns. I agree that some of the issues, like using an inhibitor versus a genetic experiment are slight concerns, but there are off target effects in genetic models as well. The authors use a combination of in vivo, culture, and behavioral assays. The cellular and molecular mechanisms identified in culture may or may not contribute to the in vivo effects, but they report what they see and cleaned up their language to say that other mechanisms could be involved in vivo they are not able to address at this point. I think most studies would have this issue. EMBO's guide for reviewers says that physiological and functional significance is required, but not detailed mechanism. I thought it was a nice contribution and don't agree with the other reviewer.

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We concur with the cross-comments from referee #2, so no response is provided.

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As per the cross-comments, we have added text to the Materials and Methods section to indicate that validated siRNA pools are comprised of multiple siRNAs that were first individually confirmed to efficiently knockdown target gene expression in cortical neurons.

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Cross-comment: This additional analysis would be informative

The referee is correct that we already had the required data. We previously plotted the % of Ctip2 and Satb2-positive neurons as a % of the total cells (DAPI+) in Fig. 1B-E and former Fig. S2 C-F, now EV2C-F. We have now added the plot of total number of cells which demonstrates that the overall number of cells (ie DAPI+) at later stages (ie P2 and P7) in the knockouts is similar to that of WT (Appendix Fig. S2).

3) The authors favor the idea that defects in neuronal polarity are responsible for the behavioral deficits in the double knockout. They attribute the absence of behavioral deficits in the single knockouts to unspecified compensatory mechanisms. The loss of Alk and Ltk results in a delay of neuronal migration but does not abolish it. Neuronal migration appears to catch up at later stages since the distribution of Satb2- and Ctip2-positive cells does not show a significant difference between wild type and knockout at P7 (Fig. 1,B-E). Do the adult mice still display structural defects when they were

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5) The number of embryos analyzed in Fig. 3F and G should be included.

Cross-comment: Figure 3G is has Golgi images from adult cortices, and there is no Fig. 3G.

We confirmed that all figure legends include the number of mice/embryos analyzed.

6) The Alkal2 knockdown induces the formation of additional axons. Is the efficiency of the knockdown sufficient to reduce the amount of Alkal2 in the medium or does Alkal2 act in an autocrine manner?

Cross-comment: This should be easy for the authors to address or discuss.

As the ligands for Alk/Ltk have only recently been discovered, many aspects of how these secreted ligands act remain to be determined. As positively charged, unstable proteins, the ligands are likely to act locally and several studies have indeed suggested an autocrine signalling mechanism (PMID: 35608912; PMID: 35412887). Accordingly, we have now added additional text to the discussion section to elaborate on the potential for autocrine signalling.

7) The total number of positive cells in the SVZ/VZ is shown in Figure 1 and S2 but the area or size of the bin is not indicated.

Cross-comment: Easy enough for the authors to indicate.

As requested, we now clarify in the legend that the entire SVZ/VZ image shown, corresponding to a 100 µm x 100 µm region was quantitated. Although not requested, we also added to the legends, the area quantitated for all of the other panels in these figures.

Referee 2:

The manuscript by Christova et al. provides novel insight into the functions of Alk and Ltk in cortical patterning, neuronal polarization and migration, and behavior. Their studies using Alk/Ltk double knockout mice in vivo, complemented with morphological and biochemical studies in cultured cortical neurons, provide physiological and functional evidence for these genes/proteins in these processes, as well as potential cellular and molecular mechanisms that might contribute to the phenotypes observed. In addition, the involvement of Alk and Ltk in neurodevelopment and cognition, and the elucidation of a potential ALK/LTK/IgF-1R mechanism, will not only appeal to the neuroscience community, but also to cancer biologists, given the association of ALK and LTK dysregulation with cancers, and clinicians utilizing ALK/LTK small molecular inhibitors as part of their treatment regimen. Overall, the manuscript is improved by the changes made, and it is now suitable for publication. However, there are a few minor points that need to be addressed.

1.) Do Alk/Ltk double knockout mice have defects in the radial glial scaffold that could contribute to the delayed migration phenotype? Defects in the radial glia fibers could result from primary effects in the glia or secondary effects from neurons or other cells types and could impact neuronal migration. The authors should provide an image of the radial glial fibers at appropriate time points, and if they are aberrant, they should comment on this in the discussion.

We agree with the reviewer, that it is possible that ALK/LTK might also cause an alteration in radial glia fibres. Our data has shown that in isolated primary neurons, ALK/LTK regulate neuronal polarization which is essential for migration so whether these receptors also have a role in other cell types, including radial glia will require additional studies. We feel this is most appropriate for future work but have now included comments on a possible role for ALK/LTK outside of neurons in the discussion.

In general, since these are not conditional KOs/DKO, the authors should discuss the potential impact of other cell types with potential loss of Alk/Ltk on the neuronal phenotypes observed.

Our analysis indicates that loss of ALK/LTK within the isolated neurons results in the observed changes in neuronal morphology. However, we agree with the reviewer that given we did not use conditional KOs, that a role in other cell types may contribute to the observed in vivo phenotypes. This is now mentioned in the discussion.

2.) Page 5, "ittle" should be "little" in the following: "ALK and LTK have been extensively studied in cancer, but ittle is ..."

This typo has been corrected.

3.) In Figure 3E, the outlined ROI in the left DKO panel does not correspond to the Layers IV-V images for the DKO in the center panels.

In Figure 3E, a new widefield Golgi stained image of the DKO has been inserted that shows the layers (Layers IV-V) similar to WT. The corresponding higher magnification image (indicated by red box) was also inserted.

4.) In the higher magnification images of WT and DKO neurites, right panels for Figure 3E, one arrow points to a dendrite in the WT, and the other arrow points to a potential axon in the DKO image, but

the legend says "higher magnification images of WT and DKO neurites". Why point to a dendrite in the WT image, if you are trying to highlight potentially aberrant axons?

We agree with the reviewer that this was confusing and unnecessary so the arrow pointing at the dendrite has been removed.

From Figure 3E golgi images and others, do you think there is a difference in spines between your WT and DKO mice that could contribute to the behavioral phenotypes observed? While quantitating spine differences is beyond the scope of this manuscript at this point, it could provide a potential explanation for behavioral differences that manifest in adults. Perhaps you can comment on this in the discussion.

This is a great observation. We agree and have added text in the discussion speculating that this might contribute to the behavioral defects.

5.) Define red arrows versus red arrowheads for Figure 3E in the text and the figure legend. For Figure 3E, you state: "Examples of disrupted directional growth and extension of projections in pyramidal neurons in knockout mice are marked (red arrowheads)." In the text, you state: "In contrast, pyramidal neurons in single and double knockout mice exhibited an abnormal pattern of neurites that included aberrant horizontal projections across the cortical layers (**red arrows**) that were more prominent in DKOs." Why are there arrowheads pointing to aberrant projections in most panels of Figure 3E, but then arrows in the higher magnification panels for WT and DKO, with one pointing to a dendrite with spines in the WT panel?

We have corrected the text and figure legends to indicate ‘arrowheads’ which correctly match the figure panels. The arrow pointing to the dendrite was removed.

6.) For Figure 5E and S6G, the pAKT should be normalized to total AKT. Also, for Figure S7A, pIgF-1R should be normalized to total IgF-1R.

We agree with this suggestion and now present quantitation in Figure 5E and S6G, in which pAkt levels were normalized to total Akt. For supplementary figure (Fig. S7A), we showed the levels of pIgflr in a large panel of multiple individual mice of all genotypes. Unfortunately, we no longer have lysates to reblot for total IgF-1r. However, we note that the pIgF-1R levels normalized to total IgF-1R, is shown in the main paper, in Fig. 7A for WT versus DKOs.

Response to Reviewer Comments.

We thank the referees for their helpful comments and especially appreciate referee #2's *cross-comments* and for considering our manuscript a nice contribution.

Referee #1.

Our response to the main outstanding comments from Referee #1, that Referee #2 concurred should be addressed are provided below.

Cross-comments from referee 2:

I think this reviewer is being unfair and is requesting things that were not asked for in the original review. The reviewer who could not do read the revision was more positive and would, I think agree with me. In my view, they addressed most of the concerns. I agree that some of the issues, like using an inhibitor versus a genetic experiment are slight concerns, but there are off target effects in genetic models as well. The authors use a combination of in vivo, culture, and behavioral assays. The cellular and molecular mechanisms identified in culture may or may not contribute to the in vivo effects, but they report what they see and cleaned up their language to say that other mechanisms could be involved in vivo they are not able to address at this point. I think most studies would have this issue. EMBO's guide for reviewers says that physiological and functional significance is required, but not detailed mechanism. I thought it was a nice contribution and don't agree with the other reviewer.

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Cross-comment: *Easy enough to fix the figure.*

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2) The Alk/Ltk knockout results in a defect in neurogenesis, visible by a severe reduction in the number of Sox2- and Tbr2-positive progenitors. A reduction in the number of progenitors is expected to decrease the number of neurons in the Alk/Ltk knockout, in addition to the defects in migration. The authors state "there are a similar number of cells in WT and mutant SVZ/VZ, consistent with the notion that there is a change in distribution rather than in reduction in the number of neurons due to defective neurogenesis". A premature onset of neurogenesis can deplete the pool of progenitors without a severe reduction in the number of cells in the SVZ/VZ at this stage. The analysis of this phenotype remains incomplete although most of the data should already be available. To address this point the total number of Ctip2- or Satb2-positive neurons has to be determined together with the number of deeper layer (e.g. Tbr1-positive) neurons.

Cross-comment: This additional analysis would be informative

The referee is correct that we already had the required data. We previously plotted the % of Ctip2 and Satb2-positive neurons as a % of the total cells (DAPI+) in Fig. 1B-E and former Fig. S2 C-F, now EV2C-F. We have now added the plot of total number of cells which demonstrates that the overall number of cells (ie DAPI+) at later stages (ie P2 and P7) in the knockouts is similar to that of WT (Appendix Fig. S2).

5) The number of embryos analyzed in Fig. 3F and G should be included.

Cross-comment: Figure 3G is has Golgi images from adult cortices, and there is no Fig. 3G.

We confirmed that all figure legends include the number of mice/embryos analyzed.

6) The Alkal2 knockdown induces the formation of additional axons. Is the efficiency of the knockdown sufficient to reduce the amount of Alkal2 in the medium or does Alkal2 act in an autocrine manner?

Cross-comment: This should be easy for the authors to address or discuss.

As the ligands for Alk/Ltk have only recently been discovered, many aspects of how these secreted ligands act remain to be determined. As positively charged, unstable proteins, the ligands are likely to act locally and several studies have indeed suggested an autocrine signalling mechanism (PMID: 35608912; PMID: 35412887). Accordingly, we have now added additional text to the discussion section to elaborate on the potential for autocrine signalling.

7) The total number of positive cells in the SVZ/VZ is shown in Figure 1 and S2 but the area or size of the bin is not indicated.

Cross-comment: Easy enough for the authors to indicate.

As requested, we now clarify in the legend that the entire SVZ/VZ image shown, corresponding to a 100 µm x 100 µm region was quantitated. Although not requested, we also added to the legends, the area quantitated for all of the other panels in these figures.

Referee 2:

Our response to all of Referee #2 comments is provided below.

The manuscript by Christova et al. provides novel insight into the functions of Alk and Ltk in cortical patterning, neuronal polarization and migration, and behavior. Their studies using Alk/Ltk double knockout mice in vivo, complemented with morphological and biochemical studies in cultured cortical neurons, provide physiological and functional evidence for these genes/proteins in these processes, as well as potential cellular and molecular mechanisms that might contribute to the phenotypes observed. In addition, the involvement of Alk and Ltk in neurodevelopment and cognition, and the elucidation of a potential ALK/LTK/IgF-1R mechanism, will not only appeal to the neuroscience community, but also to cancer biologists, given the association of ALK and LTK dysregulation with cancers, and clinicians utilizing ALK/LTK small molecular inhibitors as part of their treatment regimen. Overall, the manuscript is improved by the changes made, and it is now suitable for publication. However, there are a few minor points that need to be addressed.

1.) Do Alk/Ltk double knockout mice have defects in the radial glial scaffold that could contribute to the delayed migration phenotype? Defects in the radial glia fibers could result from primary effects in the glia or secondary effects from neurons or other cells types and could impact neuronal migration. The authors should provide an image of the radial glial fibers at appropriate time points, and if they are aberrant, they should comment on this in the discussion.

We agree with the reviewer, that it is possible that ALK/LTK might also cause an alteration in radial glia fibres. Our data has shown that in isolated primary neurons, ALK/LTK regulate neuronal polarization which is essential for migration so whether these receptors also have a role in other cell types, including radial glia will require additional studies. We feel this is most appropriate for future work but have now included comments on a possible role for ALK/LTK outside of neurons in the discussion.

In general, since these are not conditional KOs/DKO, the authors should discuss the potential impact of other cell types with potential loss of Alk/Ltk on the neuronal phenotypes observed.

Our analysis indicates that loss of ALK/LTK within the isolated neurons results in the observed changes in neuronal morphology. However, we agree with the reviewer that given we did not use conditional KOs, that a role in other cell types may contribute to the observed in vivo phenotypes. This is now mentioned in the discussion.

2.) Page 5, "ittle" should be "little" in the following: "ALK and LTK have been extensively studied in cancer, but ittle is ..."

This typo has been corrected.

3.) In Figure 3E, the outlined ROI in the left DKO panel does not correspond to the Layers IV-V images for the DKO in the center panels.

In Figure 3E, a new widefield Golgi stained image of the DKO has been inserted that shows the layers (Layers IV-V) similar to WT. The corresponding higher magnification image (indicated by red box) was also inserted.

4.) In the higher magnification images of WT and DKO neurites, right panels for Figure 3E, one arrow points to a dendrite in the WT, and the other arrow points to a potential axon in the DKO image, but the legend says "higher magnification images of WT and DKO neurites". Why point to a dendrite in the WT image, if you are trying to highlight potentially aberrant axons?

We agree with the reviewer that this was confusing and unnecessary so the arrow pointing at the dendrite has been removed.

From Figure 3E golgi images and others, do you think there is a difference in spines between your WT and DKO mice that could contribute to the behavioral phenotypes observed? While quantitating spine differences is beyond the scope of this manuscript at this point, it could provide a potential explanation for behavioral differences that manifest in adults. Perhaps you can comment on this in the discussion.

This is a great observation. We agree and have added text in the discussion speculating that this might contribute to the behavioral defects.

5.) Define red arrows versus red arrowheads for Figure 3E in the text and the figure legend. For Figure 3E, you state: "Examples of disrupted directional growth and extension of projections in pyramidal neurons in knockout mice are marked (red arrowheads)." In the text, you state: "In contrast, pyramidal neurons in single and double knockout mice exhibited an abnormal pattern of neurites that included aberrant horizontal projections across the cortical layers (**red arrows**) that were more prominent in DKOs." Why are there arrowheads pointing to aberrant projections in most panels of Figure 3E, but then arrows in the higher magnification panels for WT and DKO, with one pointing to a dendrite with spines in the WT panel?

We have corrected the text and figure legends to indicate ‘arrowheads’ which correctly match the figure panels. The arrow pointing to the dendrite was removed.

6.) For Figure 5E and S6G, the pAKT should be normalized to total AKT. Also, for Figure S7A, pIgF-1R should be normalized to total IgF-1R.

We agree with this suggestion and now present quantitation in Figure 5E and S6G, in which pAkt levels were normalized to total Akt. For supplementary figure (Fig. S7A), we showed the levels of pIgflr in a large panel of multiple individual mice of all genotypes. Unfortunately, we no longer have lysates to reblot for total IgF-1r. However, we note that the pIgF-1R levels normalized to total IgF-1R, is shown in the main paper, in Fig. 7A for WT versus DKOs.

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