Distinct temporal expression of the GW182 paralog TNRC6A in neurons regulates dendritic arborization

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AUTHORS: Bharti Nawalpuri and Ravi S Muddashetty
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this paper, the authors identify an interesting temporal specificity of GW182 expression levels in developing neurons. They demonstrate that this is involved in appropriate development of the dendritic arbor, and also they demonstrate expected effects of GW182 expression level on protein translation. Unexpectedly, loss of GW182 function caused a decrease in Limk1 expression, which the authors suggest is the underlying cause for the effect on dendritic morphology. This is important work, since the specific role of RISC proteins in neurons is poorly characterised.

Comments for the author

This is an interesting study, but there are some technical issues as well as conceptual questions that should be addressed before publication.

GW182 mRNA appears unchanged between P7 and 15, suggesting translational control of GW182 protein levels. This should be discussed.

In reference to Figure 2, it is not correct to refer to cell body staining as “cytosolic”. This should be changed.

Picrotoxin causes a decrease in GW182 protein and mRNA, yet normal development reduces only protein levels, indicating a distinct mechanism. This should be discussed.

Why does the DN-GW182 have a different distribution pattern compared to WT-GW182? Presumably, in order that it can function as a dominant negative, it would need to occupy the same location (Ago2-positive sites of translational repression) as the WT protein? Indeed, the authors state that the DN contains the Ago2 binding domain. If the DN has diffuse localization, does it also cause a change in Ago2 localization? This should be tested. Given this uncertainty, the results using this construct can’t be attributed to loss of GW182 function per se, but possibly to some other disruption of RISC function. The discrepancy in FUNCAT results (Fig S6) also suggest that DN-GW182 is having some other effect on translation that may be distinct from GW182 function. Since the authors also use GW182 siRNA for this experiment (which also has shortcomings; see below), I think these DN-GW182 data should be moved to supplementary figures.

The authors use co-transfection of plasmid expressing GFP and non-tagged siRNA and then assume green fluorescent cells will also carry the siRNA. Can the authors provide evidence that all GFP-expressing cells are also transfected with siRNA and vice versa? A better way of doing this would be to express shRNA from a plasmid that also expresses GFP.

A brief explanation of the FUNCAT technique should be included in the text, since not all readers may be familiar with it.

How does FUNCAT signal change through development, comparing stages that correspond to the greatest change in GW182 expression level? This is an essential experiment to support the model. In the data presented here, GW182 loss of function causes an increase in overall translation, which would be expected since miRNA activity would presumably be compromised. The decrease in Limk1 expression therefore seems counter-intuitive. While the authors briefly acknowledge this discrepancy in the discussion, they don’t offer any explanation. Since Limk1 is a well-characterised target of miR-134, and previous work has suggested that an increase in Ago2-GW182 interaction causes miR-134 dependent repression of Limk1 translation (Rajgor et al., EMBO J 2018), some explanation for the unexpected data needs to be given.

Not all the references in the list are in the text. The authors should check this and ensure all relevant papers are appropriately discussed.

Reviewer 2

Advance summary and potential significance to field

In this interesting manuscript, Nawalpuri & Muddashetty show for the first time that the RISC component GW182 positively impacts dendritic arborization in primary neurons in a temporally controlled manner. Further, they propose that this is achieved through GW182 dependent regulation of translation and actin cytoskeleton remodeling. The results on GW182 overexpression and KD are clear and result in a convincing model that nicely integrates with the literature. Overall, the data are well-displayed and the text is easy to follow.

Comments for the author

Before publication, I recommend that the authors reevaluate the data generated by overexpression of the dominant-negative GW182 (DNGW182). In the remainder, there are more detailed Comments for the authors.

Major comments:

1. The authors discuss the differences in between the effects of DNGW182 and siRNA on global translation/actin remodeling. However, there is no discussion on why DNGW182 still phenocopies siGW182 in dendritic arborization. The current deduction that the effect of GW182 on dendritic arborization is mediated via translation/actin cannot be made if translation is oppositely affected by DNGW182.

2. In general, the number of neurons analyzed per experiment is on the lower end throughout the paper. For example, in Fig. 6FG, the displayed neuron seems unhealthy and the analysis has been performed on only two independent experiments. I strongly suggest to include at least a third replicate and add more neurons into your analysis to exclude a possible strong influence of technical variation on your results. This may also help to clarify the influence of DNGW182 (see first comment).

3. Please use the same statistical test, when comparing different conditions from the same experiment type (e.g. Fig. 3D vs. 3H Mann-Whitney vs. unpaired T-test with Welsh correction, same for Fig. 4).

Minor comments:

- For better comparability of your in vivo (Fig.1) and in vitro data (Fig. 2BC), please include a Western Blot and analysis of Ago2 protein signal with maturation of your hippocampal cultures.
- Proper quantification of your siRNA efficiency in hippocampal neurons in the timescale of your experiments is important (Figs. 4 and 6). The images currently displayed in Sup. Fig. 4 are not (yet) convincing and are missing quantification. In the results section, a difference in GW182 intensity in between dendrites and soma upon siRNA transfection is described, however, this is not visible in the current display. Lastly, the Western Blot for GW182 has been performed in N2A cells and not in primary neurons.
- Sup.Fig. 5: can you validate the expression pattern of GFP-GW182 and GFP-DNGW82 in hippocampal neurons? RISC protein expression patterns often differ in between cell lines and neurons at different developmental stages.
- Fig. 2D,E,G: I suggest merging the images into one panel as you are referring to different regions of the same cell. This way also one grey value bar would be sufficient. In addition, the DAPI signal is barely visible.
- Fig. 3C,D: please keep the DIV label consistent with Fig. 3 K,L.
- Fig. 3I: the DN label is missing for GW182
- Fig. 6D/F: Map2 and GFP labels are swopped.
- Page 3: I assume Stufen means Staufen.
- Abstract: Spatio-temporal should be spatio-temporal (also throughout the manuscript)
- Last paragraph of introduction is repetitive, could be sharpened.
- Bartel (2018) reference is included twice

Reviewer 3

Advance summary and potential significance to field

Nawalpuri and Muddashetty studied spatiotemporal regulation of the key miRNA effector protein, GW182 in the rat brain. They observed that in the cerebellum and hippocampus, GW182 shows expression peaks at certain ages while the level of Ago2 keeps increasing during postnatal development. The GW182 expression peaks coincide with the timings of dendritogenesis. Such temporal changes could be recapitulated with cultured hippocampal neurons. An anticorrelation between the neuronal activity and the GW182 level was observed when neuronal activity was altered by toxins. Inhibition, knockdown or overexpression of GW182 altered dendritic arborization of hippocampal neurons, but the effect of GW182 could only be seen when expression was changed in certain time windows. FUNCAT analysis in hippocampal neurons revealed that GW182 levels determine global translation levels in dendrites. Finally, they showed that GW182 knockdown and overexpression caused changes in the F-actin level, and proposed that LIMK1 mediates regulation of F-actin by GW182, by demonstrating that LIMK1 expression could be altered by overexpression or knockdown of GW182.

In general, the experiments were performed carefully and the results are reasonably interpreted. While a previous report already pointed to involvement of GW182 in regulating dendritic growth (DOI 10.1016/j.cell.2012.01.036), this manuscript still adds important information. As regulation of miRNA effectors is an important topic, roles for such mechanisms in important biological processes like dendrite formation would draw attention of broad readership. With some improvement, I think this manuscript is a good candidate for publication in Journal of Cell Science.

Comments for the author

Major points:

1) How many GW182 paralogs are there in the rat genome? Mammals often have three GW182 paralogs with overlapping functions. Do the materials used here (antibodies and siRNAs) recognize all paralogs or a subset of them? Whether the observed phenomena are associated with particular paralogs or reflecting the total amounts of GW182 homologs needs to be clarified.

2) Throughout the manuscript, it was unclear whether the effects of GW182 overexpression or knockdown on dendritic morphology was related to alteration in the miRNA activity. The authors should perform luciferase sensor assays. Using a sensor containing miRNA target sites and a control sensor whose seed target sites are mutated, effects of GW182 KD, DNGW182 and GW182 overexpression on miRNA activity should be tested, ideally in the exact experimental settings where dendritic complexity was quantified. These experiments might clarify which of the observed phenomena (dendrite morphology and global translation activity) are related to miRNA activity.

3) With the results showing that neurons expressing DNGW182 still responded to BDNF (Figure 3I-L), the authors concluded that “GW182 does not modulate BDNF induced dendritic growth in DIV3-7 neurons.”. However, it is difficult to draw a clear conclusion from the present data because the change in the complexity by BDNF seems smaller in the presence of DNGW182 (with the p-values of <0.0001 vs 0.0146 as shown in Figure 3L). I do not think this claim is supported.

First revision

Author response to reviewers' comments

Response to reviewer’s comments
We sincerely thank all the reviewers for providing critical and constructive evaluation of our manuscript. We are encouraged by the overall positive response of the reviewers towards our study. Now we have performed additional experiments and modified the manuscript to address the reviewer's concern. We are hereby providing a detailed point-to-point response to the reviewers’ comments. We believe that the revision has substantially enhanced the quality of our manuscript and hope that the reviewers find the modified manuscript along with the additional data, satisfactory for publication in the journal of cell science.

Reviewer 1 Advance Summary and Potential Significance to Field:
In this paper, the authors identify an interesting temporal specificity of GW182 expression levels in developing neurons. They demonstrate that this is involved in appropriate development of the dendritic arbor, and also they demonstrate expected effects of GW182 expression level on protein translation. Unexpectedly, loss of GW182 function caused a decrease in Limk1 expression, which the authors suggest is the underlying cause for the effect on dendritic morphology. This is important work, since the specific role of RISC proteins in neurons is poorly characterized.

We thank the reviewer for the positive assessment of our manuscript.

Reviewer 1 Comments for the Author:
This is an interesting study, but there are some technical issues as well as conceptual questions that should be addressed before publication.
1) GW182 mRNA appears unchanged between P7 and 15, suggesting translational control of GW182 protein levels. This should be discussed.
We agree with the reviewer's suggestion. We had indicated the regulation of GW182 in our discussion, now we have expanded the same in the revised manuscript **(P16, L446-L450)** and also mentioned it in the related result section **(P6, L150-L151)**.

2) In reference to Figure 2, it is not correct to refer to cell body staining as “cytosolic”. This should be changed.
We apologize for the misunderstanding. In the given analysis, we have only measured the cytosolic levels of GW182 by creating a region of interest with tubulin staining inclusion and exclusion of DAPI stained region. In the revised manuscript, we have included the analysis protocol in the image analysis subsection of the methods section **(P24, L671-L673).**

3) Picrotoxin causes a decrease in GW182 protein and mRNA, yet normal development reduces only protein levels, indicating a distinct mechanism. This should be discussed.
We thank the reviewer for raising this critical point. We have now included the discussion about the same in the discussion section **(P16, L462-L471).**

4) Why does the DN-GW182 have a different distribution pattern compared to WT-GW182? Presumably, in order that it can function as a dominant negative, it would need to occupy the same location (Ago2-positive sites of translational repression) as the WT protein? Indeed, the authors state that the DN contains the Ago2 binding domain. If the DN has diffuse localization, does it also cause a change in Ago2 localization? This should be tested.
The DNGW182 mutant used in this study has been previously shown to have diffuse localization, similar to our observation (Jakymiw et al., 2005). We speculate that the diffused localization of the DN GW182 is primarily due to the absence of the middle poly Q rich domain required for P-body/GW182 body localization, along with the complete absence of the C- terminal silencing domain. The DNGW182 retains AGO2 interacting domains (Eulalio et al, 2009) and with our new data **(Figure S4E, S4F and S4G) (P8-9, L225-L235),** we found that AGO2 localization is not affected in cells where DNGW182 is overexpressed compared to WTGW182 overexpressed cells. We do not observe any changes in the localization or puncta properties of endogenous AGO2 in the presence of the DNGW182 mutant.

5) Given this uncertainty, the results using this construct can’t be attributed to loss of GW182 function per se, but possibly to some other disruption of RISC function. The discrepancy in FUNCAT results (Fig S6) also suggests that DN-GW182 is having some other effect on translation that may be distinct from GW182 function. Since the authors also use GW182 siRNA for this experiment (which also has shortcomings; see below), I think these DN-GW182 data should be moved to supplementary figures.
We agree with the reviewer's concern regarding the function of DNGW182 function as a functional dominant negative of GW182. Hence as per the reviewer's suggestion, we have now moved all the data with the dominant-negative mutant from the main figure to the supplementary figures. **Figure 3** from the old manuscript is now part of supplementary **Figure S3** in the revised version. Further, the FUNCAT data from DIV5 dominant-negative mutant present as **Figure 6F** and **6G** is now a part of supplementary **Figure S5D** in the revised manuscript. Similarly, the phalloidin and LIMK1 data (DNGW182) from **Figure 7** of the older version of the manuscript have been moved to

supplementary **Figure S6** in the revised manuscript.

6) The authors use co-transfection of plasmid expressing GFP and non-tagged siRNA, and then assume green fluorescent cells will also carry the siRNA. Can the authors provide evidence that all GFP-expressing cells are also transfected with siRNA and vice versa? A better way of doing this would be to express shRNA from a plasmid that also expresses GFP.
As per the reviewer's suggestion, we have now quantified (**Figure S3R)** the levels of GW182 in GFP transfected cells in scrambled versus GW182 siRNA groups **(P10, L277-282).** We found that GFP and GW182 siRNA transfected neurons showed an average of 40 percent reduction in GW182 levels as compared to GFP and scrambled siRNA transfected neurons. We would further like to emphasize that due to the small size, siRNA transfection efficiency in cultured neurons is greater as compared to the transfection efficiency of an overexpression construct such as GFP (Karra and Dahm, 2010). Hence, we do expect most of the GFP transfected neurons to be co- transfected with siRNA as well. We agree with the reviewer's comment that using shRNA plasmid with GFP overexpression is a better approach for such an experiment. Unfortunately, due to the difficulty in acquiring reagent in the current COVID -19 situation in India with multiple lockdowns, we were unable to perform the suggested experiment with shRNA.

7) A brief explanation of the FUNCAT technique should be included in the text, since not all readers may be familiar with it.
We thank the reviewer for this suggestion. We have now included a brief introduction of the FUNCAT technique in the corresponding result section **(P12, L327-L333).**

8) How does FUNCAT signal change through development, comparing stages that correspond to the greatest change in GW182 expression level? This is an essential experiment to support the model.
As per the reviewer's suggestion, we have now examined the FUNCAT signal at different stages of neuronal development in cultured neurons, starting from DIV3 till DIV12 **(P12, L336-L343)** (**Figure S5B**, new). We observed a significant reduction in FUNCAT signal from DIV6 to DIV9, which remained low till DIV12. This is in accordance with a recent study where the authors report a reduction in translation from DIV7 to DIV14 as measured by tRNA FRET (Koltun et al., 2020). To compare it with the GW182 function, we observed a reduction in GW182 levels as well from DIV6 to DIV9. However, this is not surprising, as we did not expect the FUNCAT signal to be anti-correlated with GW182 expression. It is important to emphasize here that global neuronal translation at any given developmental stage is determined by a multitude of factors such as expression and availability of translation factors, ribosomes, different RNA binding proteins, and an array of cellular signaling pathways. Hence GW182 is likely to regulate only a part of the total translation response in neurons.

9) In the data presented here, GW182 loss of function causes an increase in overall translation, which would be expected since miRNA activity would presumably be compromised. The decrease in Limk1 expression therefore seems counter-intuitive. While the authors briefly acknowledge this discrepancy in the discussion, they don't offer any explanation. Since Limk1 is a well-characterised target of miR-134, and previous work has suggested that an increase in Ago2-GW182 interaction causes miR-134 dependent repression of Limk1 translation (Rajgor et al., EMBO J 2018), some explanation for the unexpected data needs to be given.
We thank the reviewer for highlighting this point. As the reviewer correctly pointed out, it has been shown that the increase in AGO2-GW182 interaction causes miR-134 mediated LIMK1 repression (Rajgor et al., 2018). However, it is important to highlight that the levels of miR-134 miRNA are reported to be low during the examined developmental stages in our experiment (DIV3-7) (Schratt et al., 2006). Due to such low expression of miR134, we hypothesize that the effect of GW182 on LIMK1 is not directly via miR134, but indirectly through other regulators of LIMK1 expression. For example, ubiquitin ligase RNF6 is shown to be involved in the degradation of LIMK1 in neurons (Tursun et al., 2005). The level of RNF6 is regulated by miR- 26a-5P (Huang et al., 2019) which is further shown to be predominantly expressed during early neuronal development (Lucci et al., 2020). SiRNA mediated knockdown of GW182 can potentially lead to the de-repression of RNF6 from miR-26a-5P. The increased RNF6 might target LIMK1 for degradation, explaining the reduced LIMK1 levels observed upon GW182 knockdown. We have now described these possibilities in the discussion section of the revised manuscript **(P18, L508-L520).**

10) Not all the references in the list are in the text. The authors should check this and ensure all relevant papers are appropriately discussed.
We appreciate the reviewer for pointing this issue. We have now ensured that all the references in the list are included in the text and vice-versa.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this interesting manuscript, Nawalpuri & Muddashetty show for the first time that the RISC component GW182 positively impacts dendritic arborization in primary neurons in a temporally controlled manner. Further, they propose that this is achieved through GW182 dependent regulation of translation and actin cytoskeleton remodeling. The results on GW182 overexpression and KD are clear and result in a convincing model that nicely integrates with the literature. Overall, the data are well displayed and the text is easy to follow.

We thank the reviewer for the overall positive response to our manuscript.

Reviewer 2 Comments for the Author:
Before publication, I recommend that the authors reevaluate the data generated by overexpression of the dominant-negative GW182 (DNGW182). In the remainder, there are more detailed comments for the authors.
Major comments:
1) The authors discuss the differences in between the effects of DNGW182 and siRNA on global translation/actin remodeling. However, there is no discussion on why DNGW182 still phenocopies siGW182 in dendritic arborization. The current deduction that the effect of GW182 on dendritic arborization is mediated via translation/actin cannot be made if translation is oppositely affected by DNGW182.
We thank the reviewer for this critical comment. In the revised manuscript, we have added the discussion about how DNGW182 photocopies GW182 siRNA induced changes in the dendritic development "An important question arising from this data is how the DNGW182 mutant still appears to phenocopy GW182 knockdown in term of dendritic morphology. We speculate that even though upon gross evaluation, the dendritic morphology in GW182 knockdown and DNGW182 mutant might appear similar, in-depth analysis of dendritic morphology with more refined techniques is required to confidently conclude if the two are indeed yielding the same outcome with respect to dendritic arborization. For example, we did find some differences in dendritic morphology of DNGW182 and GW182 knockdown in our analysis of the length of the longest dendrite (Figure S3F and S3V), where the DN GW182 causes a reduction in the length of the longest dendrite, unlike GW182 knockdown where the length of the longest dendrite remains unchanged. Hence, an in-depth analysis of dendritic arborization parameters, like the number and length of primary, secondary, and tertiary dendritic branching upon GW182 knockdown and DNGW182 transfected neurons, to understand the discrepancies associated with the DNGW182 mutant. Further, it is important to emphasize here that the DNGW182 mutant does not behave like GW182 knockdown in every aspect and should be used with caution "**(P19, L536-550)**.

2) In general, the number of neurons analyzed per experiment is on the lower end throughout the paper. For example, in Fig. 6FG, the displayed neuron seems unhealthy and the analysis has been performed on only two independent experiments. I strongly suggest to include at least a third replicate and add more neurons into your analysis to exclude a possible strong influence of technical variation on your results. This may also help to clarify the influence of DNGW182 (see first comment).
As per the reviewer's suggestion, we have now included one more experimental replicate; with 8 more analyzed neurons for each column in **Figure S5D** new (earlier **Figure 6G**). Moreover, we have also changed the representative image for **Figure S5D** (new, earlier **Figure 6F**). We have now made sure to include a minimum of 25 neurons from 3 independent experiments in all experiments from main figures and in the majority of experiments from supplementary figures except few involving validations.

3. Please use the same statistical test, when comparing different conditions from the same experiment type (e.g. Fig. 3D vs. 3H Mann-Whitney vs. unpaired T-test with Welsh correction, same for Fig. 4).
We thank the reviewer for making this important alert. In the revised manuscript, we have modified the figure legends to incorporate reviewer suggestions, present as legends against

figure **S3D (**older **Figure 3D) & S3I (**older **Figure 3H)**. Further, we would like to emphasize that we have always determined whether the data follows normal distribution or not, and have chosen the corresponding test accordingly. The same is described in the statistical analysis subsection of materials and methods.

Minor comments:

1) For better comparability of your in vivo (Fig.1) and in vitro data (Fig. 2BC), please include a Western Blot and analysis of Ago2 protein signal with maturation of your hippocampal cultures.

As per the reviewer's suggestion, we have now included the developmental expression profile of AGO2 protein in hippocampal neuronal culture in the revised manuscript **(P7, L182-L185)** (**Figure 2C**, new). We found that the AGO2 expression profile in cultured neurons is similar to that of hippocampal tissue, where the level of AGO2 significantly increased from DIV3 to DIV15.

2) Proper quantification of your siRNA efficiency in hippocampal neurons in the timescale of your experiments is important (Figs. 4 and 6). The images currently displayed in Sup. Fig. 4 are not (yet) convincing and are missing quantification. In the results section, a difference in GW182 intensity in between dendrites and soma upon siRNA transfection is described, however, this is not visible in the current display. Lastly, the Western Blot for GW182 has been performed in N2A cells and not in primary neurons.

We thank the reviewer for raising these points. We have now quantified the levels of GW182 in scrambled and GW182 siRNA transfected neurons **(P10, L277-282)**, data present as **Figure S3R** (new) in the revised manuscript. Further, we have changed the representative images for the corresponding data as per the reviewer's suggestion. We have also provided the representative immunoblots and corresponding quantification for GW182 siRNA validation in neurons, data present as **Figure S3Q** (new) in the revised manuscript **(P10, L274-L277).**

3) Sup.Fig. 5: can you validate the expression pattern of GFP-GW182 and GFP-DNGW82 in hippocampal neurons? RISC protein expression patterns often differ in between cell lines and neurons at different developmental stages.

In the revised manuscript, now we have presented the validation of DNGW182 and GFP GW182 expression in neurons in **Figure S4D** (new).

4) Fig. 2D,E,G: I suggest merging the images into one panel as you are referring to different regions of the same cell. This way also one grey value bar would be sufficient. In addition, the DAPI signal is barely visible.

We have merged the required subpanels as per the reviewer's suggestion, merged panel present as **Figure 2D** in the revised manuscript. Further, we agree with the reviewer that the DAPI signal was not visible in the Full image for DIV6. We have now corrected the same by replacing the representative image.

5)Fig. 3C, D: please keep the DIV label consistent with Fig. 3 K, L.

We have made the labels consistent in the corresponding subpanels of the revised manuscript, **Figure S3C & S3D** (new, earlier **Figure3 C, D**) and **Figure S3M & S3N** (new, earlier **Figure 3K, L**).

6) Fig. 3I: the DN label is missing for GW182.

We thank the reviewer for pointing this out. We have now inserted the label in the revised manuscript **Figure S3K** (new, **earlier Figure 3I**).

7) Fig. 6D/F: Map2 and GFP labels are swopped.

We apologize for the mistake. Now we have corrected the labels in the revised manuscript, **Figure 5D** (new, earlier **Figure 6D**) and **Figure S5C** (new, earlier **Figure 6F**).

8) Page 3: I assume Stufen means Staufen.

We thank the reviewer for pointing this out. We have corrected the above-mentioned spelling in the revised manuscript **(P3, L75).**

9) Abstract: Spatio-temporal should be spatio-temporal (also throughout the manuscript).

We have made the required changes in the revised manuscript.

10) Last paragraph of introduction is repetitive, could be sharpened.

We have changed this part of the introduction

11) Bartel (2018) reference is included twice.
We apologize for the mistake. We have now corrected this mistake in the revised version of our manuscript.

Reviewer 3 Advance Summary and Potential Significance to Field:
Nawalpuri and Muddashetty studied spatiotemporal regulation of the key miRNA effector protein, GW182 in the rat brain. They observed that in the cerebellum and hippocampus, GW182 shows expression peaks at certain ages while the level of Ago2 keeps increasing during postnatal development. The GW182 expression peaks coincide with the timings of dendritogenesis. Such temporal changes could be recapitulated with cultured hippocampal neurons. An anticorrelation between the neuronal activity and the GW182 level was observed when neuronal activity was altered by toxins. Inhibition, knockdown or overexpression of GW182 altered dendritic arborization of hippocampal neurons, but the effect of GW182 could only be seen when expression was changed in certain time windows. FUNCAT analysis in hippocampal neurons revealed that GW182 levels determine global translation levels in dendrites. Finally, they showed that GW182 knockdown and overexpression caused changes in the F-actin level, and proposed that LIMK1 mediates regulation of F-actin by GW182, by demonstrating that LIMK1 expression could be altered by overexpression or knockdown of GW182.
In general, the experiments were performed carefully and the results are reasonably interpreted. While a previous report already pointed to involvement of GW182 in regulating dendritic growth (DOI 10.1016/j.cell.2012.01.036), this manuscript still adds important information. As regulation of miRNA effectors is an important topic, roles for such mechanisms in important biological processes like dendrite formation would draw attention of broad readership. With some improvement, I think this manuscript is a good candidate for publication in Journal of Cell Science.

We sincerely thank the reviewer for the positive response.

Reviewer 3 Comments for the Author:

Major points:
1) How many GW182 paralogs are there in the rat genome? Mammals often have three GW182 paralogs with overlapping functions. Do the materials used here (antibodies and siRNAs) recognize all paralogs or a subset of them? Whether the observed phenomena are associated with particular paralogs or reflecting the total amounts of GW182 homologs needs to be clarified.
We thank the reviewer for highlighting this important point. The rat genome consists of three GW182 paralogs, namely TNRC6A, TNRC6B, and TNRC6C. The antibody and siRNA used in our manuscript specifically recognizes TNRC6A. Further, the overexpression construct also is specifically for TNRC6A. Now we have clarified the same in material and methods as well as in the discussion. The results from our manuscript can only be interpreted as the role of TNRC6A in neuronal development. We agree that it is important to determine if different GW182 paralogs have binding specificity against selected miRNA-mRNA pairs and also have overlapping role in dendritogenesis. We have included these aspects in the discussion section of our manuscript **(P19, L556-L560)**.

2) Throughout the manuscript, it was unclear whether the effects of GW182 overexpression or knockdown on dendritic morphology were related to alteration in the miRNA activity. The authors should perform luciferase sensor assays. Using a sensor containing miRNA target sites and a control sensor whose seed target sites are mutated, effects of GW182 KD, DNGW182 and GW182 overexpression on miRNA activity should be tested, ideally in the exact experimental settings where dendritic complexity was quantified. These experiments might clarify which of the observed phenomena (dendrite morphology and global translation activity) are related to miRNA activity.
We agree with the reviewer this would be an important step to further understand the role of GW182 in dendritogenesis. But we want to highlight that to perform the luciferase sensor assays as suggested by the reviewer, it is essential to first identify the miRNA candidates expressed, and associated with GW182 during early development in neurons, and design the luciferase sensor against those specific miRNA or one has to take up a high throughput screening. We did attempt to perform immunoprecipitation experiments with GW182 to characterize associated miRNA

candidates, but we had several technical issues likely due to the unique role of GW182 in miRISC. We are regret that we were unable to perform this experiment due to technical limitations, limited time, and most importantly due to the constraints imposed by the second wave of the COVID-19 pandemic in India which has resulted in multiple lockdowns. However, we have modified our discussion to include limitations of our study **(P19, L560-561)** and future direction for identifying miRNA candidates involved in GW182 mediated regulation of dendritic development.

3) With the results showing that neurons expressing DNGW182 still responded to BDNF (Figure 3I-L), the authors concluded that "GW182 does not modulate BDNF induced dendritic growth in DIV3-7 neurons". However, it is difficult to draw a clear conclusion from the present data because the change in the complexity by BDNF seems smaller in the presence of DNGW182 (with the p-values of <0.0001 vs 0.0146 as shown in Figure 3L). I do not think this claim is supported.
We thank the reviewer for the critical comment. On the reviewer's suggestion, we have calculated the fold change of increase between the sum length and sum intersection of unstimulated and BDNF treated groups for GFP and DNGW182. We found the fold change for the calculated parameters to be comparable between GFP versus DNGW182 groups. For dendritic length, the GFP BDNF group showed a 1.86 times increase as compared to GFP basal, while the DNGW182 BDNF group showed a 1.72 times increase as compared to the DNGW182 basal group. For the sum intersection parameter, the GFP BDNF group showed a 1.42 times increase as compared to GFP basal, while the DNGW182 BDNF group showed a 1.53 times increase as compared to the DNGW182 basal group. Hence, we cannot certainly comment on the difference in BDNF response from the current data. Further, we think that the observed differences in p-values are primarily due to high variability in DNGW182 data points. To this end, we want to highlight that we have moved this data to **supplementary Figure 3** due to the anomaly associated with DNGW182 mutant results with respect to FUNCAT and phalloidin analysis.

List of changes made in the figures (The figure represents new figures)

Figure 1
Main: No change.
Supplementary: No change

Figure 2:
Main:
a) Figure 2B and 2C from old figure is now merged as 2B in new version.
b) AGO2 cultured neuron expression profile western blots and corresponding quantification included in new version as subpanel 2C.
c) Earlier subpanels 2D, 2E and 2F containing images of GW182 staining in different regions of neurons is now merged into single subpanel 2D as per reviewer's suggestion. Further the images now have one common calibration bar. The graph earlier labeled as 2F and 2H has now become 2E and 2F.
d) All figure subpanels afterwards, labeled as 2I, 2J, 2K, 2L, 2M, 2N and 20 have now become 2G, 2H, 2I, 2J, 2K, 2L and 2M.
Supplementary: No change

Figure 3
Main:
a) Data from old figure 4 has been moved to figure 3 in the new version.
Supplementary:
a) Data from subpanels A, B, C, D, E, F, G, H, I, J, K, and L from old figure 3 are now moved to supplementary figure 3 as panels A, B,C, D, G, H, I, J, L, M, N and O respectively.
b) Data from subpanels A, B, and C from old supplementary figure 3 are now moved to subpanels E, K and P respectively, in the new version of supplementary figure 3.
c) The new supplementary figure 3 also contains data additional data in subpanel S3F (Length of longest dendrite DNGW182/GFP DIV7), S3R (GW182 siRNA western blot validation neuron), S3T (GW182 siRNA immunostaining quantification neurons) and S3V (Length of longest dendrite Scr/GW182 siRNA DIV7).
d) Data from subpanels A, B, C, and D from old supplementary figure 4 is now moved to supplementary figure 3 as panels S3Q,S3S, S3U and S3W respectively.

Figure 4

Main:

a) Data from subpanels A, B, C, and D from old figure 5 has been moved to figure 4 as subpanels A, B, C and D in the new version.

Supplementary:

a) Data from subpanels A, B, C and D from old supplementary figure 5 are now moved to supplementary figure 4 as subpanels A, B, C and H respectively.

b) The new supplementary Figure 4 contains additional data with validation of DNGW182 and full length GW182 constructs in neurons as subpanels D.

c) The new supplementary Figure 4 contains additional data with Effect of overexpression of DNGW182 on AGO2 puncta as subpanels E, F, and G.

Figure 5

Main:

a) Data from subpanels A, B, C, D and E from old figure 6 has been moved to figure 5 as subpanels A, B , C and D in the new version.

Supplementary:

b) Data from subpanels F and G from old figure 6 has been moved to new supplementary figure 5 as subpanel S5D.

c) Data from subpanels A, B, and C from old figure 6 supplementary are now moved to subpanels S5A, S5C and S5E respectively, of figure 5 supplementary in the new version.

d) The new supplementary figure 5 contains new data in subpanel B showing the FUNCAT signal and corresponding quantification at different stages of cultured neurons.

Figure 6

Main:

a) Data from subpanels A, B, C, D, E, H , I , L ,M , N and O from old figure 7 has been moved to figure 6 as subpanels A, B , C, D, E, F, G, H, I, J, and K in the new version.

Supplementary:

b) Data from subpanels F, G, J, and K from old figure 7 has been moved to new supplementary figure 6 as subpanel A, B, C and D.

c) Data from subpanels A, B, C, D, and E from old supplementary figure 7 are now moved to subpanels E, F, G, H and I respectively, of supplementary figure 6 in the new version.

Figure 7

Main

Model present earlier as figure 8 has now become figure 7.

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Second decision letter

MS ID#: JOCES/2021/258465

MS TITLE: Distinct temporal expression of GW182 in neurons regulates dendritic arborization

AUTHORS: Bharti Nawalpuri, Arpita Sharma, Sumantra Chattarji, and Ravi S Muddashetty
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but two of them raised some critical points related to the original review that need to be properly addressed and will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Same as for the previous review

Comments for the author

In this revised manuscript, the authors have addressed most of my previous concerns. However, point 6 has not been satisfactorily addressed. The authors have not provided quantification of the

overlap between siRNA and GFP transfected neurons. There demonstration of an average ~40% reduction in expression is useful but it does not account for neurons that express GFP and no siRNA. Indeed, a 40% reduction in expression seems quite weak, and probably does reflect some cells showing no silencing. This is important, since many experiments involve imaging of individual neurons. In the absence of further analysis, it is essential that the authors include an acknowledgement of this potential weakness in the manuscript.

Reviewer 2

Advance summary and potential significance to field

Investigating the function of GW182 in neurons is important as there is little functional insight so far especially on dendritic growth. The manuscript provides new insight into dendritic morphogenesis and especially focuses on translation control and actin rearrangement.

Comments for the author

The authors made a serious effort to revise their interesting study according to the concerns of all three referees. Overall, the manuscript improved and I now suggest publication.

Reviewer 3

Advance summary and potential significance to field

Same as the previous version.

Comments for the author

The revised manuscript by Nawalpuri et al. addressed most of my concerns that were raised previously. However, regarding the descriptions about GW182 paralogs, the presence of GW182 paralogs is only discussed at the end of the discussion section. This information should be given in introduction. The title and abstract should also clarify which paralog was studied. The current manuscript still remains ambiguous about this point. (e.g. “We have identified a distinct brain region specific spatio-temporal expression pattern of GW182 during rat postnatal development.”- in abstract. It should be made clear that the expression patterns have been only examined with TNRC6A.)

With edits to satisfactorily clarify these points, I think the manuscript is ready for publication.

Second revision

Author response to reviewers' comments

Response to reviewer’s comments

We thank the reviewers for their comments and now we have modified our manuscript to include the revisions recommended by them. We hope the reviewer and the editor finds our revised manuscript suitable for publication in Journal of Cell Science.

Reviewer 1 Advance Summary and Potential Significance to Field:
Same as for the previous review

Reviewer 1 Comments for the Author:
In this revised manuscript, the authors have addressed most of my previous concerns. However, point 6 has not been satisfactorily addressed. The authors have not provided quantification of the

overlap between siRNA and GFP transfected neurons. There demonstration of an average ~ 40% reductions in expression is useful, but it does not account for neurons that express GFP and no siRNA. Indeed, a 40% reduction in expression seems quite weak, and probably does reflect some cells showing no silencing. This is important, since many experiments involve imaging of individual neurons. In the absence of further analysis, it is essential that the authors include an acknowledgement of this potential weakness in the manuscript.
We thank the reviewer for the positive assessment of our revised manuscript.
We agree with the reviewer's point regarding technical limitation of the co-transfection method used to for knockdown experiments in our study. As per the reviewer's suggestion, we have now mentioned this technical limitation in the result (P10, L279-281) and discussion **(P15, L459-464)** sections of our revised manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:
Investigating the function of GW182 in neurons is important as there is little functional insight so far, especially on dendritic growth. The manuscript provides new insight into dendritic morphogenesis and especially focuses on translation control and actin rearrangement.

Reviewer 2 Comments for the Author:
The authors made a serious effort to revise their interesting study according to the concerns of all three referees. Overall, the manuscript improved and I now suggest publication.
We thank the reviewer for the positive assessment of our revised manuscript and suggesting it for publication.

Reviewer 3 Advance Summary and Potential Significance to Field:
Same as the previous version.

Reviewer 3 Comments for the Author:
The revised manuscript by Nawalpuri et al. addressed most of my concerns that were raised previously. However, regarding the descriptions about GW182 paralogs, the presence of GW182 paralogs is only discussed at the end of the discussion section. This information should be given in introduction. The title and abstract should also clarify which paralog was studied. The current manuscript still remains ambiguous about this point. (e.g. "We have identified a distinct brain region specific spatio-temporal expression pattern of GW182 during rat postnatal development."- in abstract. It should be made clear that the expression patterns have been only examined with TNRC6A. With edits to satisfactorily clarify these points, I think the manuscript is ready for publication.
We thank the reviewer for the positive assessment of our revised manuscript and for highlighting the point regarding the inclusion of GW182 paralog information in other sections of the manuscript. As per the reviewer's suggestion, we have now included the information about GW182 paralog used in the "Title, abstract **(P2, L46)**, introduction **(P4, L106-107)**, result **(P4, L113-114)**" sections of our manuscript, in addition to the previously included discussion section **(P19, L544-550)**.

Third decision letter

MS ID#: JOCES/2021/258465

MS TITLE: Distinct temporal expression of GW182 (TNRC6A) in neurons regulates dendritic arborization

AUTHORS: Bharti Nawalpuri, Arpita Sharma, Sumantra Chattarji, and Ravi S Muddashetty
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.