THE EMBO JOURNAL

Manuscript EMBO-2012-84049

Ral mediates activity-dependent growth of postsynaptic membranes via recruitment of the exocyst

Rita O. Teodoro, Gulçin Pekkurnaz, Abdullah Nasser, Misao E. Higashi-Kovtun, Maria Balakireva, Ian G. McLachlan, Jacques Camonis and Thomas L. Schwarz

Corresponding author: Rita Teodoro, Boston Children's Hospital/Harvard Medical School

Review timeline:

Submission date:	28 November 2012
Editorial Decision:	20 December 2012
Revision received:	26 April 2013
Editorial Decision:	14 May 2013
Revision received:	23 May 2013
Accepted:	28 May 2013

Transaction Report:

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

Editor: David Del Alamo

1st Editorial Decision 20 December 2012

Thank you for the submission of your manuscript to The EMBO Journal and for your patience while it has been reviewed. We have just now received the full set of reports from the referees, which I copy below.

As you can see from their comments, all three referees are very positive about both the general interest and the novelty of your findings. In general, they believe that the evidence presented properly supports your conclusions, although some technical concerns have arisen with which you will have to deal before your manuscript is ready for publication. In addition, besides these technical concerns which are explicitly mentioned in the referee reports and I will not repeat here, referee #2 suggests a few more experiments to better characterize the roles of the exocyst in hippocampal neurons. While we acknowledge that your manuscript is centered on the Drosophila neuromuscular junction as a model system, and we understand that a complete characterization in vertebrate neurons is out of the scope of the manuscript, we also believe that not only these few experiments are relatively simple and can be performed during our standard revision time, but they will increase the general interest of the manuscript and further uncover a functional evolutionary conservation of the exocyst in synapse regulation.

I would therefore like to invite you to submit a revised version of the manuscript. Please be aware

that your revised manuscript must address the referees' concerns and their suggestions should be taken on board. It is 'The EMBO Journal' policy to allow a single round of revision only and, therefore, acceptance or rejection of your study will depend on the completeness of your responses included in the next, final version of the manuscript.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact me as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you again for the opportunity to consider your work for publication in The EMBO journal. I look forward to your revision.

Please, do no hesitate to contact me in case you have any further question, need further input or you anticipate any problem during the revision process.

REFEREE REPORTS:

Referee #1 (General Remarks):

In this work, Teodoro et al study subsynaptic reticulum (SSR) development at the fly NMJ and spine development in mammalian neurons and find that the SSR expands under influence of neuronal activity. Using genetics, fluorescent protein labeling and electron microscopy they implicate an exocyst-Ral dependent pathway in the process.
This paper is a well-put together piece of work that is of interest, probing into the role of the SSR at the fly NMJ, and revealing key physiological and molecular events that are at the basis of controlling its post synaptic structure. While I am following the logic and experiments presented, there are also a number of important shortcomings to this paper, mostly at the technical level or at the level of lacking controls. However, if appropriately revised, I believe that this paper will make an interesting contribution to the EMBOj.

Specific comments:

-The authors generate a ral antiserum but only test the specificity of this tool on western blots. Specificity needs to be shown at the NMJ as well. If the ral null larvae are large enough for EM they should be large enough for immunohistochemistry as well. It is known that many antisera non-specifically recognize the post synaptic side of the NMJ while being specific on western blot.

-The CD8-GFP experiment showing more intense GFP labeling in RalCA expressing animals is in my view not a solid argument for a larger SSR. CD8-GFP is delivered to the membrane by vesicle trafficking and the differences reported may be the result of defects in CD8-GFP trafficking rather than a larger SSR. Moreover, a positive control is lacking; ie a mutant where we know the SSR is enlarged (over expression of syndapin), does this mutant also show increased CD8-GFP labeling? An alternative would be to remove these data and rely on the TEM.

-Related to the previous point: the CD8-GFP data are also undermining the interpretation of the enriched Sec5 localization at the SSR in my view: the authors claim that Sec5 enriches at the post synaptic side in RalCA expressing animals (and also in several other conditions); however, if Sec5 were to be evenly distributed in the membrane (as is CD8-GFP) and the SSR increases in size and

density upon expression of RalCA (or other manipulations), this enrichment may be the result of the larger SSR rather than a specific enrichment of Sec5 in the SSR. Given that this phenotype is central to the paper, can this issue be clarified?

-In figure 3, TEM is performed on sec5 and ral mutants to find evidence that these genes are needed for SSR development. As indicated by the authors, the SSR develops while the larva grows and a developmental delay, rather than the genes being essential for SSR development, may yield boutons with little SSR. I would be much more confident if TEM of Ral(inact) that presumably acts dominantly, were included. Such animals develop until the third instar stage and delayed developmental staging in ral mutants could then be excluded as a reason for the phenotype.

-In those experiments where sec5 or ral mutants were used, rescue experiments are needed, definitely for the ral mutants that were homozygous.

-I am not sure the calcimycin application allows the authors to simply test post synaptic Ca/calmodulin-dependent activation of Ral. This treatment will also result in a rise in presynaptic calcium that will result in massive transmitter release. Given that neuronal activity also activates the ral/exocyst pathway (figure 5), the calcimycin experiment does in my view not distinguish between the post synaptic activation of calmodulin or an indirect effect of presynaptic transmitter release. Hence, the data are consistent with the model, but they do not add more than what is already shown in figure 5. One possibility may be to use a presynaptic blockade of release (TNT, UAS-shi,...) and then apply the calcimycin.

-Activity results in increased sec5 immunoreactivity in the SSR (figure 5). Do the manipulations that result in increased activity also result in increased levels of the other exocyst compnents, as well as in altered levels of DLG, Syndapin and GluRIIB?

-Chronic increased activity (using a temperature sensitive Trp channel) or chronic decreased activity (using shits1) results in increased or decreased SSR size. Importantly, do these manipulations results in a concomitant chance in mini amplitude?

-What happens if wild type Ral is over expressed? Does this yield a RalCA phenotype? I would also encourage a consistent use of the Ral null mutant where possible.

-Overexpression of RalCA in sec5 mutants does not rescue or result in an expansion of the SSR that is small in sec5 mutants. For the genetic pathway proposed to be fully tested, it would be good to include data that test if over expression of Sec5 can rescue ral mutants.

Minor
-I would include quantification of the data in fig S1C-F in the Figure 1.

-I would prefer to see TEVC recordings that are much more sensitive to detect subtle changes in synaptic transmission. This technology also enables a more accurate estimate of the quantal content as a correction for non-linearity would not need to be applied as is the case now.

-Is the time-scale for the mini traces shown correct?

-Given that the GluRIIB receptor abundance is changed upon expression of RalCA, is this defect detectable in the appearance of the minis?

-For completeness it would be good if other features (mitochondira, synaptic vesicles, active zones etc) visible in the TEM are quantified as well and included in the supplemental data section.

-In RalCA mutants the quantified DLG intensity is less. This is not very visible in the picture the authors provide in Figure S2. Given the larger SSR one would have expected an increase rather than a decrease in DLG. Can this be discussed?

-In Figure 2F the abbreviations in the legends are missing in the figure.

Referee #2 (General Remarks):

In this article Teodoro et al. analyze the function of the Ras-like GTPase Ral and one of its effectors, the exocyst complex, in activity-dependent postsynaptic membrane growth using the Drosophila neuromuscular junction (NMJ) model system. They show that expression of constitutively active Ral (RalCA) in muscle recruits several components of the exocyst complex (Sec5, Sec8, Sec3 and Sec15) to the postsynaptic sub-synaptic reticulum (SSR) and increases SSR size in a Sec5-dependent manner. Consistent with these results, SSR growth in sec5 and ral mutant larvae is disrupted. They subsequently use different approaches to propose that Ca2+ influx recruits Sec5 at postsynaptic membranes in a Ral-dependent way, leading to SSR growth. They finally suggest that a similar mechanism may function in mammalian neurons, since expression of RalACA in cultured hippocampal neurons increases the density of PSD95-positive dendritic spines through the exocyst complex.

Overall this is an interesting, well-written manuscript. The study in the Drosophila NMJ is carefully conducted and highlights a novel, potentially very relevant function for Ral and the exocyst complex. However, in my opinion the final section in cultured hippocampal neurons would need further investigation to strengthen and extend the importance of the authors' findings.

Major points
1) The authors nicely show that the increase in postsynaptic membranes at the Drosophila NMJ is activity-dependent and propose a role for Ral and the exocyst complex in this process. In parallel with these experiments, it would be worth testing whether exocyst components accumulate in spines after depolarization of hippocampal neurons. If so, is this accumulation dependent on RalA? (They could use the RalA inactive mutant or RalA RNAi to test this).

2) Is there a specific localization of the myc-tagged RalA constructs in the hippocampal neurons (Fig. S7)? The authors only show the ds-Red signal in Fig. S7. Does neuronal depolarization affect endogenous RalA localization in hippocampal neurons? (Also, did they test in Drosophila whether endogenous Ral is recruited in an activity-dependent fashion at the postsynaptic NMJ membranes using their anti-Ral antibody?)

3) The authors state that neuronal morphology was not grossly altered by expression of the RalA constructs and that only RalACA caused a small decrease in the number of distal branches (pag. 14, Fig. S7). However, in Fig. S7 the dendrites of neurons transfected with RalACA appear to be much shorter compared to the other constructs (this could be easily quantified). Also, the same picture (RalACA in Fig. S7) seems to show an increased branch density - I wonder whether quantifying the amount of branches/µm would reveal an effect underestimated by the Scholl analysis.

4) It would be important to confirm the increase in the PSD95-positive spines caused by RalACA using immunostaining of endogenous PSD95 instead of PSD95-YFP transfection.

Minor points
5) The Ral immunostaining shown in Fig. S1B is very faint. It is difficult to see the slight concentrations of Ral around the boutons, as the authors mention. The quality of this figure should be improved.

6) Is there a particular reason why the authors limited their analysis to muscles 6/7 and segments A2/A3 (pag. 6)?

7) The authors should show OK6-Gal4-driven motor neuron expression of Ral (for example using their anti-Ral antibody) for Fig. 1F.

8) I find the data in Fig. S2D-I quite redundant, since the same point (increase in SSR width caused by expression of RalCA in muscle) is more convincingly supported by the TEM study presented in Fig. 2D-G.

9) The abbreviations listed in Figure 2F (SSR, b, sv, m) are not used to label the corresponding features in the EM pictures shown in Fig. 2D-F.

10) Labeling should be added to Fig. 5A-H to explain what the staining is for (Sec5 and Hrp).

11) In Fig. 5E-F, the integrity of the muscle tissue appears to be disrupted for both unstimulated and stimulated samples and subsequently the Sec5 accumulation is not so evident. These pictures should be substituted.

12) Specify the value of the scale bar in Fig. S7 A-D.

13) Spell out SSR in the abstract.

Referee #3 (General Remarks):

This is an elegant and well-done study describing a novel neuronal activity-dependent pathway that promotes the growth of the subsynaptic reticulum (SSR) in Drosophila muscle. The authors use gain and loss-of-function manipulations to probe the interplay between postsynaptic calcium-influx, Ral activation, exocyst complex recruitment, and SSR membrane growth.

The results are well documented and convincing. In particular the EM analysis is instrumental in illustrating the phenotypes. One (relative) weakness of the work is the fact that it is actually not clear what the physiological consequences of SSR expansion (or shrinkage) will be with respect to synaptic function. However, I would consider the present piece of work an important step forward that might facilitate answering this question in the future.

I have one concern that the authors should address: The Sec5 and ral mutants used for the morphological analysis in Fig.3 die close to the time that the analysis of SSR elaborations was performed. This raises the concern that reductions in SSR in these mutants might be a secondary consequence of more widespread defects in the mutants. The authors need to demonstrate that loss of SSR represents a specific phenotype, e.g. the authors could extract quantitative parameters of other pre- and/or postsynaptic structures from these mutants to explore this.

1st Revision - authors' response 26 April 2013

We thank the reviewers for their close reading and helpful comments and this revision addresses each of their concerns with additional experiments and changes to the text. The additions address the specificity of the pathway and expand our analysis of the potential role of the pathway in mammalian neurons. Key new experiments include the analysis of larvae expressing sec5 RNAi in muscle and a previously uncharacterized allele of Ral that survives into the pupal stage. These genotypes allowed us to perform analysis in 3rd instar larvae and demonstrate the selective effect of Ral and Sec5 on SSR (subsynaptic reticulum) formation, rather than general developmental delay.

Additionally, we have expanded the study in mammalian neurons and showed that both RalA^{CA} and neuronal depolarization increases the levels of Sec5 in dendritic spines. As detailed below, we have done our best to answer all the reviewers comments and hope that it is now suitable for publication.

Referee #1:

In this work, Teodoro et al study subsynaptic reticulum (SSR) development at the fly NMJ and spine development in mammalian neurons and find that the SSR expands under influence of neuronal activity. Using genetics, fluorescent protein labelling and electron microscopy they implicate an exocyst-Ral dependent pathway in the process.
This paper is a well-put together piece of work that is of interest, probing into the role of the SSR at the fly NMJ, and revealing key physiological and molecular events that are at the basis of

controlling its post synaptic structure. While I am following the logic and experiments presented, there are also a number of important shortcomings to this paper, mostly at the technical level or at the level of lacking controls. However, if appropriately revised, I believe that this paper will make an interesting contribution to the EMBOj.

Specific comments:

-The authors generate a ral antiserum but only test the specificity of this tool on western blots. Specificity needs to be shown at the NMJ as well. If the ral null larvae are large enough for EM they should be large enough for immunohistochemistry as well. It is known that many antisera non-specifically recognize the post synaptic side of the NMJ while being specific on western blot.

We agree with the reviewer that the immunoreactivity of endogenous Ral may not be genuine and have removed that panel from Figure S1. At the time of our previous submission, no null allele of ral had been reported that survived to the 3rd instar, the only stage at which we could detect Ral immunoreactivity at the synapse. We have now characterized an allele of Ral that survives to pupation but has no detectable protein product by Western blot (new Figure S3A). Using this allele as a control and trying many fixation conditions, we could not unambiguously establish the specificity of the immunoreactivity around the boutons in the absence of transgene expression. In the presence of the Ral transgenes, however, the signal (shown in Figure 1A-B) is clearly specific since it depends entirely on overexpression of Ral. Moreover, as discussed below, the abundance or Ral immunoreactivity in these genotypes is not a mere reflection of the extensive membrane infolding because Ral^{CA} concentrates surrounding boutons even in sec5 mutants which have no SSR (Figure 3K-L).

-The CD8-GFP experiment showing more intense GFP labelling in RalCA expressing animals is in my view not a solid argument for a larger SSR. CD8-GFP is delivered to the membrane by vesicle trafficking and the differences reported may be the result of defects in CD8-GFP trafficking rather than a larger SSR. Moreover, a positive control is lacking; i.e. a mutant where we know the SSR is enlarged (over expression of syndapin), does this mutant also show increased CD8-GFP labelling? An alternative would be to remove these data and rely on the TEM.

We agree that, if we only based our conclusion of Ral^{CA} expressing animals having a larger SSR on the CD8-GFP data without any EM analysis, that would not be a solid argument because there could selectively be an increase in CD8-GFP and no other membrane component. But we think that the combination of EM and light microscopy is the strongest possible case since EM cannot sample as many boutons as the light microscopy. We therefore decided to keep the CD8-GFP in the paper, although we shortened the section considerably, because that experiment shows that the effect is true across the entire NMJ and not a subset of boutons. Regarding the positive control you suggested, yes, if we over-express Synd-YFP in the muscles, together with CD4-tomato, CD4-tom is also increased at the NMJ (we used the tomato to distinguish it from Synd-YFP).

-Related to the previous point: the CD8-GFP data are also undermining the interpretation of the enriched Sec5 localization at the SSR in my view: the authors claim that Sec5 enriches at the post synaptic side in RalCA expressing animals (and also in several other conditions); however, if Sec5 were to be evenly distributed in the membrane (as is CD8-GFP) and the SSR increases in size and density upon expression of RalCA (or other manipulations), this enrichment may be the result of the larger SSR rather than a specific enrichment of Sec5 in the SSR. Given that this phenotype is central to the paper, can this issue be clarified?

This is a good point and definitely worth clarifying in the text. Briefly, the Sec5 accumulation cannot be due to an even distribution across the membrane because:

1. After our acute stimulation protocols in Fig. 4 and 5, we see Sec5 accumulation occurring in a time scale of minutes, which is not long enough to promote significant SSR growth.

2. To address the possibility quantitatively, we compared the enrichment of synaptic CD8-GFP, which does reflect the extent of membrane enlargement, to Sec5 recruitment. To quantify the enrichment, we compared levels of each protein at the synapse to non-synaptic regions of the muscle. This was done for muscle Ral^{CA} expression and control muscles. We calculated the intensity/µm2 of each protein in the 2 regions in control and Ral^{CA} conditions and took a ratio of $Sec^{syn}/CD8^{syn}$ and $Sec5^{muscle}/CD8^{muscle}$. If both CD8-GFP and Sec5 were passively reflecting the distribution of membrane, the ratio of $Sec^{syn}/CD8^{syn}$ and $Sec5^{muscle}/CD8^{muscle}$ would both be identical in both control and Ral^{CA} expressing muscles. On the other hand, if Sec5 is specifically enriched at the synapse, the ratio of $Sec^{syn}/CD8^{syn}$, but not $Sec5^{muscle}/CD8^{muscle}$,would be significantly higher in Ral^{CA} and this is what we observed: control($Sec^{syn}/CD8^{syn}$)=0.51 ± 0.03, Ral^{CA}($Sec^{syn}/CD8^{syn}$)=0.85 ± 0.03, control($Sec5^{muscle}/CD8^{muscle}$)= 0.87 ± 0.05 and Ral^{CA}($Sec5^{muscle}/CD8^{muscle}$)=1.01± 0.1. If we do a t-test between control and Ral^{CA}, p($Sec^{syn}/CD8^{syn}$)<0.0001, while p($Sec5^{muscle}/CD8^{muscle}$) is n.s. (p=0.3). Or, to put it differently, the enrichment or Sec5 at the synapse is several fold greater than the enrichment of CD8-GFP upon Ral^{CA} expression. We hope this analysis alleviates the reviewer's concern. We have included a clarification of this issue in the main text.

-In figure 3, TEM is performed on sec5 and ral mutants to find evidence that these genes are needed for SSR development. As indicated by the authors, the SSR develops while the larva grows and a developmental delay, rather than the genes being essential for SSR development, may yield boutons with little SSR. I would be much more confident if TEM of Ral(inact) that presumably acts dominantly, were included. Such animals develop until the third instar stage and delayed developmental staging in ral mutants could then be excluded as a reason for the phenotype.

First a technical point: Ral^{inact} is not a dominant negative for this pathway. The mutation introduced in Ral^{inact} may indeed interfere with the RalGEF in a dominant fashion, but Ral activation by Ca^{2+} and calmodulin is direct and not via the RalGEF. A dominant effect on Sec5 recruitment was therefore neither anticipated nor observed.

Regarding the important question of a specific arrest of SSR growth vs. a developmental delay, we have included additional experiments to exclude the latter explanation more conclusively.

1) The SSR that should appear before 60 h is largely absent in sec5 null animals and yet those animals can live till 96 h and move about quite well at that stage. As we described in an earlier publication (Murthy et al, 2003), synaptic transmission continues at the NMJ until 96 h and transmitter release per active zone appears normal.

2) To further separate SSR growth from larval development, we have now added experiments with a Sec5 genotype that lives to third instar. We tested all the available Sec5 RNAi lines and found that, when coexpressed with the RNAi processing enzyme Dicer, and using a strong muscle driver (G14-Gal4) at 29^{o}C, we could knock down Sec5 enough to analyse the SSR size in 3rd instar. In these older animals where the SSR should have been fully formed, the SSR was in fact no more developed than in the null mutants.

3) For Ral, we accomplished a similar dissociation of SSR formation from development by using the null allele that survives to pupation, already mentioned above. In this new mutant, ral^{G0501}, the SSR was still lacking in 3rd instar larvae, unchanged from its size in 60 h larvae of either Ral genotype. These results have been included in Figure 3.

-In those experiments where sec5 or ral mutants were used, rescue experiments are needed, definitely for the ral mutants that were homozygous.

The requested rescues were done by expression of UAS-Sec5 using the ubiquitous driver da-Gal4, and by expressing UAS-Ral-WT in the muscle. Both rescued completely SSR development and are included with quantification as supplementary figure S4C-E.

-I am not sure the calcimycin application allows the authors to simply test post synaptic Ca/calmodulin-dependent activation of Ral. This treatment will also result in a rise in presynaptic calcium that will result in massive transmitter release. Given that neuronal activity also activates the ral/exocyst pathway (figure 5), the calcimycin experiment does in my view not distinguish between the post synaptic activation of calmodulin or an indirect effect of presynaptic transmitter release. Hence, the data are consistent with the model, but they do not add more than what is already shown in figure 5. One possibility may be to use a presynaptic blockade of release (TNT, UAS-shi,...) and then apply the calcimycin.

We agree that the calcimycin experiment, in isolation, does not determine whether this is due to pre or postsynaptic Ca^{2+} influx. The experiments with glutamate and with nerve stimulation, in conjunction with the calcimycin experiments, are strong support for the pathway we propose. In particular, the fact that glutamate recruits Sec5 only in the presence of Ca^{2+} indicates that presynaptic Ca^{2+} influx is not necessary.

-Activity results in increased sec5 immunoreactivity in the SSR (figure 5). Do the manipulations that result in increased activity also result in increased levels of the other exocyst components, as well as in altered levels of DLG, Syndapin and GluRIIB?

The effect of neuronal activity on SSR expansion was, understandably, not as pronounced as the effect of constitutive expression of Ral^{CA}. This probably means that the biochemical changes seen with Ral^{CA} would also be less pronounced and harder to detect. Nevertheless, we analysed the markers requested. We decided to do it only in the protocol with cycling of the temperature between 20^0C and 30^0C, because there are very rare cases of escapers of neuronal TrpA1 that were raised at 30^0C for 48 h (as it was mentioned in the original manuscript). After 48 h of cycling temperatures in the PCR machine, we do see a significant increase in the levels of Syndapin in the TrpA1 condition, but the control is not significantly different from shi^{ts}; gluRIIB is slightly higher in TrpA1 compared to control, but not compared to shi^{ts} and dlg is slightly lower in shi^{ts}, not different in TrpA1. Thus, as anticipated, the data trend as would be expected from the Ral^{CA} condition, but changes are smaller. Similarly, we expect that the biochemical changes in DLG, Syndapin and GluRIIB that appear after constant exposure to Ral^{CA} would be even less apparent in the short time scale of our acute nerve stimulation experiments. The dynamics of recruitment of these components are definitely worth studying but it is beyond the scope of this manuscript.

-Chronic increased activity (using a temperature sensitive Trp channel) or chronic decreased activity (using shi^{ts1}) results in increased or decreased SSR size. Importantly, do these manipulations results in a concomitant chance in mini amplitude?

We performed this analysis in the alternating temperature protocol and saw no differences in mini amplitude amongst the genotypes. Even with muscle expression of Ral^{CA}, the change in mini amplitude is small and barely reaches statistical significance and so we were not surprised that we could not see a change with less potent activators. There is also the additional concern that, whereas the samples could be processed for EM immediately after the temperature cycling, there was necessarily some time lag between the end of the cycling and the electrophysiological recordings. In any case, the observed changes in mini amplitude and frequency may be very indirect consequences of the highly significant anatomical change that is the main finding in our study. We therefore have never placed any emphasis on the physiological phenomena but include it in the characterization of the Ral^{CA} genotype for completeness.

-What happens if wild type Ral is over expressed? Does this yield a RalCA phenotype? I would also encourage a consistent use of the Ral null mutant where possible.

*Expression of wild-type Ral in the muscle results in Sec5 recruitment to the NMJ. The amount of Sec5 recruited is significant but, as expected, lower than with Ral^{CA}. This is likely because only a fraction of Ral^{WT} is activated. The quantification has been included in Fig.*1.

-Overexpression of RalCA in sec5 mutants does not rescue or result in an expansion of the SSR that is small in sec5 mutants. For the genetic pathway proposed to be fully tested, it would be good to include data that test if over expression of Sec5 can rescue ral mutants.

We have done this experiment by expressing UAS-Sec5 in the muscles of ral^{PG89}. Like with the converse experiment, Sec5 expression was not sufficient to compensate for Ral loss. This experiment has been included in Fig. S4A-B and is consistent with a model in which the exocyst needs to be activated by Ral to drive SSR expansion.

Minor
-I would include quantification of the data in fig S1C-F in the Figure 1.

I'm afraid we had not quantified the relocalization of Sec3 and Sec8 transgenes because the change was so obvious in each experiment. We trust the dramatic effect apparent in these representative images will suffice since it so precisely mimics that pattern quantified for Sec5.

-I would prefer to see TEVC recordings that are much more sensitive to detect subtle changes in synaptic transmission. This technology also enables a more accurate estimate of the quantal content as a correction for non-linearity would not need to be applied as is the case now.

As mentioned above, we do not focus on the electrophysiological phenotype because it is likely to be minor and possibly indirect. A more complete analysis of what the SSR means to the functioning of the muscle is clearly called for – it is a large gap in our understanding of this preparation despite 3 decades of its use – but it is beyond the scope of our manuscript. TEVC, trains of stimuli, measurements of muscle Ca^{2+} currents and many other properties should definitely be a part of that study!

-Is the time-scale for the mini traces shown correct?

Thanks! I don't know where that was messed up, but it is now fixed.

-Given that the GluRIIB receptor abundance is changed upon expression of RalCA, is this defect detectable in the appearance of the minis?

GluRIIBs have a smaller unit conductance than GluRIIAs (DiAntonio et al., 1999) and we see increased GluRIIB levels in muscle Ral^{CA}: while the effect in minis is small, we think that it might be due to a shift towards GluRIIB receptors.

-For completeness it would be good if other features (mitochondira, synaptic vesicles, active zones etc.) visible in the TEM are quantified as well and included in the supplemental data section.

We have quantified the number of active zones, bouton diameter and bouton area and have included it as Table I in supplemental material. We saw no significant changes. Because we are expressing Ral^{CA} only in the muscle, we are not sure what we would make of changes in these parameters or the others mentioned – they would necessarily be indirect sequelae.

-In RalCA mutants the quantified DLG intensity is less. This is not very visible in the picture the authors provide in Figure S2. Given the larger SSR one would have expected an increase rather than a decrease in DLG. Can this be discussed?

The reviewer correctly notes the dissociation of Dlg staining and SSR size. The explanation, we feel, lies in the fact that Dlg is not a component of the entire SSR but rather resides in its inner shells, just below the receptors. The membrane that is added expands the deeper layers that contain Syndapin immunoreactivity, but not Dlg. Even in control muscles, with good resolution we can observe Syndapin extending further from the bouton than the narrow zone with Dlg. Regarding the figure, the decrease in Dlg is only 20% and the image in Fig. S2 is representative of the difference which is almost impossible to detect by eye. We've resisted the temptation to put in a more extreme example. Dlg abundance at the NMJ is actively regulated. In particular, Zhang et al. (2007) have shown that Par-1 phosphorylation of Dlg promotes its detachment from the NMJ. In Ral^{CA} *expressing muscles, Par-1 levels are increased, which can lead to a reduction in synaptic Dlg. We have included the Par-1 data in the graph in Fig.3.*

-In Figure 2F the abbreviations in the legends are missing in the figure.

Thank you, we fixed this.

Referee #2:

In this article Teodoro et al. analyse the function of the Ras-like GTPase Ral and one of its effectors, the exocyst complex, in activity-dependent postsynaptic membrane growth using the Drosophila neuromuscular junction (NMJ) model system. They show that expression of constitutively active Ral (RalCA) in muscle recruits several components of the exocyst complex (Sec5, Sec8, Sec3 and Sec15) to the postsynaptic sub-synaptic reticulum (SSR) and increases SSR size in a Sec5-dependent manner. Consistent with these results, SSR growth in sec5 and ral mutant larvae is disrupted. They subsequently use different approaches to propose that Ca2+ influx recruits Sec5 at postsynaptic membranes in a Ral-dependent way, leading to SSR growth. They finally suggest that a similar mechanism may function in mammalian neurons, since expression of RalACA in cultured hippocampal neurons increases the density of PSD95-positive dendritic spines through the exocyst complex.

Overall this is an interesting, well-written manuscript. The study in the Drosophila NMJ is carefully conducted and highlights a novel, potentially very relevant function for Ral and the exocyst complex. However, in my opinion the final section in cultured hippocampal neurons would need further investigation to strengthen and extend the importance of the authors' findings.

Major points
1) The authors nicely show that the increase in postsynaptic membranes at the Drosophila NMJ is activity-dependent and propose a role for Ral and the exocyst complex in this process. In parallel with these experiments, it would be worth testing whether exocyst components accumulate in spines after depolarization of hippocampal neurons. If so, is this accumulation dependent on RalA? (They could use the RalA inactive mutant or RalA RNAi to test this).

We agree that this is an interesting question and we have addressed it. We now show that both neuronal depolarization and expression of $RalA^{CA}$*, but not* Ral^{inact} *or* $Ral^{CA\Delta exo}$ *increase levels of HA-tagged Sec5 selectively in the spines of hippocampal dendrites. The effect is highly significant and we saw no significant change in Sec5 levels in cell bodies and only a minor effect in dendrite shafts. Thus the mammalian phenomenon parallels the findings in Drosophila also in this regard. To ask whether the depolarization-mediated increase in Sec5 in spines requires RalA, we would need a potent RNAi construct and, despite considerable effort, we do not yet have one that is adequate. Moreover, the* Ral^{inact} *does not act as a dominant negative for this pathway. It locks Ral in the GDP bound form, therefore titrating away the GEF for Ral. However, Ral activation by* Ca^{2+} *is direct and GEF-independent. Therefore, we must for now rely on the fact that the effect of depolarization is mimicked by* $RalA^{CA}$*, but not* Ral^{inact} *or* $Ral^{CA\Delta exo}$*.*

2) Is there a specific localization of the myc-tagged RalA constructs in the hippocampal neurons (Fig. S7)? The authors only show the ds-Red signal in Fig. S7. Does neuronal depolarization affect endogenous RalA localization in hippocampal neurons? (Also, did they test in Drosophila whether endogenous Ral is recruited in an activity-dependent fashion at the postsynaptic NMJ membranes using their anti-Ral antibody?)

Myc-tagged RalA constructs localize throughout the dendrite. This may reflect the fact that the ability to form a new spine upon stimulation is a broad property of the dendrites, unlike the very localized zones for SSR in Drosophila muscle. We have included the myc panels in the Figure. Endogenous Ral staining has proven itself to be very challenging and I am sorry to say that we could not get this data in a satisfactory manner. In our dense neuronal cultures, the mammalian RalA antibody stains pretty much everything and it is impossible for us to discern what's real from background. Without an adequately potent RNAi, we can't verify that the endogenous staining is real. In Drosophila, endogenous staining is extremely low and did not appear to change with depolarization.

3) The authors state that neuronal morphology was not grossly altered by expression of the RalA constructs and that only RalACA caused a small decrease in the number of distal branches (pag. 14, Fig. S7). However, in Fig. S7 the dendrites of neurons transfected with RalACA appear to be much shorter compared to the other constructs (this could be easily quantified). Also, the same picture (RalACA in Fig. S7) seems to show an increased branch density - I wonder whether quantifying the amount of branches/µm would reveal an effect underestimated by the Scholl analysis.

We want to thank the reviewer for this suggestion; the reviewer was quite correct. We did the suggested analysis, which showed a significant increase in branches/µm. We have added this quantification to the Figure (previously Fig. S7, now Fig. S9D)

4) It would be important to confirm the increase in the PSD95-positive spines caused by RalACA using immunostaining of endogenous PSD95 instead of PSD95-YFP transfection.

We have quantified endogenous PSD95 in $RalA^{CA}$ vs. $RalA^{inact}$ and have confirmed the increase in PSD95-positive spines. This data has been added as a supplemental figure (Fig. S10).

Minor points
5) The Ral immunostaining shown in Fig. S1B is very faint. It is difficult to see the slight concentrations of Ral around the boutons, as the authors mention. The quality of this figure should be improved.

We agree and removed the figure (see response to Reviewer 1, first specific point).

6) Is there a particular reason why the authors limited their analysis to muscles 6/7 and segments A2/A3 (pag. 6)?

Muscle 6/7 are probably the best studied muscle group for NMJ analysis in the fly and has become quite standard. It is highly stereotypical from animal to animal. That said, the phenotypes we describe are not unique to this muscle group, but analysing a single type of NMJ decreases variability. Since this was not clear, we added an explanatory sentence in the text.

7) The authors should show OK6-Gal4-driven motor neuron expression of Ral (for example using their anti-Ral antibody) for Fig. 1F.

Neuronal expression of Ral constructs is very intriguing: Ral^{inact} accumulates at the NMJ (and in the dendrites) but both Ral^{WT} and Ral^{CA} do not accumulate at the pre-synaptic NMJ: instead, it appears that it is localized to the dendrites of motor neurons, which are very hard to identify. Therefore,

given that we know that all the flies used are of the correct genotype and since we don't see any specific staining of neuronal expression of Ral (except for the inactive), we decided to not include this.

8) I find the data in Fig. S2D-I quite redundant, since the same point (increase in SSR width caused by expression of RalCA in muscle) is more convincingly supported by the TEM study presented in Fig. 2D-G.

We completely agree that TEM is more convincing, but, as we answered reviewer #1, who also raised this point, we decided to keep the CD8 data mainly because it shows that the effect is true across the entire NMJ and not a subset of boutons (which could happen by TEM, due to sampling errors). We reduced the space dedicated to its explanation in the main text.

9) The abbreviations listed in Figure 2F (SSR, b, sv, m) are not used to label the corresponding features in the EM pictures shown in Fig. 2D-F.

Thank you, we fixed this.

10) Labelling should be added to Fig. 5A-H to explain what the staining is for (Sec5 and Hrp).

Thank you, we fixed this.

11) In Fig. 5E-F, the integrity of the muscle tissue appears to be disrupted for both unstimulated and stimulated samples and subsequently the Sec5 accumulation is not so evident. These pictures should be substituted.

Again, we do agree with the reviewer. However, if we substitute these images, they would have to be replaced by some others very similar. Unfortunately, stimulation of the nerve at 10Hz for 5 min, does lead to massive muscle contraction and compromises the morphology of the prep. Regardless of these caveats, Sec5 recruitment is significantly increased in the stimulated side, compared to the unstimulated side, and this is why we have kept the data in the paper, although we now include the less traumatic TrpA stimulation in the main text and have relegated the data from electrical stimulation to the supplement (Fig. S7).

12) Specify the value of the scale bar in Fig. S7 A-D.

Thank you, we fixed this.

13) Spell out SSR in the abstract.

We fixed this.

Referee #3:

This is an elegant and well-done study describing a novel neuronal activity-dependent pathway that promotes the growth of the subsynaptic reticulum (SSR) in Drosophila muscle. The authors use gain and loss-of-function manipulations to probe the interplay between postsynaptic calcium-influx, Ral activation, exocyst complex recruitment, and SSR membrane growth.

The results are well documented and convincing. In particular the EM analysis is instrumental in illustrating the phenotypes. One (relative) weakness of the work is the fact that it is actually not clear what the physiological consequences of SSR expansion (or shrinkage) will be with respect to synaptic function. However, I would consider the present piece of work an important step forward that might facilitate answering this question in the future.

I have one concern that the authors should address: The Sec5 and ral mutants used for the morphological analysis in Fig.3 die close to the time that the analysis of SSR elaborations was performed. This raises the concern that reductions in SSR in these mutants might be a secondary consequence of more widespread defects in the mutants. The authors need to demonstrate that loss of SSR represents a specific phenotype, e.g. the authors could extract quantitative parameters of other pre- and/or postsynaptic structures from these mutants to explore this.

We really appreciate this comment and, while we think that the failure of the SSR to form in the sec5 and ral mutants is unlikely to arise from poor viability of the larvae, we found an alternative way of showing the specificity of Sec5 and Ral in SSR formation. To circumvent this problem, instead of characterizing other parameters of the mutants that could very well be altered, but being unrelated to SSR development and plasticity, we tested all the available Sec5 RNAi lines and found that, when coexpressed with the RNAi processing enzyme, Dicer2, and using a strong muscle driver (G14-Gal4) at 29^{o}C, we could knock down Sec5 enough to analyse the SSR size in 3rd instar, a time point significantly after the development of the SSR starts. These results showed that the muscle specific muscle RNAi of Sec5 allowed larvae to develop to the 3rd instar but it hampered SSR development (the SSR was not different from the null mutants). For Ral, none of the RNAi lines knocked down sufficiently Ral but, we did find a previously uncharacterized ral mutant that appears null by Western Blot, and that survives to late pupal stages. We therefore analysed the SSR of this new mutant, ral^{G0501}, at both 60 h and in 3rd instar. We found that the SSR failed to develop in both cases, and was no different from the previously analysed mutant. In summary, we have now analysed SSR development in sec5 and ral mutant muscles in the 3rd instar and confirmed our previous results. These results have been included in Figure 3. We hope that these results satisfy the reviewer's concern of it being a developmental delay.

2nd Editorial Decision 14 May 2013

Thank you for the submission of your revised manuscript to The EMBO Journal and for your patience while it has been evaluated. It has been sent back to two of the original reviewers, who now consider that their major concerns have been properly addressed and your manuscript is almost ready for publication.

Notwithstanding their positive view, referees still point out to some issues, as you will see below, that will need your attention before your manuscript can be finally accepted. Although most of their points are essentially addressable by further discussion or clarifications, I would like to particularly draw your attention to points 2 and 3 of referee #2, which may require further (albeit minor) experimental evidence.

Thank you very much again for your patience. Do not hesitate to contact me in case you need further input or you anticipate any potential issues. I am looking forward to seeing the revised, final version of your manuscript.

REFEREE REPORTS:

Referee #1 (General Remarks):

The revised manuscript by Teodoro et al has significantly improved. I thank the authors for the clear explanations to my questions and for their willingness to include the new data pieces that in my view were essential to support the claims. I am happy to recommend the paper for publication but hope that the 3 points below can be dealt with in the final version of the manuscript:

-I am fine with the explanation on ral antibody specificity and that it can only be detected upon ral overexpression but I would like the authors to include a statement to that effect in the results section so this is made clear to the reader and colleagues wishing to use this tool in the future.

-I understand that the 'weaker' stimuli to increase activity only result in a trend for altered levels of

Synd or GluRIIB. Nonetheless I believe the authors should include these data in the text of the results section for completeness.

-I agree with the authors that a complete electrophysiological analysis of the SSR in synaptic transmission would be useful and that this is not the point of this paper. The authors are however calculating quantal content based on non-linear excitatory junctional potentials (fig S2A). For quantal content to be properly measured TEVC recordings are needed. I suggest to either remove the QC calculation or include simple TEVC recordings to assess the QC properly.

Referee #2 (General Remarks):

In this revised manuscript, Teodoro et al have satisfactorily addressed a series of points raised by different reviewers by adding a considerable amount of data, which have improved and completed their work. The experimental approaches in Drosophila are elegant, solid and very convincing. I only have some concerns regarding some additional results obtained in hippocampal cultures, which in my opinion should be addressed to conclusively prove the validity of the authors' interpretation.

Specifically:

1) Figure 7A shows images of dendrites apparently expressing GFP and YFP-PSD95. However, the legend mentions that neurons were transfected with DsRed instead of GFP (consistent with the description in Extended Experimental Procedures). The images in Figure 7A are slightly cropped versions of the pictures shown in the original version of the manuscript (former Figure 7A), but now the red channel corresponding to DsRed has been changed to green for GFP. Can the authors please clarify this.

2) The authors state that "The increase in PSD95-positive spines after expression of RalACA relative to RalAinact is also present when endogenous PSD95 rather than the YFP-tagged transgene is used to identify those spines" (page 15 second line, related to Fig. S10). However, they should include a control in their experiments (for example, co-transfection of GFP with an empty vector or wild-type RalA) to conclusively prove that RalACA increases the amount of endogenous PSD95-containing spines, similarly to what they have done in the case of co-transfection experiments with YFP-PSD95 (Fig. 7B-D). If one compares the results shown in Fig. S10B with the values of the control condition shown in Fig. 7B (transfection of DsRed plus YFP-PSD95) RalACA does not increase protrusion density/µm, while RalAinact decreases them.

3) Similarly, on page 15 the authors state: "RalACA expression increased levels of Sec5 in spines, whereas RalAinact and RalACA Exo expression did not". To prove this they need to include appropriate controls (such as co-transfection of GFP with an empty vector), especially since HA-tagged Sec5 appears to partially localize in spines also in control conditions (Fig. 8E, bottom left panel) and because in Fig. 8F-H the control values for Sec5 localization are in a different range compared to those presented for the RalA constructs in Fig. 8B-D, which could potentially undermine the validity of their conclusions.

Minor point:

The label in Fig. 3G should read ralG0501, not ralG050

2nd Revision - authors' response 23 May 2013

Referee #1

The revised manuscript by Teodoro et al has significantly improved. I thank the authors for the clear explanations to my questions and for their willingness to include the new data pieces that in my view were essential to support the claims. I am happy to recommend the paper for publication but hope that the 3 points below can be dealt with in the final version of the manuscript:

-I am fine with the explanation on ral antibody specificity and that it can only be detected upon ral overexpression but I would like the authors to include a statement to that effect in the results section so this is made clear to the reader and colleagues wishing to use this tool in the future.

We have now included a sentence in the Results section saying that we could not specifically discern endogenous dRal staining with our antibody.

-I understand that the 'weaker' stimuli to increase activity only result in a trend for altered levels of Synd or GluRIIB. Nonetheless I believe the authors should include these data in the text of the results section for completeness.

As requested, we included a summary of these data in the text of the results section.

-I agree with the authors that a complete electrophysiological analysis of the SSR in synaptic transmission would be useful and that this is not the point of this paper. The authors are however calculating quantal content based on non-linear excitatory junctional potentials (fig S2A). For quantal content to be properly measured TEVC recordings are needed. I suggest to either remove the QC calculation or include simple TEVC recordings to assess the QC properly.

We have removed the panel from the figure because we do not think it a major point. However, for the record, while we agree that TEVC is the best way to analyse quantal content, a great many papers on this preparation have used single electrode current clamp and corrected for non-linearity of summation. In saline that supports a moderate rate of release, the method works rather well. We chose to use this method rather than voltage clamp so that we could see any changes in active membrane properties that might correlate with the change in SSR.

Referee #2 (General Remarks):

In this revised manuscript, Teodoro et al have satisfactorily addressed a series of points raised by different reviewers by adding a considerable amount of data, which have improved and completed their work. The experimental approaches in Drosophila are elegant, solid and very convincing. I only have some concerns regarding some additional results obtained in hippocampal cultures, which in my opinion should be addressed to conclusively prove the validity of the authors' interpretation.

Specifically:
1) Figure 7A shows images of dendrites apparently expressing GFP and YFP-PSD95. However, the legend mentions that neurons were transfected with DsRed instead of GFP (consistent with the description in Extended Experimental Procedures). The images in Figure 7A are slightly cropped versions of the pictures shown in the original version of the manuscript (former Figure 7A), but now the red channel corresponding to DsRed has been changed to green for GFP. Can the authors please clarify this.

This was just a labelling error we have now corrected. The images are exactly the same that were used in the first version of the manuscript; they were simply pseudo-coloured to be colour-blind friendly. We also decided to keep the dendrite label green throughout all the manuscript images, for consistency. Both the legend and the methods are correct, only the figure label was wrong.

2) The authors state that "The increase in PSD95-positive spines after expression of RalACA relative to RalAinact is also present when endogenous PSD95 rather than the YFP-tagged transgene is used to identify those spines" (page 15 second line, related to Fig. S10). However, they should include a control in their experiments (for example, co-transfection of GFP with an empty vector or wild-type RalA) to conclusively prove that RalACA increases the amount of endogenous PSD95-containing spines, similarly to what they have done in the case of co-transfection experiments with YFP-PSD95 (Fig. 7B-D). If one compares the results shown in Fig. S10B with the values of the

control condition shown in Fig. 7B (transfection of DsRed plus YFP-PSD95) RalACA does not increase protrusion density/µm, while RalAinact decreases them.

We thank the reviewer for such a thorough reading of our manuscript, but in this matter we respectfully disagree. The observation that appears to have raised the specter of doubt in the reviewers mind is that the absolute values for protrusion density/um of dendrite are different between Figure 7 and Supp. Fig 10. But this is hardly surprising; spine density is very dependent on the culture conditions and density of neurons. Within each experimental data set, the different transgenes were always compared in parallel in cultures prepared in tandem. The data sets that make up Fig 7 and Supp Figure 10, on the other hand, were not done in at the same time. Thus, while the absolute values of protrusion density differ, the fold change seen upon expression of RalCA is the same whether we were using the standard approach of a PSD95 transgene (Figure 7) or immunostaining for endogenous PSD95 (Supp Fig 10) as requested by the reviewer. Moreover, as demonstrated in Figure 7, a control expressing GFP alone was identical to the controls expressing either RalA inact or RalA CA delta exo. To make this point more clear in the manuscript, we changed our statistical test from and "ANOVA comparing all the groups to control" to an "ANOVA comparing all the groups to each other". Thus it is clear that the change in PSD95 spines is due to Ral activity. Indeed, the inactive Ral is arguably a superior control to GFP alone as it is equivalent to RalCA in everyway except two point mutations that render it inert in our pathway.

3) Similarly, on page 15 the authors state: "RalACA expression increased levels of Sec5 in spines, whereas RalAinact and RalACAΔExo expression did not". To prove this they need to include appropriate controls (such as co-transfection of GFP with an empty vector), especially since HA-tagged Sec5 appears to partially localize in spines also in control conditions (Fig. 8E, bottom left panel) and because in Fig. 8F-H the control values for Sec5 localization are in a different range compared to those presented for the RalA constructs in Fig. 8B-D, which could potentially undermine the validity of their conclusions.

Again, we must respectfully disagree with the reviewer on this point, as explained in the response above, we think that we have included the most suitable controls and in this case they include the RalCA delta exo which is identical in all regards to RalCA except for its ability to interact with the exocyst. This is very much like the "kinase dead" control that is standard in the phosphorylation field and, together with the RalA inact, leaves no doubt the alteration in Sec5 distribution requires active RalA and an interaction of the active RalA with the exocyst complex.

With regard to the question about HA-Sec5 localization, we completely agree that some Sec5 is present in spines even in control conditions. We apologize if it wasn't clear in the main text that Sec5 is a ubiquitous protein that is also present in spines (especially when over-expressed). But this is not surprising and quite consistent with what we saw in Drosophila. As in Drosophila, the redistribution of Sec5 is not a black-to-white switch, but a quantitative enrichment. Sec5 is present at modest levels in the vicinity of the synapse at the fly NMJ and in hippocampal spines. Activation of the RalA pathway causes a quantitative increase in Sec5 in these regions relative to the rest of the cell. That is why in our experiments in Figure 8, we quantified the intensity change in the spines upon RalACA expression or after neuronal depolarization. The reviewer notes also that the range of the values on the axes differ in the different panels in Figure 8, particularly between those in which we examine the effects of RalA constructs and those in which we examine the effects of high KCl. This has a trivial explanation: these are arbitrary units of fluorescence that come from two separate experiments imaged at different times and with different gain settings and therefore cannot be directly compared to each other. Within a given experiment, we of course used cultures prepared and analysed in parallel and imaged with identical settings so that the values could be compared. However, the two unrelated protocols were not meant to be compared and necessarily involved different imaging settings. This should now be clear from the figure legend.

Minor point:

The label in Fig. 3G should read ralG0501, not ralG050

This has been corrected.