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Wnt/Ca^{2+} pathway is involved in interneuronal communication mediated by tunneling nanotubes

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

1st Editorial Decision 7th Jan 2019

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see from the comments the referees find the analysis interesting but also that some further data is needed to support the key conclusions. The referees' comments are constructive and reasonable. Given the input from the referees, I would like to invite you to submit a suitably revised manuscript that addresses the concerns raised. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the key concerns at this stage.

REFEREE REPORTS:

Referee #1:

The intercellular spreading of prion-like proteins, such as α-synuclein (α-syn), is a well-documented process in the pathogenic progression of neurodegenerative diseases. Tunnelling nanotubes (TNTs) offer a mechanism by which vesicles and α-syn aggregates can be transferred between cells and neurons. In the manuscript "Wnt/Ca2+ pathway is involved in interneuronal communication mediated by tunnelling nanotubes", Vargas et al. identified the Wnt/Ca2+ pathway as a regulator of TNT formation and stability, with the β isoform of CaMKII as a critical effector in this process. While this work produces some convincing evidence for the role of Wnt/Ca2+ in influencing TNT formation and α-syn spreading, there are some assumptions with regards to the role of this pathway here, which need addressing.
Major comments:
The authors claim that any effect on the intercellular transfer of vesicles should occur only due to

alterations in TNT formation and not filopodia, as filopodia are closed, while TNTs are open-ended connections. This assumption may not hold true, as there is increasing evidence that filopodia are capable of transporting cargo between cells, including membranous structures such as exosomes.
Furthermore, as stated in the introduction, filopodia and TNTs are primarily regulated by the same molecular players: the authors focus on the Wnt/Ca2+ pathway in regulating TNTs, claiming that this pathway has been "shown to have a role in actin remodelling, thereby controlling filopodial formation in neurons". However, the Wnt/JNK (aka Wnt/PCP) pathway has also been shown to control filopodial dynamics in neurons.
Furthermore, one of the main components of the Wnt/Ca2+ pathway is the receptor tyrosine kinase Ror2. As mentioned in the discussion, Ror2 has been shown to influence filopodia formation and somehow Wnt transport. Therefore, a more extensive analysis differentiating TNTs from filopodia and their activation by the Wnt/Ca2+ pathway; e.g. assessing levels or activation of other components of the pathway, would be beneficial.
Throughout the paper, the authors use the percentage (%) of TNT-connected cells as a measure of TNT formation. However, through time-lapse imaging, the authors also report that, e.g. βCamKII increases the stability of TNTs and thus their lifetime. One could argue that measuring the % of TNT-connected cells is not sufficient to conclude that βCamKII increases TNT formation, as the increase in connected cells could be due to the greater persistence of pre-existing TNTs and thus increasing the appearance of TNTs at a given time point. The authors need to address this point.
Minor comments:
Fig. 1: The authors should explain how they define TNTs versus filopodia and other protrusions. How can the authors claim that they count only TNTs? One characteristic is the transport of vesicles - so do all investigated protrusions identified as TNTS transport vesicles?
The concentration of H2O2 needs stating (in the figure and the main text)
Fig. 3 To my knowledge - according to the literature, T287D should function as a dominate-negative construct; however, such an effect is not observed. Is this CAD specific?
Figure 4 - The time-lapse images are 10 mins apart. This could lead to an error in calculating the lifetime of TNTs. More frequent imaging would be preferable.
In Figure 4B, only 6 to 10 TNTs were averaged per condition. The sample size needs to be increased.
In the image of Fig. 4C, CaMKII-EGFP seems to cluster in the cytoplasm. Are these vesicular structures, protein clusters, etc.? Does endogenous CaMKII behave/localize similarly? How is the interaction between the actin-cortex and CaMKII regulated?
Figure 5 - In the previous figures, WT CaMKII activates the number of TNTs significantly. However, in Fig 5A-C CaMKII does not influence the ration of F-/G-Actin. This is counter-intuitive. The authors need to explain this finding. Why is in this setting Wnt7a required to form more TNTs whereas in the previous experiments it is not.
The author needs to provide a control experiment showing that Wnt7a function in influencing F-/G-Actin ratio depends on CaMKII function - this should be easily done as the authors have non-functional mutant forms of CaMKII.
The images in 5A are rather small and difficult to see.
Figure 6: The authors find that in primary cortical neurons Wnt5a is the activator of TNTs and Wnt7a has a minor role instead. However, in previous experiments the author use Wnt7a. Therefore, it would be interesting to investigate if Wnt5a has a similar function in CAD cells?
If the CTG neurons were incubated with media of the DiI treated neurons neither Wnt5a nor Wnt7a has an effect. Is this due to a release of Wnt inhibitory factors from DiI neurons. The authors should comment on this finding.
Figure 8: The authors claim that Wnt5a treatment leads to an increase of a-syn fibril transport between primary neurons. However, the pictures do not show this. Especially in A & C, it is difficult to see an effect of the treatments as the images are rather small.

Referee #2:

The manuscript entitled "Wnt/Ca2+ pathway is involved in interneuronal communication mediated by tunneling nanotubes." demonstrates the evidence that wnt CaMKII/Ca pathway mediates tunneling nanotube formation/stabilization enhancing the intercellular transfer of cargo between CAD cells and primary neurons. They further show that the actin binding activity of CaMKII is important, further the CaMKII KO primary neuron decreases the fibrils transfer. This work is

important for the TNT field as most of the studies are still heavily relying on the in vitro system and completely lacking mutant mice study. This is also an important piece of work to elucidate potential therapeutic target of neurodegenerative diseases. Overall, the manuscript is logical, and the experiments are carefully done. There are a few issues that should be addressed prior to publication.

-specific major concerns;

1. Because authors treat cells with wnt7 or 5 at the ex vivo culture, it is still unclear whether wnt is biological ligand influencing the intercellular transfer or not. Is there any potential experiment can be done to inhibit endogenous wnt (or receptor)?

2. There are still possibility considerable amount of secretion-based transfer is occurring (20% vs 40% control in Figure1 D vs F.
In Fig 1 and 6, conditioned media experiments must be done more carefully. Especially, donor cells are exposed to vesicles longer for co-culture experiment as this case has extra 20hours of co-culture time. This is much longer than condition media experiment (4-hour treatment). Also, it is not clear how long donor cells were cultured before collection of the conditioned media. Even if longer treatment makes more transfer, it doesn't affect authors argument because wnt7a seems to specifically affect TNT mediated transfer (fig1 D F). However, I think more accurate comparison would be appreciated for this point. Corning Transwell (instead of using conditioned media) might be useful to clear this point.

3, TNT connection frequency was not scored in primary neuron experiments. Is there any reason?

-minor concerns;
Figure1.
If there is no connection found between recipient cell and donor, does it still mean transfer happened through TNT? How frequently these cells are attaching/detaching?

Figure2. As KN-93 inhibit the basal level of TNT connection (Fig2B) and adding wnt7a eliminate this effect. Does it mean there is the possibility of involvement of another pathway utilizing CaMKII?

Fig8. Fig8B and 8D shows huge difference in control value. What is causing the difference? I might be missing the information about these experiments.

Expanded View Figure 2
Immunoblot pictures A and B are too different. They should show these with similar exposure? Also, why one use GAPDH and the other total b-catenin for the control? 45min point in B looks positive to me.

-additional suggestions;

It could be a future publication, but it is great if they can use Parkinson model mouse system introducing CaMKII KO to see whether it can reduce the disease progression. Or KN93 treatment can delay the disease onset?

1st Revision - authors' response 7th May 2019

Thank you for reviewing our manuscript titled "Wnt/Ca^{2+} pathway is involved in interneuronal communication mediated by tunneling nanotubes" (EMBOJ-2018-101230). According to your suggestion, we have undertaken a comprehensive revision of our study, based on the reviewers' comments.

We think the reviewers provided insightful suggestions, which have contributed to improve our work. In this new version, we have performed a major revision of our manuscript and included new experiments to fully address the reviewers' questions.

Below you will find a detailed summary of the changes/additions made to the manuscript and figures, followed by our point-by-point response to the reviewers' comments (in blue). Also, the changes in the manuscript has been highlighted (in red).

List of changes:

Figure 2

A-D Data obtained using the JNK inhibitor (TAT) have been added.

E Vinculin staining images were now included.

F Quantification of vinculin puncta per cell was added.

Figure 4

A New time lapse images comprising a shorter interval of time were added.

B New quantification of TNT duration including data of 15-20 TNT per condition is shown.

D Confocal images showing endogenous βCaMKII expression in CAD cells are now included.

Figure 6

A Top: the schematic was changed to include the setting used to study secretion-based transfer of vesicles. Bottom: contrast and brightness of the images were adjusted to allow a better visualization of the small puncta inside of acceptor cells.

Figure 7

A Confocal images showing vesicle transfer in primary neurons treated with the Wnt scavenger protein, sFRP-2, have been now included.

B Vesicle transfer quantification was added.

Figure 8

A Contrast and brightness of the images were adjusted.

C Bigger images and of better quality are now provided.

D New quantification of α-syn transfer is shown.

E Contrast and brightness of the images were adjusted.

Figure EV1

B Confocal images of CAD cells treated with Wnt5a and Wnt7a are now included.

C Quantification of the % of TNT-connected cells was added.

D Former Fig EV2A.

E Active β-catenin blot is coming from former Fig EV2B. A new pS9-GSK3β blot and the loading control GAPDH blot, are now added.

F Former Fig EV2C.

G Former Fig EV2D.

Figure EV2

A, B Former Fig EV3.

Figure EV3

A, C Former Fig EV4.

D A G-/F-actin ratio assay for comparing the effect of βCaMKII T287D mutant against βCaMKII WT, was now added.

Figure EV4

A Confocal images showing endogenous βCaMKII expression in primary cortical neurons have been included.

B, C Former Fig EV5.

Point-by-point answers to the reviewers' comments:

Referee #1:

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molecular players: the authors focus on the Wnt/Ca2+ pathway in regulating TNTs, claiming that this pathway has been "shown to have a role in actin remodelling, thereby controlling filopodial formation in neurons". However, the Wnt/JNK (aka Wnt/PCP) pathway has also been shown to control filopodial dynamics in neurons.

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Answer:

We thank the reviewer for raising these important points, which enrich the discussion.

We have recently demonstrated that TNTs and filopodia in CAD and SH-SY5Y cells, exhibit different features at the ultrastructural level (Sartori-Rupp *et al*, 2019). By using cryo-electron microscopy (cryo-EM) and correlative FIB-SEM, we have shown that the TNT-like structures observed by fluorescent microscopy in these neuronal cell lines, in fact, correspond to open-ended connections that can transport vesicles and organelles (Sartori-Rupp *et al*, 2019). In these cell lines we do not observe cargoes being transported in classical filopodial structures (i.e. structures with close ends and attached to the substratum). Filopodia in general have not been described to actively participate in the intercellular transfer of cargoes (Delage *et al*, 2016; Dilsizoglu Senol *et al*, 2019; Gallo, 2013; Jacquemet *et al*, 2015; Mattila & Lappalainen, 2008; Sartori-Rupp *et al*, 2019), and different to what the reviewer suggested, there are few papers in the literature that present evidences involving filopodia in the transfer of cellular materials. Interestingly, the transfer mediated by filopodia seems to be restricted to melanosomes (Singh *et al*, 2010; Scott *et al*, 2002). The filopodia-like protrusion described by Singh and collaborators, as melanosome transfer-mediator, has not been yet studied at the nanometric resolution to determine whether it is a closed- or open-ended structure. Furthermore, the functionality of the structure was measured using the inhibitor Cytochalasin B (Singh *et al*, 2010), which has also been shown to affect TNTs (Bukoreshtliev *et al*, 2009). Thus, it is not possible to be sure that the intercellular transfer of melanosomes is really mediated by filopodia or by TNT-like structures. In addition, there is no evidence that supports the potential involvement of filopodia in the transfer of pathogenic aggregates, as α-synuclein.

Nonetheless, following the reviewer's suggestion, in this revised version of the manuscript we have addressed the question of whether Wnt7a through the activation of Wnt/β-catenin-independent signaling can also regulate filopodia formation in CAD cells. Therefore, we have now included the staining and quantification of vinculin puncta (Fig 2E, F). The quantification of vinculin puncta as a method to quantify filopodia has been extensively reported in the literature (He *et al*, 2017; Huang *et al*, 2017). Our lab has previously used this method to quantify attached filopodia (Delage *et al*, 2016; Zhu *et al*, 2018; Dilsizoglu Senol *et al*, 2019), and has reported that TNTs and filopodia in CAD cells, are differentially regulated by the same actin regulators (Delage *et al*, 2016). In fact, we have previously reported that the overexpression of the actin-bundling protein Eps8, can increase the formation of TNTs, while decreasing vinculin-positive filopodia (Delage *et al*, 2016). Therefore, we

took advantage of this method and quantified vinculin positive puncta in Wnt7a treated CAD cells. In the new Fig 2E, F, we show that vinculin-positive puncta significantly increase under Wnt7a treatment, and this effect is dependent on the Wnt/JNK pathway **and not on the Wnt/Ca^{2+} pathway**. Moreover, we also examined the role of the Wnt/JNK pathway in TNT formation, by using a JNK inhibitor. In contrast to the KN-93-mediated down regulation of TNTs, JNK inhibitor alone did not significantly reduce TNT formation nor TNT-mediated vesicle transfer (Fig 2A-D). These results suggest that Wnt7a-induced increase of TNTs was indeed due to the Wnt/Ca^{2+} pathway activation. Altogether, these data suggest that Wnt7a in CAD cells, can activate both Wnt/JNK and Wnt/Ca^{2+} pathway, but each pathway is specifically involved in modulating different cellular structures. This is a very interesting hypothesis that should be studied further in the future.

Here, we also ruled out that the transfer of vesicles between cells could be mediated by a secretory mechanism, such as exosome release. We performed experiments using the conditioned media of donor cells on pure acceptor cells. In the data already presented in Fig 1E, 1F and 6C, we showed that Wnt-induced increase in vesicle transfer does not seem to occur through vesicle secretion, but instead through a cell-to-cell contact-mediated mechanism, such as TNTs.

Throughout the paper, the authors use the percentage (%) of TNT-connected cells as a measure of TNT formation. However, through time-lapse imaging, the authors also report that, e.g. βCamKII increases the stability of TNTs and thus their lifetime. One could argue that measuring the % of TNT-connected cells is not sufficient to conclude that βCamKII increases TNT formation, as the increase in connected cells could be due to the greater persistence of pre-existing TNTs and thus increasing the appearance of TNTs at a given time point. The authors need to address this point.

Answer:

We would like to thank this reviewer to raise this very important point that has now been clarified in the discussion. Please see page 17, lines 13 to page 18 line 2. More precisely, we added this comment: "The increase in the stabilization of TNT could produce an increase in the number of TNT-connected cells that is not necessarily linked to *de novo* formation of TNTs, as the increase in the number of connected cells could be due to the greater persistence of pre-existing TNTs".

Indeed, we agree with the reviewer's remark on that the changes on the % of TNT-connected cells (in fix conditions) is not enough to conclude that there is an effect on TNT formation, since also changes on TNT stability could affect this %. To avoid this bias, experiments should be performed by live imaging, thus de novo formation of the tubes can be tracked. However, there are several technical limitations that make this technique unreliable to measure TNT formation. In the first place, TNTs seem to be photosensitive as they are not formed frequently when observed live, and cells exhibit a lot less TNTs than in fix conditions, which makes TNT formation a phenomenon difficult to catch by live imaging. Secondly, automated tracking of TNTs on live imaging acquisitions are more difficult to achieve than in fix conditions, since there is not a current available software that allows tracking of those structures while forming, which makes TNT counting by live imaging, a very challenging task. Here, we have used live imaging for the quantification of TNT duration, since no all connections in the micrograph need to be counted to obtain reliable results, as

for TNT counting. Moreover, the method that we have used here to identify and count TNTs is a well-established method (Abounit *et al*, 2015) that has allowed us produce consistent data on TNTs formation and function. Our lab is currently working in developing new and more reliable tools to detect and count TNTs, as FIB-SEM and cryo-EM (Sartori-Rupp *et al*, 2019), however these techniques do not allow monitoring TNT formation.

Minor comments:

Fig 1: The authors should explain how they define TNTs versus filopodia and other protrusions. How can the authors claim that they count only TNTs? One characteristic is the transport of vesicles - so do all investigated protrusions identified as TNTS transport vesicles?

Answer:

Based on the publications from our group and others (Rustom *et al*, 2004; Gousset *et al*, 2009; Delage *et al*, 2016; Hanna *et al*, 2017), we counted thin, continuous and straight connections that were hovered above the substrate (Abounit *et al*, 2015). We now included a more detailed explanation in the Materials and Methods section, clarifying the criteria used to identify TNTs (see page 20, line 19 to page 21 line 7). We are aware that the lack of a specific marker is a problem; but we have been as much rigorous as possible in our counting; and most importantly, we have always coupled our results from TNT counting to those from the transfer experiments. Moreover, by using correlative cryo-EM, we have demonstrated that practically all the protrusions we identify as TNTs by fluorescent microscopy, and using the above-mentioned criteria, are indeed open-ended structures that contain vesicles and organelles in their interior (Sartori-Rupp *et al*, 2019). As also mentioned before, filopodia in CAD cells do not contain cargoes as observed by EM.

The concentration of H2O2 needs stating (in the figure and the main text)

Answer:

Thanks, we have now stated the concentration both in the figure legends and the main text (see page 5, line 24).

Fig 3 To my knowledge - according to the literature, T287D should function as a dominate-negative construct; however, such an effect is not observed. Is this CAD specific?

Answer:

Indeed, in the literature this mutant has been shown to function as a dominant-negative construct. One of the reasons why T287D is considered as a dominant negative construct is that it leads to the phosphorylation on the residues T305/T306, which in turn result in the inhibition of the kinase activity of the molecule and therefore inducing an impairment of long-term potentiation in hippocampal neurons (Pi *et al*, 2010).

In the current study, we aimed to address how the different properties of βCaMKII (kinase activity, calmodulin-dependent activation, actin-binding property) can influence TNT formation. For this purpose we used: 1) a kinase dead mutant, which can still bind Calcium/Calmodulin, hence can be

transiently released from actin (K43R), 2) a mutant that cannot bind Calcium/Calmodulin, preventing it from becoming activated and therefore cannot be released from actin (A303R) and 3) a mutant that mimics phosphorylation at the T287 site, which prevents it from binding to actin (T287D) (Shen & Meyer, 1999). Our results with the K43R and A303R mutants sustain the hypothesis that the kinase activity is not involved in the increased TNT formation (see our data in Fig 3). From our results with the T287D mutant, we can conclude that the F-actin binding of βCaMKII is the driving force behind the increased TNT formation. Therefore, the dominant-negative effect of T287D on the kinase activity does not play role on TNTs.

As for the specificity in CAD cells, T287D mutant has been shown to severely reduce actin-binding activity of βCaMKII also in neurons (Lin & Redmond, 2008) and in human umbilical vein endothelial cells (Khan *et al*, 2016), indicating that it is unlikely that the TNT modulation we described here is specific to CAD cells only.

Figure 4 - The time-lapse images are 10 mins apart. This could lead to an error in calculating the lifetime of TNTs. More frequent imaging would be preferable.

Answer:

Thanks for pointing this out. However, those images were acquired every 100 s, as showed in the original Movie EV1, but we selected frames from 10 min apart to show in the time-lapse images of original Fig 4A. Now, in addition, we have acquired the videos every 25 s up to 50 s interval. New videos are shown in Movie EV1 and selected frames of shorter intervals, are presented in the new Fig 4A. Quantification of TNT duration using these more frequently acquired videos gave similar results to the ones obtained with 100 s apart videos. New quantification is presented in Fig 4B.

In Figure 4B, only 6 to 10 TNTs were averaged per condition. The sample size needs to be increased.

Answer:

Although challenging now we have analyzed between 15 to 20 TNTs per condition.

In the image of Fig 4C, CaMKII-EGFP seems to cluster in the cytoplasm. Are these vesicular structures, protein clusters, etc.? Does endogenous CaMKII behave/localize similarly? How is the interaction between the actin-cortex and CaMKII regulated?

Answer:

Similar kind of clusters when overexpressing GFP-βCaMKII WT have been seen in literature before (Shen *et al*, 1998), and are thought to be protein clusters, formed by βCaMKII binding to F-actin. This is supported by the notion that overexpression of the T287D mutant (which has severely reduced actin-binding properties), does not show this clustering. We have now performed endogenous βCaMKII staining in both CAD cells (Fig 4D) and primary neurons (Fig EV4A). We found that endogenous βCaMKII in CAD cells exhibits a similar staining pattern as βCaMKII overexpression. As shown in Fig 4D, endogenous βCaMKII in CAD cells, seems to form clusters all

over the cytoplasm, although less clear than in the overexpression condition. This could be merely due to the amount of protein expressed.

Many papers have studied how the interaction between βCaMKII and F-actin is regulated (Shen *et al*, 1998; Shen & Meyer, 1999; Kim *et al*, 2015). βCaMKII is shown to bind F-actin in its inactive state. Then upon activation, Calcium/Calmodulin enters the cell, activates the CaMKII holoenzyme, resulting in T287 autophosphorylation in *trans*. Upon this autophosphorylation βCaMKII is released from the F-actin. As soon as βCaMKII gets dephosphorylated, it will again bind to F-actin. Thus, in basal conditions, βCaMKII would colocalize with F-actin, forming the kind of clusters seen in Fig 3A, 4C and 5A, C.

Figure 5 - In the previous figures, WT CaMKII activates the number of TNTs significantly. However, in Fig 5A-C CaMKII does not influence the ration of F-/G-Actin. This is counter-intuitive. The authors need to explain this finding. Why is in this setting Wnt7a required to form more TNTs whereas in the previous experiments it is not. The author needs to provide a control experiment showing that Wnt7a function in influencing F-/G-Actin ratio depends on CaMKII function - this should be easily done as the authors have non-functional mutant forms of CaMKII.

Answer:

The increase in the % of TNT-connected cells induced by the overexpression of βCaMKII WT (Fig 3A, B), could be due (at least in part) to an increase on TNT's stabilization as observed by live imaging (Fig 4A, B). This possibility is hypothesized in our model presented in Fig 9 (see Fig 9 legend). If this is the case, no significant increases on F-actin are expected to take place. When compared to non-transfected condition, overexpression of βCaMKII WT, indeed increased the G-actin ratio (Fig 5B), which could be due to its ability to sequester monomeric actin (Sanabria *et al*, 2009).

As shown below, in the absence of Wnt7a, no striking changes on G-/F-actin ratio were observed among βCaMKII WT and the different βCaMKII mutants.

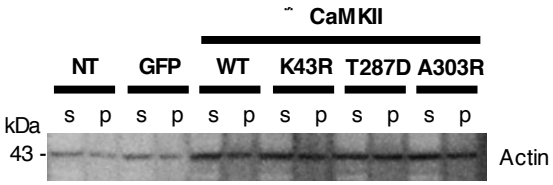

Fig 1. Anti-actin immunoblot showing changes in the ratio between G-actin (in supernatant fraction, s) and F-actin (in the pellet fraction, p) of CAD cells non-transfected (NT) or transfected with either GFP vector, GFP-βCaMKII WT or GFP- βCaMKII mutant plasmids.

We also tested the effect of Wnt7a in non-transfected CAD cells. We expected to observe a marked increase on the F-actin ratio upon Wnt7a treatment, as an increase on TNT formation will require an increase on actin polymerization events. Surprisingly, as shown in the figure below, no striking changes on G-/F-actin ratio were observed between control and Wnt7a treated cells.

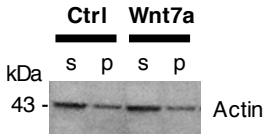

Fig 2. Anti-actin immunoblot showing changes in the ratio between G-actin (s) and F-actin (p) of CAD cells treated or not with Wnt7a ligand.

Thus, in order to see more evident changes on G-/F-actin ratio, we decided to potentiate the system by overexpressing βCaMKII WT and then exposing these cells to Wnt7a (Fig 5). The data shown in Fig 5B suggest that βCaMKII WT does not influences *de novo* formation of TNTs, as the levels of F-actin were reduced in comparison to control. Instead, Wnt7a, which induced an increase on F-actin levels of βCaMKII WT-overexpressing cells, is most probably influencing TNT number by increasing its *de novo* formation. Thus, an increase in the % of TNT-connected cells could be because: 1) an increase in the de novo formation of TNTs (the most probable effect of Wnt7a), or 2) an increase in the stability of pre-existent TNTs (the most probable effect of βCaMKII WT and mutants in which the actin-binding activity is not affected).

A control experiment showing that Wnt7a-induced effect on the G-/F-actin ratio depends on the actin-binding activity of βCaMKII, has now been included in Fig EV3D. For this, we treated βCaMKII T287D-expressing CAD cells with or without Wnt7a and demonstrated that the G-/F-actin ratio was not significantly altered.

The images in 5A are rather small and difficult to see.

Answer:

We now have increased the size of the micrographs to allow a better visibility.

Figure 6: The authors find that in primary cortical neurons Wnt5a is the activator of TNTs and Wnt7a has a minor role instead. However, in previous experiments the author use Wnt7a. Therefore, it would be interesting to investigate if Wnt5a has a similar function in CAD cells?

Answer:

Based on the literature, Wnt5a is mainly activating the Wnt/Ca^{2+} pathway in the rodent hippocampal and cortical neurons, while Wnt7a is known to activate Wnt/β-catenin pathway in these cells (Hirabayashi *et al*, 2004; Zhou *et al*, 2017). For this reason, we used Wnt5a instead of Wnt7a in the experiments for the primary cortical neurons.

We tested both Wnt5a and Wnt7a ligands in CAD cells. We found that both ligands significantly increase TNT in these cells, however Wnt7a produced a stronger effect (Fig EV1B, C) and therefore we preferred Wnt7a over Wnt5a for CAD experiments.

If the CTG neurons were incubated with media of the DiI treated neurons neither Wnt5a nor Wnt7a has an effect. Is this due to a release of Wnt inhibitory factors from DiI neurons. The authors should comment on this finding.

Answer:

Indeed, it is possible that endogenous Wnt inhibitory factors are being released to the culture medium. However, if those factors are released, they could be equally affecting co-cultured and conditioned medium-treated cells. Since CTG neurons (acceptors) in both experimental conditions were exposed for the same amount of time to either donor neurons or the conditioned medium of donor neurons, any inhibitory factor released to the medium would affect DiI transfer in the same way, in both settings.

Figure 8: The authors claim that Wnt5a treatment leads to an increase of a-syn fibril transport between primary neurons. However, the pictures do not show this. Especially in A & C, it is difficult to see an effect of the treatments as the images are rather small.

Answer:

Thanks. The quality of the images has now been improved and bigger images for Fig 8C were incorporated to allow a better observation of the puncta in the cells.

Referee #2:

The manuscript entitled "Wnt/Ca2+ pathway is involved in interneuronal communication mediated by tunneling nanotubes." demonstrates the evidence that wnt CaMKII/Ca pathway mediates tunneling nanotube formation/stabilization enhancing the intercellular transfer of cargo between CAD cells and primary neurons. They further show that the actin binding activity of CaMKII is important, further the CaMKII KO primary neuron decreases the fibrils transfer. This work is important for the TNT field as most of the studies are still heavily relying on the in vitro system and completely lacking mutant mice study. This is also an important piece of work to elucidate potential therapeutic target of neurodegenerative diseases. Overall, the manuscript is logical, and the experiments are carefully done. There are a few issues that should be addressed prior to publication.

-specific major concerns;

1. Because authors treat cells with wnt7 or 5 at the ex vivo culture, it is still unclear whether wnt is biological ligand influencing the intercellular transfer or not. Is there any potential experiment can be done to inhibit endogenous wnt (or receptor)?

Answer:

We greatly appreciate the reviewer for his/her insightful comments.

To tackle this important point, we have now performed experiments using sFRP-2, a Wnt ligand scavenger, and examined its effect on vesicle transfer in primary cortical neurons. The data presented in Fig 7A, B, showed that in the presence of sFRP-2, Wnt5 failed to induce DiI transfer between primary cortical neurons. Moreover, we observed a significant decrease of DiI transfer when using only sFRP-2 compared to the control. This suggest that endogenous Wnt pathway could play a role in the interneuronal transfer of vesicles.

2. There are still possibility considerable amount of secretion-based transfer is occurring (20% vs 40% control in Figure1 D vs F.

In Fig 1 and 6, conditioned media experiments must be done more carefully. Especially, donor cells are exposed to vesicles longer for co-culture experiment as this case has extra 20hours of co-culture time. This is much longer than condition media experiment (4-hour treatment). Also, it is not clear how long donor cells were cultured before collection of the conditioned media. Even if longer treatment makes more transfer, it doesn't affect authors argument because wnt7a seems to specifically affect TNT mediated transfer (fig1 D F). However, I think more accurate comparison would be appreciated for this point. Corning Transwell (instead of using conditioned media) might be useful to clear this point.

Answer:

Although it is true that we detected a considerable amount of vesicle transfer taking place in CAD cells and in primary neurons, via a secretion-based mechanism (close to 20 % and 10 %, respectively), we did not find that any assayed Wnt ligand, had an effect on this type of transfer. Instead, we found that Wnt treatment specifically influenced a cell-to-cell contact-mediated mechanism of vesicle transfer (Fig 1C-F and 6A-C). Besides, co-culture and conditioned medium experiments were performed using similar conditions. We apologize for the misunderstanding and we have modified the text to be clearer (please see page 12 lines 23-24 and the legend of Fig 6A). We have also included a new schematic in Fig. 6A to show the settings used for co-culture and conditioned medium experiments. The transfer experiments in CADs were performed for 4 h, while in neurons, experiments were performed for 24 h, because these cells attach and develop more slowly than CADs. Since we do not compare the effect of Wnt ligands between both cell types (CADs and neurons), but the mechanisms taking place in each cell type, co-culture and conditioned medium experiments were performed for the same amount of time on each cell type. Therefore, acceptor cells in both experimental conditions were exposed with a similar duration to the "DiI-containing medium" than co-cultured cells. On the other hand, donor cells were exposed for 4 h to treatments. We did not performed transfer experiments using Transwell inserts, although we had already used this approach with CAD cells (Abounit *et al*, 2016), because primary neurons do not grow well on those inserts, and this can cause cellular death, which could in turn affect the results of the transfer.

3. TNT connection frequency was not scored in primary neuron experiments. Is there any reason?

Answer:

Neurons display complicated axonal and dendritic networks. Since there is no specific marker for TNTs by far, it is difficult to accurately count TNTs in primary neurons. For these reasons, we preferred to evaluate the effect of Wnts on TNTs in primary neurons by assessing the function of TNTs.

-minor concerns;

Figure1.

If there is no connection found between recipient cell and donor, does it still mean transfer happened through TNT? How frequently these cells are attaching/detaching?

Answer:

TNTs are fragile structures that are easily broken by chemical and mechanical forces, such as fixation. Therefore, even if some images showed no TNT connections, it could be due to the breakdown of TNTs during the experimental procedures.

In the Fig 4B, we have determined that the lasting duration of a TNT is about 10 min in basal conditions.

Figure2. As KN-93 inhibit the basal level of TNT connection (Fig2B) and adding wnt7a eliminate this effect. Does it mean there is the possibility of involvement of another pathway utilizing CaMKII?

Answer:

It is possible that in CAD cells, like in neurons, there is a basal level of βCaMKII activity, as we could observe phosphorylated CaMKII by WB (Fig EV2). This can explain that KN-93 alone could decrease the basal level of TNT connections, while by adding Wnt7a and KN-93, we balanced the TNT formation.

Fig8. Fig8B and 8D shows huge difference in control value. What is causing the difference? I might be missing the information about these experiments.

Answer:

We thank the reviewer for pointing out this issue. The differences in the results were due to the fact that these experiments were evaluated by independent people using different thresholds. We have now unified the evaluation and obtained similar control values between different experiments and new evaluated results are displayed in Fig 8B and 8D.

Expanded View Figure 2. Immunoblot pictures A and B are too different. They should show these with similar exposure? Also, why one use GAPDH and the other total b-catenin for the control? 45min point in B looks positive to me.

Answer:

Immunoblot images were acquired using similar exposure time, as evidenced by comparing the GAPDH blots in Fig EV1D and E. The difference in the intensity of the β-catenin bands is due to the different exposure times to Wnt7a used in the experiment in former Fig EV2A (4 h exposure) and EV2B (a time course ranging from 5 min to 1 h exposure), which caused stronger and weaker activation of the protein, respectively. We have now included a GSK3β blot to further strengthen our results on the short-term activation of Wnt/β-catenin pathway (Fig EV1E). We now show that 1 h treatment with LiCl slightly induce the phosphorylation of GSK3β at S9 site (an inhibitory GSK3β-phosphorylation associated with the activation of Wnt/β-catenin pathway), while no strong

phosphorylated GSK3β was detected upon Wnt7a treatment. In addition, we have changed total β-catenin blot for a GAPDH blot in Fig EV1E, as the same internal control than in Fig EV1D.

-additional suggestions;

It could be a future publication, but it is great if they can use Parkinson model mouse system introducing CaMKII KO to see whether it can reduce the disease progression. Or KN93 treatment can delay the disease onset?

Answer:

We greatly appreciate this comment, and it is indeed very important and interesting to address whether the disease progression could be delayed by inhibiting Wnt/Ca^{2+} pathway. We will take it into account for our future studies.

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2nd Editorial Decision 31st May 2019

Thanks for submitting your revised manuscript. I have now received the input back from the two referees on the manuscript.

As you can see from the comments below, the referees appreciate the introduced revisions, but still find that they don't fully resolved the initial raised concerns and that further experiments are needed to do so. In particular further work is needed to support that what you are seeing are TNTs and not filopodia. The remaining issues that needs to be sorted out are:

- We need more quantitative data to support the observed TNT mediated transfer of vesicles

- The number # of vesicles received in each acceptor cell needs to be quantified (ref #1)

- I also like the control experiment for Figure 1 as suggested by ref #1

- Would be good to have some data to support if Wnt is the biological relevant ligand (ref #2)

The above experiments will strengthen the findings and should be doable.

Could you also please take a careful look at the image in Figure 4C- it looks similar to EV3 A-C

REFEREE REPORTS:

Referee #1:

In general the authors should be more reluctant to call all cell protrusions TNTs. It seems to me that CAD cell form many filopodia and TNTs. Whereas there are obvious representations of TNTs in many pictures, others are less clear (for example, in Fig. 1A, C, 2A, 4a.). Therefore, I would strongly advice to name these extensions only TNTs if there is clear evidence.

Specific points:
Fig 1: It seems that in the Wnt7a experiment the donors generated a vast amount of DiD positive vesicles. Can the authors provide an experiment with equal numbers of vesicles in Ctrl donor cell and compare these to Wnt7a stimulated donor cells? Otherwise one could explain the results just by an increased number of DiD positive vesicles.

If this is not possible the author should measure the ratio of transported vesicles versus vesicles in the producing cells to give the reader a possibility of increased TNT based transport.

To complement the analysis, the authors should state how many DiD labelled vesicles were found in the acceptor cells. (Fig 1C)

Fig. 2: In F, KN-93 treatment shows a downregulation of Vinculin, however, this is not reflected in the bar chart. The authors should provide a better picture to show that the number of vinculin-positive protrusions is not altered significantly.

As states above, I would be very much interested in the amount of TNT-transported vesicles/a-syn fibrils (Fig. 6 and Fig. 8). It is informative to count the number of cells which received vesicles; however, it is even more important to give an average number of vesicles received per cell.

Referee #2:

Overall, authors added a decent amount to the study that strengthens their conclusion. They addressed basically most of reviewer's concerns, and especially the vinculin counting to distinguish effects of Wnt on filopodia vs TNTs, identifying a possible Wnt pathway specially regulating filopodia in the process. This added another interesting regulatory mechanism of different structures. One concern is still not clear whether the observed transfer was actually TNT-dependent or not. Authors added more explanation about their coculture vs conditioned media experiments in better detail in figure 6, it makes sense, but no quantitative measurement was provided to get the accurate percentage of TNT dependent vs. secretion dependent transfer. Moreover, although I understand the wnt pathway only affect TNT mediated transfer, another new experiment, inhibition of endogenous wnt pathway was still done "in vitro" setting, thus it does not fully address the question whether wnt is biological ligand or not. I would like to see more careful discussion or addition of experiment to clarify at least one of these two points.

One more minor point is that the inhibitor KN-93 was able to inhibit basal TNT formation, but adding Wnt7 seemed to rescue this. They provide an explanation to the reviewer for this but don't really mention it in their paper, which is understandable because it's a minor point but still something that could've been in the discussion.

2nd Revision - authors' response date

Thank you for reviewing our manuscript titled "Wnt/Ca^{2+} pathway is involved in interneuronal communication mediated by tunneling nanotubes" (EMBOJ-2018-101230R). Following your advice, we have made most of the changes recommended by the reviewers. All pertinent modifications were included in the manuscript and are properly highlighted in red. The detailed list of changes/additions made to the manuscript and figures are outlined below, followed by a point-by-point response to the reviewers' comments (in blue). We consider that the reviewers' comments

were very important to help us reinforce the conclusions drawn by our work and we hope that this version fully addresses their questions.

List of changes:

- Corrected color contrast in Fig 1A, Fig 2A, and in upper and lower panels of Fig 4A in order to make TNTs more visible (see explanation below)
- Increased color intensity in Fig 2E
- In Fig EV3 A, B and C, images were labelled as CODIM or Confocal
- Quantifications of the number of transferred vesicles/α-syn puncta added in new Figs EV1D and EV4D-F
- New paragraphs inserted (in red), mainly in results and discussion sections

Point-by-point answers to the reviewers' comments:

Referee #1:
In general the authors should be more reluctant to call all cell protrusions TNTs. It seems to me that CAD cell form many filopodia and TNTs. Whereas there are obvious representations of TNTs in many pictures, others are less clear (for example, in Fig. 1A, C, 2A, 4a.). Therefore, I would strongly advice to name these extensions only TNTs if there is clear evidence.

Answer:
We agree with this reviewer that caution should be taken when using the term TNT, and that is why, in the case of connections seen in primary neurons, we are using the term TNT-like connections instead of TNTs (for example, see page 4, line 22). However, having fully characterized these structures in CAD cells [1-7], we are more confident when it comes to identify TNTs in this cell line.
The representative images presented in the manuscript concerning TNTs, are projections of the upper slices of each confocal Z-stack and may show some filopodia. However, for TNT counting we do not use projected images, but the full Z-stacks. To unequivocally identify these structures, in all the experiments where TNTs were quantified, we manually examined the entire Z-stack and only counted those connections that completely fitted the defined TNT criteria (please refer to page 21, line 14 until page 22, line 4). We applied the same criteria previously used in several works from the laboratory [1-7], which consider that TNTs should fulfill the three following conditions:
i) Protrusions should be hovering above the substratum, therefore protrusions that were below the first 3-4 slices of the Z-stacks were excluded from the analysis, as they were considered as filopodia.
ii) All protrusions should be thinner than 1 µm.
iii) They should be continuous projections, clearly starting from one cell and uninterruptedly continuing towards the other cell, forming a "bridge".
Before TNT counting, all images have been pre-processed, removing the first slices from the Z-stack containing attached filopodia (as observed in Fig 2E). In addition, all our TNT counting data has been accompanied by functional data i.e. transfer experiments. Thus, we are confident that what we are counting and calling TNTs are indeed those structures. This information is detailed in the Materials & Methods section, and we also included the following sentence in the results section (Page 5, lines 21-22); "for detailed criteria used to identify and count TNTs, please refer to the Materials and Methods section". In addition, to make TNTs of Fig 1A, 2A and 4A more visible (specific cases mentioned by this reviewer), we increased the contrast of the photomicrographs. Images of Fig 1C were not modified because they are mainly showing vesicle transfer from donor to acceptor cells.

Specific points:

Fig 1: It seems that in the Wnt7a experiment the donors generated a vast amount of DiD positive vesicles. Can the authors provide an experiment with equal numbers of vesicles in Ctrl donor cell and compare these to Wnt7a stimulated donor cells? Otherwise one could explain the results just by an increased number of DiD positive vesicles. If this is not possible the author should measure the ratio of transported vesicles versus vesicles in the producing cells to give the reader a possibility of increased TNT based transport.

Answer:
We appreciate the reviewer's remark, unfortunately we cannot perform the experiment that he/she is proposing. Because DiD is a lipophilic dye that stains cell membranes and vesicles by diffusion, we cannot control the number of intracellular vesicles that are labelled in the donor cells. To perform the transfer experiments in CAD cells, DiD-labeled donor cells were detached and mixed in suspension with the acceptor cells and then, equal cell numbers were plated and assigned to different experimental conditions, that is, donor cells within each experiment were derived from the same pool of donor cells, i.e. they were exposed for the same period of time, to the same DiD concentration. Therefore, we consider that we have properly controlled this variable.

To address the reviewer's concern that Wnt7a might increase vesicles in donor cells, we re-analyzed the experiments of Fig 1C and checked whether the number of vesicles is significantly different between conditions in donor cells, and we did not find significant differences. In Fig 1 of this response, we show the raw files of the 3 experimental conditions of the experiment. The upper panels of the figure show the expanded fields of the images presented in Fig 1C of the manuscript (yellow squares). To better illustrate transferred vesicles in acceptor cells, we selected the areas of the images and the slices of the z-stack (selected projection) for projection.
In contrast, the bottom panels show the whole stack projection of the expanded fields, i.e. projections of the entire Z-stacks acquired, which covers the whole body of the donor cell (cell inside the yellow dashed circle, upper panels) without focusing on the acceptor cells. In this case it is possible to appreciate all the DiD labeled vesicles contained in the donor cells in the different experimental conditions. As evidenced in the whole stack projection, there are no striking differences in the number of DiD-labeled vesicles contained in donor cells among the different treatments. Therefore, the apparent difference observed in Fig. 1C of the manuscript comes from the fact that different slices and regions of interest were considered for making the projection corresponding to control, H_2O_2 and Wnt7a treatments.

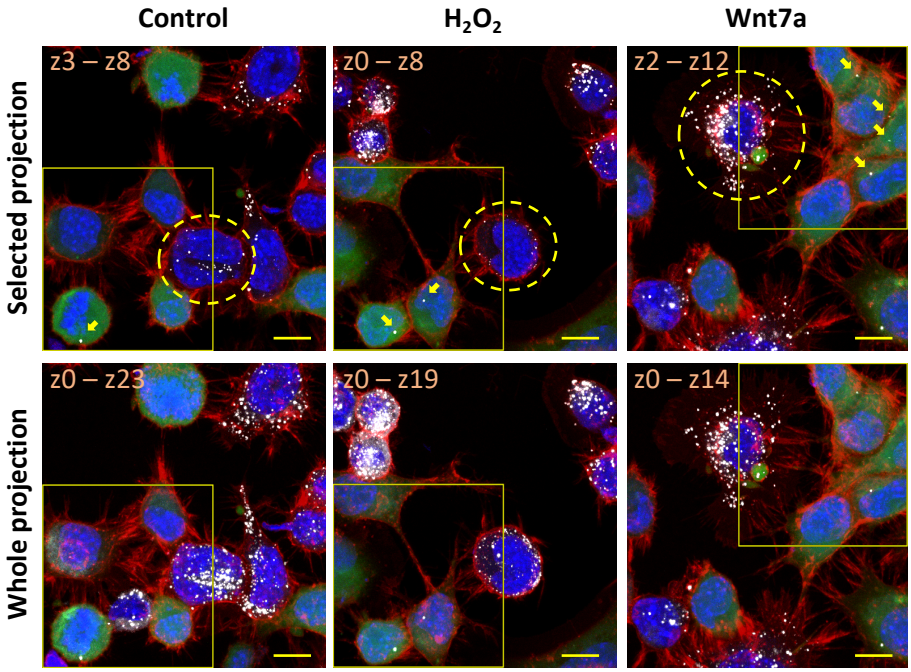

Fig 1. Expanded field of the images showed in Fig. 1C of the manuscript. The areas inside the yellow squares correspond to the images used in the figure, and the yellow arrows point to acceptor cells positive for DiD. The bottom panels show projections of all the slices acquired in the Z-stack, covering the whole body of the donor cell, contained within the yellow dashed circle of the upper panels. The number of Z-stacks considered in each case for the projections are indicated at the top left of each photomicrograph. Red is WGA, green is CTG, blue is DAPI and white is DiD. Scale bars represent 10 µm.

To complement the analysis, the authors should state how many DiD labelled vesicles were found in the acceptor cells. (Fig 1C).

Answer:

To automatically quantify the number of DiD-labelled puncta per acceptor cell in the experiments presented in Fig 1C, we used the spot detector tool of the ICY software. The results of this analysis are presented both in Fig 2 of this letter and in Fig EV1D in the manuscript. On average, we found 5.2, 4 and 5.1 puncta within acceptor cells in Control, H_2O_2 and Wnt7a conditions, respectively, and no significant differences were found between experimental conditions in the number of DiD vesicles received by acceptor cells. These results are discussed in page 6, lines 15-17.

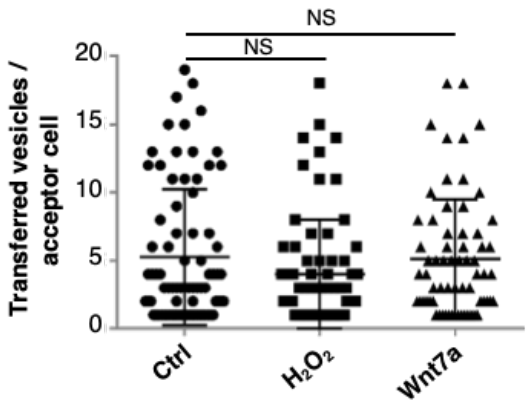

Fig 2. Quantification of the number of DiD-positive vesicles in CTG acceptor cells (CADs) after 4 h of treatment with H_2O_2 or Wnt7a. NS= not significant.

Fig. 2: In F, KN-93 treatment shows a downregulation of Vinculin, however, this is not reflected in the bar chart. The authors should provide a better picture to show that the number of vinculin-positive protrusions is not altered significantly.

Answer:
We appreciate the reviewer's remark. The results of Fig 2F show quantitative data from 3 independent experiments where KN-93 treatment did not change significantly the number of Vinculin puncta per cell. We realized that the apparent difference noticed by the referee was due to differences in the fluorescence intensity of the images shown in Fig 2E. As you could see in the images we provide now, the vinculin puncta are easily distinguished in all conditions (Fig 2E), and there is no difference in the number of puncta between control and KN-93 treatment (Fig 2F).

As states above, I would be very much interested in the amount of TNT-transported vesicles/a-syn fibrils (Fig. 6 and Fig. 8). It is informative to count the number of cells which received vesicles; however, it is even more important to give an average number of vesicles received per cell.

Answer:
Following the suggestion of this reviewer the number of DiI vesicles/α-syn puncta received per cell were counted using ImageJ. The results are shown both in Fig 3 below and in the new Fig EV4D, E and F, which correspond to the experiments of Fig 6A, Fig 8A, and Fig 8E, respectively. As shown in the graphs, on average, all acceptors cells contained ~2 puncta/cell, and we did not find significant differences in the number of received vesicles/α-syn puncta among the experimental conditions. These results are now included in the text in page 13, lines 1-3, and also in page 15 lines 9-10, 23-24 and line 1 of page 16.

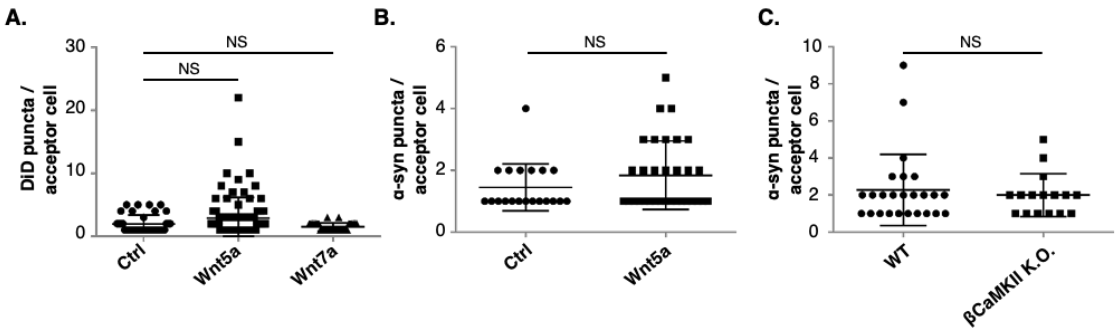

Fig 3. A) Number of DiD-positive vesicles in CTG acceptor neurons after 4 h of treatment with Wnt5a or Wnt7a (2, 2.9 and 1.5, respectively). B) Number of α-syn puncta in acceptor neurons in control conditions and after Wnt5a treatment (1.5 and 1.8, respectively). C) Number of α-syn puncta in CTG-positive acceptor neurons from wild type or βCaMKII K.O. mice (2.3 and 2, respectively). NS= not significant.

Referee #2:

Overall, authors added a decent amount to the study that strengthens their conclusion. They addressed basically most of reviewer's concerns, and especially the vinculin counting to distinguish effects of Wnt on filopodia vs TNTs, identifying a possible Wnt pathway specially regulating filopodia in the process. This added another interesting regulatory mechanism of different structures.

One concern is still not clear whether the observed transfer was actually TNT-dependent or not. Authors added more explanation about their coculture vs conditioned media experiments in better detail in figure 6, it makes sense, but no quantitative measurement was provided to get the accurate percentage of TNT dependent vs. secretion dependent transfer. Moreover, although I understand the wnt pathway only affect TNT mediated transfer, another new experiment, inhibition of endogenous

wnt pathway was still done "in vitro" setting, thus it does not fully address the question whether wnt is biological ligand or not. I would like to see more careful discussion or addition of experiment to clarify at least one of these two points.

Answer:
We are grateful with the reviewer for these comments. As we have previously explained in the first reply, due to the lack of a specific marker of TNTs, we cannot give a quantitative measurement of the number of TNTs in neurons to correlate with cell-cell contact dependent transfer. This is why we usually perform secretion experiments in parallel with co-cultures at least to exclude an effect of transfer mediated by secretion. In a previous study from our lab, we have compared the percentage of α-syn transfer that was dependent on cell-cell contact vs cells treated with conditioned medium and cultures separated by a filter in CAD cells, and found a significant decrease in transfer when cell-cell contact was impaired. Similar results were obtained with primary neurons that were cultured with conditioned media or that were cultured in the same dish but on different coverslips, separating donor/acceptor populations [6]. To obtain the ratio of transfer between cell-cell contact vs secretion in the current study, we quantitatively analyzed the data shown in Fig 6B and C, and the values are: control (80% vs. 20%), Wnt5a (92% vs. 8%) and Wnt7a (80% vs. 20%). These values were now added in page 13, lines 7-8.

Regarding the second point raised by the reviewer concerning the *in vitro* inhibition of the endogenous Wnt pathway, we agreed that we should have discussed this better and therefore added a more detailed explanations about this issue in the Results and Discussion sections of the manuscript. The following sentences were added in page 13, lines 16-24 of the results section: "Our results show that the increase on vesicle transfer induced by Wnt5a was completely abolished in the presence of sFRP-2 (Fig 7A, B). More importantly, the treatment with sFRP-2 alone resulted in a small but significant reduction of vesicle transfer in comparison to control (Fig 7B), suggesting that the activity of the endogenous Wnt pathway is required for a small proportion of basal vesicle transfer to take place in neurons. This small effect could be explained in part that sFRP-2, which is known to sequester several Wnt ligands, including Wnt5a (Galli *et al*, 2006; Godoy *et al*, 2014; Wolf *et al*, 2008), does not bind all Wnt ligands. Therefore, the small reduction on vesicle transfer observed in the presence of sFRP-2 alone could be caused by a compensatory effect of unbound endogenous Wnt ligands".

We also added the following paragraphs in Discussion, page 16, lines 19-23: "We show that in our *in vitro* conditions, the activation of Wnt/Ca^{2+} pathway (by Wnt7a in CAD cells or by Wnt5a in cortical neurons), is involved in the establishment of TNTs and that βCaMKII plays a key role in this event. We also show that by modulating this pathway, the intercellular spreading of α-syn fibrils can be affected. Whether this mechanism plays a role in the brain during development and/or in the case of α-synucleinopathies, remains to be studied".

One more minor point is that the inhibitor KN-93 was able to inhibit basal TNT formation, but adding Wnt7 seemed to rescue this. They provide an explanation to the reviewer for this but don't really mention it in their paper, which is understandable because it's a minor point but still something that could've been in the discussion.

Answer:
We agree with the reviewer that this is an important point and thank him/her for bringing it up. This information now appears on page 19, lines 16-19, of the discussion: "Both, TNT formation and TNT-meditated vesicle transfer can be blocked by the CaMKII inhibitor, KN-93. The decrease on basal TNT formation induced by KN-93 exposure, suggests that there is a basal level of βCaMKII activity, which is required for the establishment of TNT connections in control conditions".

References

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2. Delage E, Cervantes DC, Pénard E, Schmitt C, Syan S, Disanza A, Scita G & Zurzolo C (2016) Differential identity of Filopodia and Tunneling Nanotubes revealed by the opposite functions of actin regulatory complexes. Sci Rep **6**
3. Dilsizoglu Senol A, Pepe A, Grudina C, Sassoon N, Reiko U, Bousset L, Melki R, Piel J, Gugger M, Zurzolo C (2019) Effect of tolytoxin on tunneling nanotube formation and function. Sci Rep **9**:5741.
4. Gousset K, Schiff E, Langevin C, Marijanovic Z, Caputo A, Browman DT, Chenouard N, de Chaumont F, Martino A, Enninga J, Olivo-Marin J-C, Männel D & Zurzolo C (2009) Prions hijack tunnelling nanotubes for intercellular spread. Nat. Cell Biol. **11**: 328–336
5. Gousset K, Marzo L, Commere P-H & Zurzolo C (2013) Myo10 is a key regulator of TNT formation in neuronal cells. J. Cell. Sci. **126**: 4424–4435
6. Sartori-Rupp A, Cordero Cervantes D, Pepe A, Gousset K, Delage E, Corroyer-Dulmont S, Schmitt C, Krijnse-Locker J & Zurzolo C (2019) Correlative cryo-electron microscopy reveals the structure of TNTs in neuronal cells. Nat Commun **10**: 342
7. Zhu S, Bhat S, Syan S, Kuchitsu Y, Fukuda M & Zurzolo C (2018) Rab11a-Rab8a cascade regulates the formation of tunneling nanotubes through vesicle recycling. J. Cell. Sci. **131**

3rd Editorial Decision 22nd Aug 2019

Thank you for submitting your revised manuscript to The EMBO Journal. It has now been seen by the two referees and the comments are provided below. Both referees appreciate the introduced changes and support publication here. Referee #1 has two remains points that would be good to clarify.

REFEREE REPORTS:

Referee #1:

Basically, most of my comments have been addressed in the revised version. The definition of TNTs versus filopodia has been explained, as well. However, there are a few issues the authors need to address.

The authors use SFRP2 in Figure 2 to block Wnt5a signalling. However, in contrast to the cited paper (Godoy, et al., 2014), sFRP2 has also been shown to enhance Wnt/PCP signalling in other neurons, e.g. DA neurons (Kele, et al., 2002). Therefore, further evidence is needed to conclude that Wnt5a signalling is required for the regulation of vesicle transfer.

The pictures in Fig1c show an increased amount of vesicles in Wnt7a treated CAD cells, the author quantified the amount of DiD vesicles in the donor cells and could not find a significant difference (mention in the letter of response). The authors need to show this relevant low magnification pictures as well as the quantification - at least in the supplementary data.

The quantification of the acceptor vesicle has been performed well.

The quantification of the vinculin positive filopodia after KN-93 treatment has been explained.

Referee #2:

The revised version of the manuscript entitled "Wnt/Ca2+ pathway is involved in interneuronal communication mediated by tunneling nanotubes." demonstrates the evidence that wnt CaMKII/Ca pathway mediates tunneling nanotube formation/stabilization promoting the intercellular transfer of cargoes. Current version explains more clearly experimental details and information provided here well support author's conclusion. Authors addressed most of the concern raised by us and the other reviewer. We especially liked the new points added to discussion. And we think this version is now ready for publication.

3rd Revision - authors' response 8th Sep 2019

Thank you for reviewing our manuscript (EMBOJ-2018-101230R1), entitled "Wnt/Ca^{2+} pathway is involved in interneuronal communication mediated by tunneling nanotubes", by Jessica Y. Vargas, Frida Loria, Yuan-Ju Wu, Gonzalo Cordova, Takashi Nonaka, Sebastien Bellow, Sylvie Syan, Masato Hasegawa, Geeske M. van Woerden, Capucine Trollet and myself.

In this revised version, we have addressed the remaining two points of Reviewer 1 and added all the information that you have requested to us. Specifically:

1. We included and discussed the reference that Reviewer 1 mentioned on the use of sFRP-2, at page 13, line 24, until page 14, line 3.
2. We have also included, as per his/her request, the expanded fields of the images provided in Figure 1C, showing that there are not striking differences in the number of puncta per donor cell among the different experimental conditions. Please see new Figure 1C and the corresponding legend (page 35, line 27, until page 36, line 3).
3. We have added a clarification in the legend of Figure EV3 A-C to relate the images with those on Figure 4C. Please see page 44, lines 15-16.

4th Editorial Decision 12th Sep 2019

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at it and all looks good. I am therefore very happy to accept the manuscript for publication here.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Chiara Zurzolo
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-101230

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All experiments were repeated at least three times. We analyzed for each experiment at least 100 cells in order to have statistically relevant analysis and to identify trends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	The number of independent experiments performed or the number of cells analyzed per condition, as well as the statistical test used in each case (including definition of significance) is indicated in the figure legend of each figure. Also, a brief description of the statistical analysis employed can be found in Materials and Methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analysis is described in the Materials and Methods section of the manuscript. We used Student's t-test when comparing two gropus with normal distribution, and the statistical relevance was obtained by calculating the p value using the paired one-tail Student's t-test. For comparisons between more than two groups, we used one-way ANOVA with Tukey's post hoc analysis. We presented the bar graphs showed in the figures as mean ± SEM. All statistical analyses were done using GraphPad Prism version 6 software.
Is there an estimate of variation within each group of data?	For each group, we have shown the standard error of the mean.
Is the variance similar between the groups that are being statistically compared?	Statistical analysis was performed using the GraphPad Prism version 6 software. For Student`s t-test the software calculates the homogeneity of variances by means of the F-test. Since F-test for homogeneity of variances was non-significant, there was no need to change the test for a non-parametric one. For the one-way ANOVA, the software assumes that all the populations have the same standard deviation (and thus the same variance). This assumption is not very important since all the groups have the same (or almost the same) number of samples.

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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Main antibodies used are described in the Materials and Methods section. For WB we used rabbit anti-β-Catenin, Cell Signaling Technologies Cat. #8814; rabbit anti-(pan)-CaMKII, Cell Signaling Technologies Cat. #4436; rabbit anti-phospho-(α-β-γ)-CaMKII, Cell Signaling Technologies Cat. #12716; rabbit anti-SAPK/JNK, Cell Signaling Technologies Cat. #9252, rabbit anti-phospho-SAPK/JNK, Cell Signaling Technologies Cat. #4668; rabbit anti-phospho-GSK3β, Cell Signaling Technologies Cat. #5558 and rabbit anti-GAPDH, Boster Cat. #M00227-1. For IF we used mouse anti-vinculin, Sigma Cat. #V9264; mouse monoclonal anti-βCaMKII CB-beta-1, ThermoFisher Cat. #13-9800; rabbit anti-MAP-2, Sigma Aldrich Cat. #M3696; mouse anti-β-III-tubulin, Sigma Aldrich Cat. #T9026.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	CAD cells were a gift from Hubert Laude (Institut National de la Recherche Agronomique, Jouy-en-Josas, France) and were tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We did't use any animal models for the study. However, we worked with primary neurons isolated from wild type (C57BL/6) and knock out (homozygous beta-CaMKII -/- exon 2) mouse (Mus musculus) embryos (embrionic day 17). C57BL/6 wild type mice were obtained from an in-house colony at Institut Pasteur (Paris, France). beta-CaMKII K.O. mice were generated and maintained at the facilities of Erasmus Medical Center (Rotterdam, The Netherlands). This is described in the Materials and Methods section, subsections: Animals and Primary neuronal cultures. Briefly, all animals were housed in cages with filter tops in a ventilated rack and maintained on food and water ad libitum. Handling of animals was performed in compliance with the guidelines of animal care set by the European Union and approved by the Ethics Committees of Institut Pasteur and Erasmus Medical Center.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No