Pericentrosomal targeting of Rab6 secretory vesicles by Bicaudal-D related protein-1 (BICDR-1) regulates neuritogenesis

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Thank you for submitting your research manuscript for consideration to The EMBO Journal.

As you will recognize from the enclosed reports all three referees indicate an interest in your study. However, careful reading of their very detailed and considered comments also reveals that significant further experimental support would be needed to substantiate the functional proposal for BICDR1/2. This would have to include functional validation also in professional secretory cells (ref#1), further detailed elaboration on the actual mechanism of BICDR1 to regulate trafficking (ref#2) and a rather extensive list on necessary clarifications/controls that would have to close current gaps in the experimental evidence. With the already rather high density of data, the paper would therefore need more focus and re-structuring to emphasize the major novelty based on convincing results. I hope you understand that these assessments reflect the still very preliminary state of your study and can therefore NOT results in a straightforward decision. This is particularly important, as the outcome of necessary experiments might indeed change the overall message and scope of the current submission. However, all three referees recommended to at least offer you the chance to revise/redraft the paper and would be willing to assess a significantly improved version in the future. I therefore urge you to take their comments and suggestions very serious and you would have to at least aim to address all their concerns before considering submission of a revised version to our very competitive and demanding journal. I also like to add that we can easily grant additional time for extensive revisions as requested for your particular study. Finally, I also have to remind you...
that it is EMBO_J policy to allow a single round of major revisions only, and that the final decision on acceptance or rejection entirely depends on the content of the final version of your manuscript!

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This paper by Schlager et al. identifies two new proteins showing homology to the Bicaudal-D (BICD) family and investigates the possible role(s) of one of them (BICDR1) as a regulator of the secretory pathway and microtubule-dependent vesicular traffic in cell lines and neurons. Importantly, this work demonstrates the importance of BICDR1 in axonal and dendritic extension, suggesting that BICDR1 acts as a coordinator of anterograde and retrograde transport at early stages of neuronal development.

Overall, this is an interesting paper, in which the experiments are of good quality and follow a logical progression. The first part of this study resembles the work done by the same group on the two known members of the mammalian BICD family, BICD1 and BICD2. Although these experiments were necessary to characterise BICDR1/2, the outcome was expected based on the similarity between BICD family members, with the notable exception of the lack of a kinesin-1 binding domain in BICDR1. As suggested by the authors, this finding is paramount to interpret the role of BICDR1 and its functional differences with the other family members. In this light, the authors propose that BICDR1 acts by modulating the amount of dynein and kinesin associated with the secretory vesicles. BICDR1 overexpression would drive the recruitment of dynein and keep secretory carriers sequestered in the pericentrosomal area, thus preventing neurite outgrowth. This inhibitory effect would subside early during nervous system development due to the tightly regulated expression of BICDR1. The in vivo expression data shown in Fig. 5 strengthens the biochemical and cell biological work, adding a further element of interest to this manuscript. Furthermore, it is conceivable that the mechanism described in this paper is important not only in neurons, but may also apply to other cell types, such as cytotoxic T lymphocytes where delivery of secretory granules to the immunological synapse is controlled by the centrosome.

In spite of the general interest of the topic and the high quality of several of the experiments shown, a few points need clarification and/or amendment prior to publication.

1. The authors decided to test the hypothesis that BICDR1 controls the trafficking of secretory vesicles using two well established markers of secretory granules (neuropeptide Y) and BDNF. However, instead of using professional secretory cells (i.e. PC12 cells) or primary neurons (see also Fig. 6), they focussed their attention on HeLa cells, a cell line that lacks BICDR1. Whilst it is interesting to assess the effect of BICDR1 overexpression in a null background, I believe that the very strong conclusions reached (i.e. BICDR1 does not influence regulated secretion, but controls the topology of exocytic carriers and their release site; page 7) need validation in professional secretory cells prior to be taken further (see Discussion).

2. Although BICDR1 is expressed in the nervous system (Fig. 1D), it is much more concentrated in the kidney (fivefold or more). It is surprising that the authors did not comment further on this striking finding and did not investigate if this distribution is conserved also during development. In this regard, it would be useful to add to Fig. 5 a whole-mount staining for BICDR1 at later stages of development during the formation of metanephric nephrons (E11.5-12.5). The authors should also consider replacing panels 5B-D with better quality images.

3. Whilst the authors discussed in detail the binding of BICDR1 to Rab6, this referee felt that not enough attention has been paid to the specificity of this interaction. Does BICDR1 compete with BICD1 and BICD2 for the binding to Rab6? Fig. 2A and E suggest that Rab6B binds strongly than Rab6A to BiCDR1. Is this the case? Do the two Rab isoforms compete with each other for the
binding to BICDR1? Based on the binding of BICDR1 to Rab6 and the requirement of Rab6 for the recruitment of BICDR1 to the centrosome (and vice versa; see Fig. 4), it would be reassuring to include in Fig. S3A the expression levels of Rab6 as control for BICDR1 downregulation, in addition to p150. Furthermore, the observed binding of BiCDR1 to Rab11B (Fig. 2A) has not been mentioned or commented on in the text.

4. The overexpression of BICDR1 has a sort of a dominant-negative effect, clustering Rab6-positive vesicles and inhibiting neurite elongation, in striking contrast with BICD2 overexpression which is without effect. Is this the case also for BICD1 overexpression? On the other hand, what is the effect of BICDR1 downregulation in neurons? Would it increase neurite length or speed up neuronal differentiation? Along the same lines, is BICDR1 overexpression altering the neuronal network in mature neurons?

5. Many of the colocalisation experiments (i.e. Fig. 6) would benefit from a quantification of the co-distribution of the different markers. In this regard, it would be interesting to check the level of colocalisation of the Rab6/BICDR1 secretory compartment with Kif1 and Kif5, and with other cargoes independently implicated in neurite outgrowth (i.e. TI-VAMP, L1).

6. The scheme presented in Fig. 8 adds very little to paper and fails to clarify the text. It should therefore be removed.

Referee #2 (Remarks to the Author):

Authors describe a mechanism by which the protein BICDR-1 controls the localisation of secretory vesicles and thereby neuritogenesis by maintaining the Rab6 positive vesicles at the centrosome. Although this mechanism is interesting given its regulation during neuronal development and neurite outgrowth, the exact molecular mechanisms by which BICDR-1 regulate the trafficking of secretory vesicles are not provided. Most of the experiments focus on dynactin but not on the dynein motor. Authors provide with movies showing antero and retrograde movement of vesicles in neurites but no data are provided regarding the mechanism that control anterograde movement and whether BICDR-1 coordinates such movements. Authors should clarify this in the context of MT orientation during neurites & axons/dendrites elongation. Finally, there are several errors throughout the text and some described experiments are not included in the figures. Also the title mentioning a centrosomal targeting by BICDR-1 is overstated.

See also comments below.

The specificity of the binding of BICDR-1 to specific Rabs is poorly convincing. According to figure 2, BICDR-1 binds to Rab6A and Rab6B and marginally with Rab12. What's about Rab11A and Rab11B as those two Rab and especially Rab11B that seems to be pulldowned efficiently. A main issue is the fact that interactions are based only on experiments using overexpressing cells. It remained to be determined whether this specificity is achieved in more physiological conditions (e.g.: interaction between endogenous proteins in brain extracts).

Similar to the interaction of BICDR-1 with Rab6, the interaction of BICDR-1 with dynein is not convincing given the background in the IP (Figure 4A). Again, all the interactions are based on overexpressing cells. These data are particularly important as the authors show dynactin co-localisation by IF but no images of the effect of BICDR-1 overexpression or silencing on dynein localisation. Also no information is given regarding the number of cells analyzed.

Although the pericentrosomal localisation of BICDR-1 is demonstrated in Vero cells, the interaction of BICDR-1 with the MT cytoskeleton is unclear. I seems that MTs are affected in Figure 2G when cells are silenced for BICDR-1. Therefore, it would be interesting to analyze whether MT/actin depolymerisation impacts on BICDR-1 localization. Conversely, it is important to know whether BICDR-1 silencing impacts on MT nucleation and/or MT dynamics (polymerisation and depolymerisation). Finally, quantitation of BICDR-1 silencing on Rab6A dispersion is required.

Authors do not analyze the possibility of interaction between BICDR-1 and kinesin-1. This is
especially important as both anterograde and retrograde movements of Rab6A / BICDR-1 positive vesicles are visible in movie S3. This does not fit with the proposed model implying that BICDR-1 maintains Rab6A vesicles around the centrosome by a dynein/dynactin dependent mechanism and that this control is lost when BICDR-1 is absent or downregulated during neuritic development.

The relation between secretory vesicles such as NPY-containing vesicles and neuritogenesis is unclear. Markers such as VAMP7 that directly control neuritogenesis might be more demonstrative.

Other comments:

Page 4: Dynactin should rather be considered as a coordinator of transport rather than an associated factor of cytoplasmic dynein.

Page 5: The order the figures are introduced is unusual. Why citing fig S1B,C before S1A? or 1C before 1B?

Figure 1C: Does the mRNA detected in heart correspond to alternative splicing? Although BICDR-1 is detected by WB, it is poorly (almost not) detected by Northern.

Scale bars are missing in Figs 1E & 1F.

Again scale bars are missing in Figure 2F & 2G and in figures 3, 4 and some of figure 6.

Some panels in figure 6 are not labelled.

Although the effect of BICDR-1 on NPY vesicles is quite visible in Figure 3, it would be useful to know how the authors define the center of the cell.

It is unclear how authors define the cells that have been transfected with siRNA that target p150

Authors mention a role for BICDR-1 in the transport of BDNF-GFP vesicles but no experiments are provided.

Referee #3 (Remarks to the Author):

The manuscript examines the role of BicDR1 in neuronal development. The hypothesis put forward by the authors is that BicDR1 is localised in the centrosomal area where it sequestered Rab6 positive exocytic vesicles and promote their fusion at the centre of the cells.

The relevance of the BicDR1 activity in vivo is meant to be shown by the sharp decrease in BicDR1 expression in neurons in culture, and the fact that its sustained expression leads to defects in neuron outgrowth. As Rab6 depleted neurons leads to the same phenotype and because Rab6 and BicDR1 interacts, the authors propose that BicDR1 retains Rab6 vesicles in young neurons in the cell body and upon decline of its expression, the Rab6 vesicles are allowed to move to the neurites and contribute to their growth.

The manuscript presents a huge amount of data in a very compressed format, too compressed to my taste I must say, not always very elegantly and potentially leading to misunderstanding.

The hypothesis is interesting but falls short on a number of points that I list below:

1) Given the huge amount of info, it would be better if the figures were presented in the order they are described in the text. This is particularly true for Figure 2. Panel E is described before B and then come D before C. It is then very hard to follow and judge the argument presented by the authors. In this figure, there is no reason why such a randomisation has been introduced.

2) I urge the authors NOT to use the word Rab6-vesicles before they actually show that the pattern is consistent with vesicles. In the same token they should refrain to use vesicular trafficking pathway (bottom of page 5) before they clarify that it is what they are looking at.

For Figure 1 and 2, what we see is a fluorescent dot or a punctate for BicDR1 that co-localise with centrosomal markers and Rab6. There is no evidence of vesicles. As far as I know, the centrosome does not contain membrane, and the presence of Rab6 is not necessarily suggestive of vesicles.
Membrane at best, but as a cytoplasmic protein, it would be anything! So the word vesicle should be avoided!

In this regard, the authors do not play the normal game of the Rab world at showing that the centrosomal pattern for Rab6 is due to Rab6-GTP and that the BiCDR1 bins Rab6-GTP less than Rab6-GDP.

The situation could be remedied in Figure 3 where cargo NPY is introduced. True, Rab6 and NPY co-localise in transport vesicles as shown in Grigoriev (2007). But there is no evidence that Rab6 and NPY co-localise with BiCDR1 at the centrosome. True, NPY co-localise with BiCDR1 (panel A) and Rab6 co-localises with BiCDR1 (Figure 2) but it is not clear that NPY and Rab6 are present together at in the same structures. I would at least perform a triple labelling to shown that the three components are localised together in the same cell!

Same thing in neurons: Rab6 and NPY are not shown to co-localise with BiCDR1. Rab6 and NPY co-localise in the neurites on moving structures (vesicles) but do they at the centrosome? In Figure 5L and 5N, Rab6 should be localised together with BiCDR1!

This needs to be re-worded properly and experiments added to sustain the notion that exocytic vesicles are sequestered at the centrosomes. This is important.

3) it is also not clear to me where these putative vesicles come from? The authors called them exocytotic which is a term I am not sure I understand. Does it mean that there are postGogi? Is NPY modified according to a postGolgi origin? Could they be pre-Golgi?

4) The localisation of BiCDR in neurons is much more extended than in Vero or HeLa cells. Does it correspond to the centrosome as well? The authors do not discuss this in the text. The co-localisation with gamma tubulin in Figure1 is almost meaningless as the BiCDR1 labeling covers an area 10 times larger than the centrosome dot (same thing in Fig5M)

Why is the BiCDR1 pattern in neurons different from this in Vero?
In this regard, in Figure 6C, why not use the gamma tubulin antibody instead of an anti tubulin?

5) I also have trouble with the experiments using the neurons in culture.
First, this paragraph that is crucial for the paper is even more condensed than the rest of the text and would deserve more space for clarity.

Second, from the blots, it is clear than after DIV1, BiCDR1 level decreases sharply and that Rab6B is not yet expressed. There is only a little bit of Rab6A!
At DIV3, there is no BiCDR1 and plenty of Rab6A and B.
So it seems that in neurons, the two partners of the complex are not expressed at the same time, with the same dynamics. This, to me, shadows the relevance of the in vivo results.
I don't have a problem with the claim that sustained high level of BiCDR1 is detrimental to the neurites outgrowth. The expression of the mutated form of BiCDR1 (K512M) does not lead to a neurite outgrowth and that indeed suggests that the effect is mediated by Rab6.
However, given the gene expression profile, I have reservation on the relevance if this in vivo. In this experiment, BiCDR1 could compete artificially with BiCD and whose role I ma not sure I understand in this context. More experiments should be added to strengthen this point

6) What is the affinity of BiCDR1 for Rab6 and how does this compare to this of BiCD (sicne they have very similar binding sites, Figure 2B)? How do the authors envisage the competition of the two proteins for the binding and that so much?

7) The authors show that
-Rab6A RNAi leads to no BiCDR1 at the centrosome? (Is it degraded as there is no staining anywhere?). This is now a suppl but should be shown as a main figure.
-Conversely, BiCDR1 RNAi leads to no Rab6 at centrosomes
So they need each other to be located to the centrosome and therefore, BiCDR1 is not a true effector of Rab6. It is possible that they are recruited by a third partner (Dynein, P150) and then bind to each other once at the centrosome.
This is sustained by the fact that Dynein and p150 RNAi leads to no BiCDR1 at the centrosome?
What happens to Rab6 then?
Is Dynein and P150 role in localising BiCDR1 through their concentration at the centrosome? What happens when gamma tubulin (or centrins) are depleted?
8) What are the arrows and arrowheads in Figure 3C?

9) The quantitation in Figure 3D and E is interesting but suggests that BicDR1 overexpression not only concentrates the NPY "vesicles" to the cell centre but also increases their fusion. In fact, there is less NPY and more fusion. The fusion role of BicDR1 should be discussed. Is it relevant?

10) Figure 5E-J: Why choose a E13.5 mouse embryos and not a E10.5 where BicDR1 level is the highest?

11) Figure 6 is complicated and introduced in an odd manner. Till this point, the reader is geared on BicDR1 at the centrosome together with Rab6 and NPY. But in this figure, it is the dual movement of BicDR1 and Rab6 on vesicle leaving the cell centre and fusing, perhaps, to the periphery (or whatever it shows, which id hard to understand). This is confusing because I thought the message was that BicDR1 was sequestering the vesicles at the cell centre which should be released once BicDR1 level decreased. Here the authors suggests movement with them and this raises the question of the sequestration and how it is mediated.

In this regard, I don't understand the sentence Page 9: "BICDR-1 was observed on mobile Rab6-positive vesicles and tubular structures which docked and fused with larger BICDR-1/Rab6 membrane domains in the cell body (Fig. 6D, Movie S3)."

Also, as I mentioned above, there is no convincing evidence in Figure 6A and B that Rab6 co-localises with BicDR1 at the cell centre (despite claims starting the paragraph page 9)

In the same paragraph, I really do not understand the sentence "Consistently, the secretory vesicle markers NPY-GFP and BDNF-GFP are both decreased in the developing neurites and clustered with BICDR-1 and Rab6 in the cell body (Fig. 6A)"

And I do not agree with the conclusion: "Together these data indicate that BICDR-1 concentrates secretory vesicles in the neuronal cell body and prevents anterograde transport into growing neurites."

I don't see where is the data showing the prevention of anterograde transport.

Perhaps a less compact, more restful manner of presentation would lead to less ambiguity in the reasoning.

12) The authors seem to propose that the only way to deliver membrane to the neurites are carriers coming for the cell body. However, it has been shown that at least dendrites have so called Golgi outposts that can supply part of the membrane needed for growth. This is not addressed at all in the introduction.

Additional Correspondence

07 January 2010

Thank you for your email of October 12 2009 (see below). The reviewers made some very useful comments about our manuscript (EMBOJ-2009-72511). They were generally complimentary about the study, but there were two main reservations. First, our observation that BICDR-1 does not influence regulated secretion, but controls the topology of exocytic carriers and their release site needed validation in primary hippocampal neurons; and second, no data are provided regarding BICDR-1’s role in anterograde movement.

Since then we have been working intensively to improve and extend the study with additional key experiments. We are confident that we can address the specific criticisms of the reviewers and that incorporation of these new data greatly strengthens the impact and significance of the story. In addition, we plan to "re-focus" the paper to emphasize the role of BICDR-1 during neuronal differentiation and more clearly describe the novelty of our findings. We expect that finishing some of the control experiments and restructuring the paper will take a few more weeks.

In short, we would like to resubmit an improved manuscript with additional data approximately one
month from now. I hope you will grant us some extra time to finish the experiments and submit a complete story.

Response to Referees

**** Referee #1 ****

This paper by Schlager et al. identifies two new proteins showing homology to the Bicaudal-D (BICD) family and investigates the possible role(s) of one of them (BICDR1) as a regulator of the secretory pathway and microtubule-dependent vesicular traffic in cell lines and neurons. Importantly, this work demonstrates the importance of BICDR1 in axonal and dendritic extension, suggesting that BICDR1 acts as a coordinator of anterograde and retrograde transport at early stages of neuronal development. ...//... In spite of the general interest of the topic and the high quality of several of the experiments shown, a few points need clarification and/or amendment prior to publication.

We are pleased that Referee 1 thinks highly of our paper. We have worked hard to address all the comments raised – see below.

1. The authors decided to test the hypothesis that BICDR1 controls the trafficking of secretory vesicles using two well established markers of secretory granules (neuropeptide Y) and BDNF. However, instead of using professional secretory cells (i.e. PC12 cells) or primary neurons (see also Fig. 6), they focussed their attention on HeLa cells, a cell line that lacks BICDR1. Whilst it is interesting to assess the effect of BICDR1 overexpression in a null background, I believe that the very strong conclusions reached (i.e. BICDR1 does not influence regulated secretion, but controls the topology of exocytic carriers and their release site; page 7) need validation in professional secretory cells prior to be taken further (see Discussion).

In the revised manuscript we tested whether BICDR1 regulates secretory vesicle trafficking and/or exocytosis in hippocampal neurons. In these neurons secretion occurs after neuronal depolarization with 60 mM KCl (de Wit et al, J Neuroscience, 2009). Live imaging showed that both in control and BICDR1 expressing neurons transient NPY-GFP exocytotic events occur after KCl induced depolarization (Fig. 6E,F). Quantification revealed that the NPY-GFP secretion events in control and BICDR1 expressing neurons have similar kinetics (Fig. 6F). However, secretory vesicle fusion is seen at a different subcellular location in BICDR1 expressing neurons. In control cells most NPY-GFP exocytotic events take place in developing neurites, while exocytotic events mainly occur in the cell body of BICDR1 neurons. These live imaging data are consistent with the fixed data, which showed an accumulation of NPY-GFP positive secretory vesicles in the soma and decreased number of vesicles in neurites in BICDR1 neurons (Fig. 6C,D). Together these data suggest that BICDR1 does not influence secretory vesicle exocytosis but controls the distribution of secretory carriers in neurons.

These results confirm our initial observations in Hela cells (now shown in Fig. S6B-F).

2. Although BICDR1 is expressed in the nervous system (Fig. 1D), it is much more concentrated in the kidney (fivefold or more).

On the Western blot shown in Fig. 1D we loaded extracts of adult tissues. We generally find moderate levels of BICDR1 in the adult brains (Fig. 1D) but high expression levels in embryonic brain tissue (Fig. 1E-H and Fig. 1M-R). In this regard, it is interesting that the expression of BICDR1 is high in the adult kidney (Fig. 1D). We now mention this in the revised manuscript.
It is surprising that the authors did not comment further on this striking finding and did not investigate if this distribution is conserved also during development. In this regard, it would be useful to add to Fig. 5 a whole-mount staining for BICDR1 at later stages of development during the formation of metanephric nephrons (E11.5-12.5). The authors should also consider replacing panels 5B-D with better quality images.

We include in the revised manuscript an in situ hybridized cryosection (E13.5) of the developing kidney (Fig. 1I-L). Expression of BICDR-1 is observed in the metanephric tubules (Fig. 1L).

3. Whilst the authors discussed in detail the binding of BICDR1 to Rab6, this referee felt that not enough attention has been paid to the specificity of this interaction. Does BICDR1 compete with BICD1 and BICD2 for the binding to Rab6?

The revised manuscript now includes new data (Fig. S2B) that support the idea that BICDR-1 competes with BICD for Rab6 binding in in vitro pulldown assays.

Fig. 2A and E suggest that Rab6B binds strongly than Rab6A to BiCDR1. Is this the case? Do the two Rab isoforms compete with each other for the binding to BICDR1?

It is very well possible that Rab6A and Rab6B compete with each other for binding to BICDR-1 in vitro but looking at the expression profiles in developing hippocampal neurons (Fig. 5A; BICDR-1 and Rab6A are present at high levels, while Rab6B is expressed at lower levels at DIV 1-2), it seems most likely that BICDR-1 interacts with Rab6A in vivo. On the other hand, the referee is correct in his/her notion that BICDR-1 binds more strongly to Rab6B than Rab6A. We have seen this in all our GST pull down experiments (Fig. 3A,C). At this moment we have no knowledge about the exact molecular ratios of both Rab isoforms in young hippocampal neurons during the time of BICDR-1 expression and whether competition between BICDR-1 and Rab6A / Rab6B takes place. Moreover, it is not clear what the difference is between BICDR-1 function to differentially bind to Rab6A and Rab6B. We know that Rab6 isoforms are not redundant during neuronal development - both are needed for neurite outgrowth (Fig. 8I) - but additional studies are required to find the differences/similarities between the Rab6 isoforms and whether their differential binding to BICDR-1 is physiologically relevant.

Based on the binding of BICDR1 to Rab6 and the requirement of Rab6 for the recruitment of BICDR1 to the centrosome (and vice versa; see Fig. 4), it would be reassuring to include in Fig. S1A the expression levels of Rab6 as control for BICDR1 downregulation, in addition to p150.

In the revised manuscript we now include Western blots of Rab6A and p150<sup>αααα</sup> levels in control and BICDR-1 knockdown cells Fig. S4A (Fig. S3A; in the original manuscript). Both Rab6A and p150<sup>αααα</sup>-protein levels are unchanged in the absence of BICDR-1.

Furthermore, the observed binding of BiCDR1 to Rab11B (Fig. 2A) has not been mentioned or commented on in the text

To further investigate the possible interaction of BICDR-1 with Rab11B, we performed additional pulldown experiments using BICDR-1 and BICDR-1-K512M (see Fig. 1 for the referees at the end of the rebuttal letter). The experiment confirmed that wild-type BICDR-1 strongly binds Rab6 compared to the K512M mutant form. However, BICDR-1 binds rather weak to Rab11B and both wild-type and K512M mutant BICDR-1 show the same binding affinity. These data suggest that the weak binding is independent of the Rab6 binding site on BICDR-1. Future experiments are necessary to identify the potential Rab11B interaction site on BICDR-1. We now mention in the
revised text that BICDR-1 marginally binds to Rab11B

4. The overexpression of BICDR1 has a sort of a dominant-negative effect, clustering Rab6-positive vesicles and inhibiting neurite elongation, in striking contrast with BICD2 overexpression which is without effect. Is this the case also for BICD1 overexpression?

We now included new experiments testing the influence of BICD1 overexpression in developing neurons. Fig. 8G shows that, similar to BICD2, BICD1 overexpression does not adversely affect neurite outgrowth, strengthening the observation that the BICDR-1 effect on Rab6 vesicles distribution and neuron morphology is specific and remarkably distinct from other BICD family proteins.

On the other hand, what is the effect of BICDR1 downregulation in neurons? Would it increase neurite length or speed up neuronal differentiation?

Since BICDR1 is only expressed for 1-2 days in our neuronal cultures it is technically challenging to use BICDR-1-shRNA - which requires >3-4 days of expression - to down regulate BICDR-1 at these early stages in neurons. Instead, we collaborated with the lab of Iain Shepherd (Emory University, Atlanta, GA, USA) to determine the in vivo loss-of-function phenotype in zebrafish.

Two independent BICDR1-specific morpholinos (MOs) were used to block BICDR-1 expression and revealed striking neural development abnormalities in zebrafish embryos (Fig. 2B). These data strengthen our observation in cultured neurons and indicate that BICDR-1 is required for early neuronal development in vivo.

Along the same lines, is BICDR1 overexpression altering the neuronal network in mature neurons? Although further studies on BICDR-1 overexpression in more mature neurons and the potential effects on network properties are interesting, we strongly believe that these experiments are beyond the scope of this study.

5. Many of the colocalisation experiments (i.e. Fig. 6) would benefit from a quantification of the codistribution of the different markers. In this regard, it would be interesting to check the level of colocalisation of the Rab6/BICDR1 secretory compartment with Kif1 and Kif5, and with other cargoes independently implicated in neurite outgrowth (i.e. TI-VAMP, L1).

The revised manuscript now includes correlation coefficients for the colocalizations shown in Fig. 5E (Fig. 6A in the original manuscript, colocalization indicated by arrows, r_p=0.8) and Fig. 5F (Fig. 6B in the original manuscript, NPY-GFP/Rab6A r_p=0.9, NPY-GFP/Rab6B r_p=0.8; Rab6A/Rab6B r_p=0.8). In the revised manuscript, we provide new data showing the binding and colocalization of BICDR-1 and Kif1C (Fig. 7). For members of the kinesin-1/Kif5 family no interaction or colocalization was observed. Expression of mCherry-BICDR-1, GFP-VAMP7 and subsequent staining for Rab6A in hippocampal neurons did not reveal any apparent colocalization between BICDR-1/Rab6A and VAMP7 (see Fig. 2 for the referees at the end of the rebuttal letter) suggesting that BICDR-1/Rab6A and VAMP7 do not function in the same trafficking pathway.

6. The scheme presented in Fig. 8 adds very little to paper and fails to clarify the text. It should therefore be removed.

The schematic diagram is removed from the main text and is now shown in Supplementary Figure 9

**** Referee #2 ****

Authors describe a mechanism by which the protein BICDR-1 controls the localization of secretory vesicles and thereby neuritogenesis by maintaining the Rab6 positive vesicles at the centrosome. Although this mechanism is interesting given its regulation during neuronal development and neurite outgrowth, the exact molecular mechanisms by which BICDR-1 regulate the trafficking of secretory vesicles are not provided.

We are pleased that the referee finds our model interesting. We now tried to make to study more compelling according to the referee’s suggestions.

1/ Most of the experiments focus on dynactin but not on the dynein motor.

In the revised manuscript we extended our data on the dynein motor and repeated all experiments with dynein antibodies. We now show that BICDR-1 pulls down dynein heavy chain (DHC) and dynein intermediate chain (IC74) and dynactin (Fig. 4A), BICDR-1 colocalizes with dynein (and
dynamic) at the pericentrosomal region (Fig. S5B) and overexpression of BICDR-1 recruits dynein (and dynactin) to Rab6 vesicles in fibroblast cells and neurons (Fig. S5C and S7A). These results confirm our previous results with dynactin and indicate that, similar to BICD, both dynein and dynactin interact and coincide with BICDR-1.

Authors provide with movies showing antero and retrograde movement of vesicles in neurites but no data are provided regarding the mechanism that control anterograde movement and whether BICDR-1 coordinates such movements.

We thank the referee for this important comment. It is well known that direction of transport strongly depends on the net balance between microtubule plus-end (anterograde) and minus-end (retrograde) directed motors (e.g. Wang & Schwarz, Cell, 2009; Welte, Current Biol, 2004). In the BICDR-1/Rab6A movie (Movie S1), the observed retrograde movement of vesicles is most likely caused by the interaction between Rab6/BICDR-1 and the dynein dynactin complex (Fig. 4, Fig. S5, Fig. S7), while the anterograde movement is probably explained by the binding of the plus-end directed kinesin motors to Rab6 secretory vesicles, directly or indirectly via BICDR-1. We have previously demonstrated that BICD family proteins associate with kinesin-1/Kif5 (Grigoriev et al, Dev Cell, 2007). Here we show that BICDR-1 does not interact with Kif5 but associates with Kif1C (Fig. 7A,B). Interestingly, Kif1C is recruited to Rab6 positive secretory vesicles (Fig 7C,D) and neurons transfected with Kif1C-shRNA accumulate NPY-GFP positive secretory vesicles in the soma, strongly decrease the number of NPYGFP vesicles in neurites (Fig. 7E,F) and showed reduced neurite outgrowth (Fig. 8I). These data suggest that the association of BICDR-1 with Rab6, Kif1C, and dynein/dynactin is likely to contribute to the bidirectional motility of secretory vesicles in young neurons. However it is very likely that multiple other kinesin motor proteins are involved regulating anterograde secretory trafficking.

Since overexpression of BICDR-1 moves most of the secretory vesicles inward to the cell center, it is tempting to speculate that BICDR-1 is a coordinator of bidirectional transport and crucial for setting the balance in the microtubule minus-end direction. In fact, at low level expression of BICDR-1 there is a slight shift towards retrograde transport of Rab6 vesicles and slight accumulation of Rab6 vesicles in the center of the cell (Fig. S6A), while adding increasing amount of BICDR-1 changes the balance towards retrograde transport and large concentrations of Rab6 vesicles are present around the pericentrosomal region (Fig. S6B). Interestingly, BICDR-1 overexpression accumulates Kif1C in the cell body suggesting that minus-end directed motor dynein has a dominant role in mediating BICDR-1/Rab6 vesicle transport. It is possible that Kif1C is a passive motor in the context of BICDR-1 and that Kif1C has a more active role in Rab6 vesicles transport at later stages of neuronal development independent of BICDR-1. Since this study reports the first description and initial characterization of this new BICD family member, additional studies are required to discover the precise molecular mechanisms regulation bidirectional secretory vesicle transport.

2/ Authors should clarify this in the context of MT orientation during neurites & axons/dendrites elongation.

It is well known that in mature neurons axonal microtubules have uniform orientations with all plus ends pointing outward and dendritic microtubules have mixed orientations (Baas, PNAS, 1988; Conde and Caceres, Nat Rev Neurosci 2009; Dombec, et al, PNAS, 2003, Jaworski et al, Neuron, 2009). However in neurites from young neurons (<5DIV) the majority of the microtubules are oriented with their plus end outward (Baas, et al, J Cell Biol, 1989; Stepanova, et al, J Neurosci 2003). In contrast to fully developed dendrites, in young neurites the selective activation of either kinesin or dynein will immediately determine the direction of transport, anterograde and retrograde, respectively. Therefore, the bidirectional trafficking of secretory vesicles observed in young neurons is most likely a direct effect of the net balance between microtubule plus-end and minus-end directed motors and not a consequence of the mixed microtubule organization.

3/ Also the title mentioning a centrosomal targeting by BICDR-1 is overstated.

We agree with the referee and changed the title to “Pericentrosomal targeting of Rab6 secretory vesicles by Bicaudal-D related protein-1 (BICDR-1) regulates neuritogenesis”

4/ The specificity of the binding of BICDR-1 to specific Rabs is poorly convincing. According to figure 2, BICDR-1 binds to Rab6A and Rab6B and marginally with Rab12. What's about Rab11A and Rab11B as those two Rab and especially Rab11B that seems to be pulldown efficiently.

To further investigate the possible interaction of BICDR-1 with Rab11B, we performed additional pull down experiments using BICDR-1 and BICDR-1-K512M (see Fig. 1 for the referees at the end of the rebuttal letter). The experiment confirmed that wild-type BICDR-1 strongly binds Rab6.
compared to the K512M mutant form. However, wild-type BICDR-1 binds rather weak to Rab11B and both wildtype and K512M mutant BICDR-1 show the same binding affinity. These data suggest that the weak binding is independent of the Rab6 binding site on BICDR-1. Although testing physiological significance of the interaction between BICDR-1 and Rab11B/12 is of interest, we believe that these experiments are beyond the scope of this study. We now mention in the text that BICDR-1 marginally binds to Rab11B and Rab12.

A main issue is the fact that interactions are based only on experiments using overexpressing cells. It remained to be determined whether this specificity is achieved in more physiological conditions (e.g.: interaction between endogenous proteins in brain extracts).

Naturally, one of the first things we tried when we found the Rab6/BICDR-1 interaction was to communoprecipitate (IP) endogenous Rab6 and BICDR-1 from cultured Vero cells and embryonic brain extracts to document their interaction under conditions where neither of the two proteins is overexpressed or mutated. Unfortunately, we were unable to get a clear positive result, i.e. a result whereby the antibodies against BICDR-1 precipitate Rab6 and vice versa. The most likely explanations for this "failure" are: 1) that during the preparation of the cell extracts, there are conditional changes (such as disruption of membranes) that make it very hard to co-IP endogenous Rab6 and BICDR-1 (normally this interaction is expected to occur in the context of membrane association); 2) the Rab6- BICD interaction is transient, which is reported for BICD and in line with other cargo-adaptor motor proteins. These reasons likely explain why a lot of other investigators have also been unable to detect an association between endogenous Rab proteins and their effectors by co-IP. For instance, several key publications in high impact journals on Rab/Rab effector interaction (De Renzis, et al, NCB 2002; Wu et al NCB 2002; Hoogenraad, et al, Plos Biology, 2010) do not report co-IPs between endogenous proteins. Moreover, we were never successful in showing an endogous IP between BICD and Rab6 (Hoogenraad et al, EMBO J, 2001; Matanis et al, NCB, 2002; Hoogenraad et al, EMBO J, 2003). In any case, we think that GST-pull down experiments and yeast two hybrid data are supportive for a BICDR-1/Rab6 interaction and that the loss-of-function experiments added in the revised version of the manuscript strongly supports the physiological relevance of BICDR-1 in vivo (Fig. 2).

5/ Similar to the interaction of BICDR-1 with Rab6, the interaction of BICDR-1 with dynein is not convincing given the background in the IP (Figure 4A). Again, all the interactions are based on overexpressing cells. These data are particularly important as the authors show dynactin colocalisation by IF but no images of the effect of BICDR-1 overexpression or silencing on dynein localisation. Also no information is given regarding the number of cells analyzed.

We have improved the Western blots in Fig. 4A. The IP experiments show that GFP-BICDR-1 precipitates the major dynein subunits (DHC and IC74) and the dynactin subunit (p150glue), while no binding is seen with control GFP (Fig. 4A). Consistently, both dynactin (Fig. 4D) and dynein (Fig. S5B) colocalize with endogenous BICDR-1 around the centrosome in Vero cells. Overexpression of GFP-BICDR-1 caused a ~2-fold increase in both dynein (IC74) and dynactin (p150glue) fluorescent staining intensity (Fig. 4B,E and Fig. S5C), indicating that BICDR-1 recruits both the dynein and dynactin complex to the pericentrosomal region. In the revised manuscript we now show that knockdown of BICDR-1 does not affect dynein/dynactin expression (Fig. S4A) and dynein/dynactin localization around the centrosome (Fig S5A). These data strongly support our model that BICDR-1 is an motor-cargo-adaptor protein between dynein/dynactin and Rab6 secretory vesicles. We now included the number of cells analyzed (n) for all quantifications in the figure legends.

6/ Although the pericentrosomal localisation of BICDR-1 is demonstrated in Vero cells, the interaction of BICDR-1 with the MT cytoskeleton is unclear. I seems that MTs are affected in Figure 2G when cells are silenced for BICDR-1. Therefore, it would be interesting to analyze whether MT/actin depolimerisation impacts on BICDR-1 localization. Conversely, it is important to know whether BICDR-1 silencing impacts on MT nucleation and/or MT dynamics (polimerisation and depolimerisation). Finally, quantitation of BICDR-1 silencing on Rab6A dispersion is required.

To analyze the impact of microtubule or actin depolimerization on the pericentrosomal BICDR-1 localization, we treated cells with nocodazole or cytochalasin D, respectively. In the revised manuscript we show that microtubule depolimerization leads to mislocalization of BICDR-1 while actin depolimerisation has no influence on the BICDR-1 distribution (Fig. S3A). These data reveal that the localization of BICDR-1 around the centrosome is microtubule dependent, which is consistent with our model that BICDR-1 is present on Rab6 vesicles and involved in microtubule-dependent transport.
The effect of BICDR-1 knockdown on microtubule organization and dynamics was assed by measuring microtubule nucleation rate (using EB3-GFP), MT growth velocity (mCherry-Tubulin) and microtubule shrinking velocity (mCherry-Tubulin). All these parameters were analyzed in control and BICDR-1 knockdown cells, but no significant difference in any of the measured parameters was observed (Fig. S4F). These results indicate that BICDR-1 silencing does not affect microtubule organization or dynamics.

7/ Authors do not analyze the possibility of interaction between BICDR-1 and kinesin-1. This is especially important as both anterograde and retrograde movements of Rab6A / BICDR-1 positive vesicles are visible in movie S3. This does not fit with the proposed model implying that BICDR-1 maintains Rab6A vesicles around the centrosome by a dynein/dynactin dependent mechanism and that this control is lost when BICDR-1 is absent or downregulated during neuritogenic development.

As discussed above, the anterograde movement of BICDR-1/Rab6 vesicles is most likely explained by the binding of the plus-end directed kinesin. In young neurons kinesin motors may bind to Rab6 secretory vesicles via BICDR-1. We have previously demonstrated that BICD family proteins associate with kinesin-1/Kif5 (Grigoriev et al, Dev Cell, 2007). However in yeast two hybrid assays and coimmunoprecipitation experiments we never observed association between BICDR-1 and kinesin-1/KIF5 (data not shown). In the revised manuscript we now include results from our yeast-two hybrid screen for BICDR-1 partners and where we found Kif1C as interacting protein (Fig. 7A). The BICDR1/Kif1C interaction is confirmed by co-immunoprecipitation experiments and co-stainings in Vero cells and neurons (Fig. 7C,D). Moreover, neurons transfected with Kif1C-shRNA accumulate NPY-GFP positive secretory vesicles in the cell body and strongly decrease the number of secretory vesicles in neurites (Fig. 7E,F) and phenocopy the Rab6 knockdown and BICDR-1 overexpression effects on neurite outgrowth (Fig. 8f). As stated in the discussion, further studies are needed to resolve the underlying molecular mechanism of kinesin and dynein motor binding. It needs to be tested whether Kif1C is an active/passive motor in the context of BICDR-1 and whether Kif1C has a role in Rab6 vesicles transport at later stages of neuronal development independent of BICDR-1. Regardless of the precise molecular mechanism, the data suggest that the association of BICDR-1 with Rab6, Kif1C, and dynein/dynactin is likely to contribute to the bidirectional motility of secretory vesicles in young neurons.

8/ The relation between secretory vesicles such as NPY-containing vesicles and neuritogenesis is unclear. Markers such as VAMP7 that directly control neuritogenesis might be more demonstrative.

We thank the referee for this interesting suggestion. VAMP7/TI-VAMP is recently shown to involved in secretory transport and neurite growth (Burgo et al, EMBO reports, 2009) and could potentially be part of the BICDR-1/Rab6 secretory pathway. However, live cell imaging of HeLa cells expressing both GFP-VAMP7 and mStrawberry-Rab6A did not reveal any codistribution of the two markers (data not shown). Additionally GFP-VAMP7 did not colocalize with endogenous Rab6A en BICDR-1 in Vero cells and co-expression of mCherry-BICDR-1 and GFP-VAMP7 in neurons resulted in the accumulation of endogenous Rab6A but the localization of GFP-VAMP7 remained unchanged (see Fig. 2 for the referees at the end of the rebuttal letter). These data suggest that VAMP7 and Rab6/BICDR-1 function in different pathways.

Page 4: Dynactin should rather be considered as a coordinator of transport rather than an associated factor of cytoplasmic dynein.

We agree with the referee that dynactin is nowadays considered to be an accessory and regulatory factor for cytoplasmic dynein motors. We modified this in the text accordingly.

Page 5: The order the figures are introduced is unusual. Why citing fig S1B,C before S1A? or 1C before 1B?

In the revised manuscript, we reorganized the figure consistent with the flow of the text.

Figure 1C: Does the mRNA detected in heart correspond to alternative splicing?

At this moment we have no experimental data on possible BICDR-1 splice variants. The genomic organization of BICDR-1 and the EST expression databases predict that multiple splice variants might exist.

Although BICDR-1 is detected by WB, it is poorly (almost not) detected by Northern.

We do not agree, the BICDR-1 mRNA levels in brain and kidney are quit high on Northern blot (Fig. 1B).
Scale bars are missing in Figs 1E & 1F. Again scale bars are missing in Figure 2F & 2G and in figures 3, 4 and some of figure 6.

In the revised manuscript we added scale bars to the figures.

Some panels in figure 6 are not labelled.

We corrected this in the revised manuscript.

Although the effect of BICDR-1 on NPY vesicles is quite visible in Figure 3, it would be useful to know how the authors define the center of the cell.

We define the center of the cell as the region where the centrosome is located. In BICDR-1 expressing cells NPY positive vesicles accumulate in the pericentrosomal region (Fig. S6A, B). We now more clearly explained this in the text.

It is unclear how authors define the cells that have been transfected with siRNA that target p150.

Hela cells were transfected with p150glue siRNA for 4 days and stained for p150glue using the mouse monoclonal antibody from BD bioscience. The p150glue knockdown cells clearly lack the p150glue staining at the microtubule plus-ends (as reported by Landsbergen et al, JCB, 2004) and centrosome (Fig. 4F).

Authors mention a role for BICDR-1 in the transport of BDNF-GFP vesicles but no experiments are provided.

We demonstrate that BICDR-1 strongly accumulates Rab6 positive secretory vesicles in the soma (Fig. 6A). In the revised manuscript we observed very similar results with other neuronal secretory vesicle markers, such as NPY-GFP, GFP-Sema3A and BDNF-GFP (Fig. 6C, D). Images are shown in Fig. S7F, G.

**** Referee #3 ****

The manuscript examines the role of BicDR1 in neuronal development. The hypothesis put forward by the authors is that BicDR1 is localised in the centrosomal area where it sequestered Rab6 positive exocytic vesicles and promote their fusion at the centre of the cells. The relevance of the BicDR1 activity in vivo is meant to be shown by the sharp decrease in BicDR1 expression in neurons in culture, and the fact that its sustained expression leads to defects in neuron outgrowth. As Rab6 depleted neurons leads to the same phenotype and because Rab6 and BicDR1 interacts, the authors propose that BicDR1 retains Rab6 vesicles in young neurons in the cell body and upon decline of its expression, the Rab6 vesicles are allowed to move to the neurites and contribute to their growth. The manuscript presents a huge amount of data in a very compressed format, too compressed to my taste I must say, not always very elegantly and potentially leading to misunderstanding. The hypothesis is interesting but falls short on a number of points that I list below:

We are pleased that the referee finds our model interesting. We have worked hard to address all the comments raised and rewrote the paper substantially.

1/ Given the huge amount of info, it would be better if the figures were presented in the order they are described in the text. This is particularly true for Figure 2. Panel E is described before B and then come D before C. It is then very hard to follow and judge the argument presented by the authors. In this figure, there is no reason why such a randomisation has been introduced.

In the revised manuscript, we reorganized the figures and panels according to the referee’s request.

2/ I urge the authors NOT to use the word Rab6-vesicles before they actually show that the pattern is consistent with vesicles. In the same token they should refrain to use vesicular trafficking pathway (bottom of page 5) before they clarify that it is what they are looking at. For Figure 1 and 2, what we see is a fluorescent dot or a puntate for BicDR1 that co-localise with centrosomal markers and Rab6. There is no evidence of vesicles. As far as I know, the centrosome does not contain membrane, and the presence of Rab6 is not necessarily suggestive of vesicles. Membrane at best, but as a cytoplasmic protein, it would be anything! So the word vesicle should be avoided!

We agree with the referee. We removed the indicated sentences and modified the text as follows “Fluorescence microscopic analysis showed an intense perinuclear BICDR-1 staining (Fig. S2C-E) specifically localized around the γ-tubulin labeled centrosome (Fig. 3F)”.

In this regard, the authors do not play the normal game of the Rab world at showing that the centrosomal pattern for Rab6 is due to Rab6-GTP and that the BicDR1 bins Rab6-GTP less than Rab6-GDP.
Again we agree with the referee. We now show in Fig. S2A that full length BICDR-1 (amino acids 1-577) and the C-terminal part of BICDR-1 (382-577) bind to Rab6 (Rab6A-WT) and Rab6-GTP (Rab6A-Q82R) but not to Rab6-GDP (Rab6A-T27N).

The situation could be remedied in Figure 3 where cargo NPY is introduced. True, Rab6 and NPY colocalise in transport vesicles as shown in Grigoriev (2007). But there is no evidence that Rab6 and NPY co-localise with BicDR1 at the centrosome. True, NPY co-localise with BicDR1 (panel A) and Rab6 co-localise with BicDR1 (Figure 2) but it is not clear that NPY and Rab6 are present together at in the same structures. I would at least perform a triple labelling to shown that the three components are localised together in the same cell!

In the revised manuscript we provide a triple labeling of NPY/Rab6/BICR-1 (Fig. S6A).

Same thing in neurons: Rab6 and NPY are not shown to co-localise with BicDR1. Rab6 and NPY colocalise

in the neurites on moving structures (vesicles) but do they at the centrosome? In Figure 5L and 5N, Rab6 should be localised together with BicDR1! This needs to be re-worded properly and experiments added to sustain the notion that exocytic vesicles are sequestered at the centrosomes. This is important.

We now show colocalization of endogenous BICDR-1 and endogenous Rab6 on vesicles in the cell body of neurons (Fig. 5C). Additionally, we show new data to support the notion that exocytic vesicles can be sequestered in the cell body by expression of BICDR-1 (Fig. 6A-D).

3) it is also not clear to me where these putative vesicles come from? The authors called them exocytotic which is a term I am not sure I understand. Does it mean that there are postGolgi? Is NPY modified according to a postGolgi origin? Could they be pre-Golgi?

Utrastructural studies show that endogenous Neuropeptide Y (NPY) localizes to large dense core vesicles (LDCVs) in rat brain (Pelletier et al, Neuropeptides 1984; Pickel et al, Brain Res 1989). NPY tagged with GFP is a widely used secretory vesicle cargo marker (e.g. Nagai et al, Nature Biotech 2002; de Wit, et al, J Neuroscience, 2009) for visualizing secretory transport (e.g. de Wit al, Traffic 2001) and imaging calcium-triggered exocytosis of single secretory vesicles (e.g. Holroyd et al, PNAS, 2002). We have previously shown that Rab6 labeled NPY-GFP secretory vesicles (also called exocytotic carriers or postGolgi vesicles), which displayed persistent flow from the Golgi complex to the cell periphery (Movie S2 in the revised manuscript) (Grigoriev et al, Dev Cell, 2007), a typical behavior for secretory vesicles (Hirschberg et al, JCB, 1998). In this study we use NPY as a secretory vesicle marker but also use NPY-GFP to monitor transient NPY-GFP exocytotic events after KCl induced depolarization in neurons (Fig. 6E, F). Since all membrane and secreted proteins traffic through the secretory pathway (from the ER via the Golgi to the plasma membrane), we cannot exclude that some NPY-GFP positive vesicles are ER-derived/preGolgi vesicles. Previous quantification revealed ~70% overlap between Rab6 and NPY vesicles (Grigoriev et al, Dev Cell 2007).

The observed co-localization between BICDR-1 with NPY and Rab6 suggests that BICDR-1 is localized to the transport pathway of secretion in neurons (Fig. 5C,E). Moreover, all tested secretory markers, GFP-Sema3A and BDNF-GFP, NPY-GFP and Rab6 accumulate in the soma of BICDR-1 overexpression neurons (Fig. 6A-D and Fig. S7F, G).

4) The localisation of BicDR in neurons is much more extended than in Vero or HeLa cells. Does it correspond to the centrosome as well? The authors do not discuss this in the text. The co-localisation with gamma tubulin in Figure1 is almost meaningless as the BicDR1 labeling covers an area 10 times larger than the centrosome dot (same thing in Fig5M) Why is the BicDR1 pattern in neurons different from this in Vero? In this regard, in Figure 6C, why not use the gamma tubulin antibody instead of an anti tubulin?

We agree with the referee that the BICDR-1 and γ-tubulin do not precisely colocalize. We now modified the title and manuscript text and state that BICDR-1/Rab6 vesicles are present in the pericentrosomal region.

The BICDR-1 distribution is not so different in Vero cells and neurons – in both cell it labels the pericentrosomal region. Although there is a much larger variation in BICDR-1 staining in primary neurons, mostly likely caused by the temporal heterogeneity in the neuron cultures, the staining in developing neurons is generally more extended into the cell body. However, it should be noted that size of a neuron at DIV1 is much smaller than a single Vero cell, so direct comparison between these images is a bit misleading. We now separated the Vero cells and neurons staining in different figures (Fig. 3F and Fig. 5B). Another important difference between BICDR-1/Rab6 staining in
neurons versus Vero cells, is the fact that in many Vero cells the centrosome is moved away from the Golgi stacks and is situated on top of the nucleus. This makes the pericentrosomal staining quite obvious, especially when compared to cells where the centrosome/pericentrosomal region is mixed with the Golgi (Fig. S2F; cell in the upper left corner). In neurons, we only see the vesicular BICDR-1/Rab6 staining mixed with the Golgi making the pericentrosomal localization less obvious (Fig. 5C).

In Fig. 6A (in the original manuscript Fig. 6C), we use tubulin staining in this experiment to outline the morphology of the neuron.

5) I also have trouble with the experiments using the neurons in culture. First, this paragraph that is crucial for the paper is even more condensed than the rest of the text and would deserve more space for clarity.

We reorganized and re-worded the manuscript and decompressed the text where possible.

Second, from the blots, it is clear than after DIV1, BicDR1 level decreases sharply and that Rab6B is not yet expressed. There is only a little bit of Rab6A! At DIV3, there is no BicDR1 and plenty of Rab6A and B. So it seems that in neurons, the two partners of the complex are not expressed at the same time, with the same dynamics. This, to me, shadows the relevance of the in vivo results.

All three proteins are expressed in young hippocampal neurons (Fig. 5A); BICDR-1 and Rab6A are expressed at high levels, while Rab6B is expressed at low levels at DIV 1-2 (long exposure blots show clear signals at DIV 1). From these data it seems most likely that BICDR-1 interacts and coincides with Rab6A (Fig. 5C). On the other hand, BICDR-1 binds more strongly to Rab6B than Rab6A (Fig. 3A,C). At this moment we have no knowledge about the molecular ratios of both Rab isoforms (this is hard to compare since different antibodies are used) in young hippocampal neurons during the time of BICDR-1 expression and whether competition between BICDR-1 and Rab6A / Rab6B takes place. Regardless of the precise Rab6 switching mechanism, our results show that BICDR-1 can both interact with Rab6A/B and regulate dynein-dependent trafficking of these secretory vesicles in young neurons.

I don’t have a problem with the claim that sustained high level of BicDR1 is detrimental to the neuritis outgrowth. The expression of the mutated form of BicDR1 (K512M) does not lead to a neurite outgrowth and that indeed suggests that the effect is mediated by Rab6. In this experiment, BicDR1 could compete artificially with BicD whose role I ma not sure I understand in this context. More experiments should be added to strengthen this point.

We have set up an in vitro competition experiment. In the revised manuscript we show that GST-Rab6A is able to efficiently pull down GFP-BICD2C from cell lysate, while addition of increasing amounts of purified His-BICDR-1-C reduced the amount of precipitated GFP-BICD2C (Fig S2B). These data indicate that BICDR-1 and BICD2 can compete for Rab6 binding in vitro.

We have previously demonstrated that BICD binds to kinesin-1/kif5 and dynein/dynactin (Grigoriev et al, 2007; Hoogenraad et al, 2001) and here we show that BICDR-1 associates with Kif1C and dynein/dynactin. Since both BICDR-1 and BICD regulate Rab6 secretory transport (this study and Matanis, NCB, 2002), and only BICDR-1 overexpression results in pericentrosomal accumulation of Rab6 vesicles it is tempting to speculate that BICDR-1 interaction on Rab6 vesicles changes the transport fate of secretory vesicles. In this model, BICDR-1 could compete with BICD on Rab6 on secretory vesicles, which then switches the balance in the microtubule minus-end direction. We strongly believe that by changing the molecular ratios of BICDR-1/BICD on the Rab6 vesicles during neuronal differentiation, the direction and distribution of secretory vesicle transport can be altered. However, additional studies are required to test the relevance in vivo.

I have reservation on the relevance if this in vivo.

We collaborated with the zebrafish lab of Iain Shepard (Emory University, Atlanta, GA, USA) to determine physiological relevance of BICDR-1. Two independent BICDR-1 specific morpholinos (MOs) where used to block BICDR-1 expression and revealed striking neuronal development abnormalities in zebrafish embryos (Fig. 2B). These data strengthen our observations in cultured neurons and indicate that BICDR-1 is required for early neuronal development in vivo.

6) What is the affinity of BicDR1 for Rab6 and how does this compare to this of BicD (since they have very similar binding sites, Figure 2B)? How do the authors envisage the competition of the two proteins for the binding and that so much?

As discussed above, we show that GST-Rab6A pull downs GFP-BICD2C from cell lysate. However, after addition of purified His-BICDR-1 the amount of precipitated GFP-BICD2C is reduced suggesting that BICDR-1 and BICD2 compete for Rab6A binding (Fig. S2B).
7) The authors show that Rab6A RNAi leads to no BicDR1 at the centrosome? (Is it degraded as there is no staining anywhere?). This is now a suppl but should be shown as a main figure.

We now include new Western blots showing that expression levels of BICDR-1, dynactin (p150Glued), dynein (IC74) and actin (control) remain constant after knockdown of Rab6A (Fig. S3B). These data suggest that BICDR-1 is not degraded but most likely mislocalized and randomly distributed throughout the cytoplasm of the cell. This phenomenon is also seen in γ-tubulin knockdown cells (Fig. S3C)

-Conversely, BicDR1 RNAi leads to no Rab6 at centrosomes. So they need each other to be located to the centrosome and therefore, BicDR1 is not a true effector of Rab6. It is possible that they are recruited by a third partner (Dynein, P150) and then bind to each other once at the centrosome. This is sustained by the fact that Dynein and p150 RNAi leads to no BicDR1 at the centrosome? What happens to Rab6 then?

BICDR-1 is a true effector of Rab6 since BICDR-1 binds to wild type Rab6 (Rab6A-WT) and Rab6-GTP (Rab6A-Q82R) but not to Rab6-GDP (Rab6A-T27N) (Fig. S2A), furthermore Rab6 knockdown leads to loss of pericentrosomal BICDR-1 (Fig. S3D). This suggests that Rab6 activity is necessary to recruit BICDR-1 to secretory vesicles. Additional experiments show that the pericentrosomal localization of Rab6 depends on the presence of BICDR-1 (BICDR-1 knockdown leads to loss of pericentrosomal Rab6; Fig. S4D, E), indicating that BICDR-1 is necessary to connect motor proteins to Rab6 vesicles in order to transport these vesicles towards the centrosome. These results are consistent with our model in which BICDR-1 is a motor-cargo-adapter that links the dynein/dynactin complex to Rab6 positive secretory vesicles and facilitates Rab6 vesicle transport to the microtubule minus-end. It is very well possible that Rab6-BICDR-1-dynein/dynactin form a “triple” complex and that all the components are necessary for the stability of the complex. Interesting in this respect are the data from the Neefjes lab, which demonstrate that Rab7-RLIP-dynein/dynactin forms a complex but require additional regulatory proteins to fully assemble in a functional endosomal motor-adapter complex (Johansson et al, JCB, 2007)

Is Dynein and P150 role in localising BicDR1 through their concentration at the centrosome?

Dynein/dynactin functions primarily as a retrograde motor complex to drive Rab6/BICDR-1 vesicles to the minus-end of the microtubules. Since it has been shown that dynein/dynactin is concentrated at the centrosome, it is very well possible that Rab6/BICDR-1 vesicles bind to dynein/dynactin localized at the centrosome Future experiments are necessary to test whether dynein/dynactin are directly involved in the stabilization secretory vesicles around the centrosome

What happens when gamma tubulin (or centrins) are depleted?

We thank the referee for this interesting point and performed the suggested experiment. Knockdown of γ-tubulin disrupts the pericentrosomal localization of BICDR1. This is most likely caused by the disrupted microtubule organization in the cell, which in turn leads to mistargeting of Rab6/BICDR-1 vesicles and loss of pericentrosomal BICDR-1 (Fig S3C). Moreover, microtubule depolymerization by nocodazole leads to a similar phenotype (Fig. S3A)

8) What are the arrows and arrowheads in Figure 3C?

- The arrow-head indicates a “stable” control secretory vesicles (to show that the focus is not changed) and the arrow shows a vesicle that undergoes exocytosis. This is now clearly indicated in the figure legends.

9) The quantitation in Figure 3D and E is interesting but suggests that BicDR1 overexpression not only concentrates the NPY "vesicles" to the cell centre but also increases their fusion. In fact, there is less NPY and more fusion. The fusion role of BicDR1 should be discussed. Is it relevant?

In the revised manuscript we quantified the effect of BICDR-1 on vesicle fusion/exocytosis in neurons (Fig. 6E,F). We did not observe a significant difference in rate of NPY vesicle fusion (Fig. 6F). Further studies should be done to test other parameters.

10) Figure 5E-J: Why choose a E13.5 mouse embryos and not a E10.5 where BicDR1 level is the highest?

We did this for technical reasons. E10 mouse embryos are well suited for whole mount in situ hybridization because of the efficient penetration and subsequent hybridization of the probe in a whole embryo. Sections are more appropriate to study the precise cellular distribution of BICDR-1. Since in E13.5 mouse embryos the organs/tissues are further developed, it allows us to better recognize the various structures in the developing brain / kidney positive for BICDR-1.
11) Figure 6 is complicated and introduced in an odd manner. Till this point, the reader is geared on BicDR1 at the centrosome together with Rab6 and NPY. But in this figure, it is the dual movement of BicDR1 and Rab6 on vesicle leaving the cell centre and fusing, perhaps, to the periphery (or whatever it shows, which is hard to understand).

We agree with the referee that this part in the original manuscript is a bit confusing. We significantly reorganized the manuscript in order to improve clarity. It is well known that almost all transport vesicles exhibit bidirectional movement. Therefore it is not unusual for BICDR-1/Rab6 vesicles to move away from (anterograde) and towards (retrograde) the cell body. We found a strong correlation between the level of BICDR-1 expression, retrograde transport direction and the accumulation of NPY/Rab6 vesicles in the cell body. At low levels of BICR-1 there is a slight shift towards retrograde transport of Rab6 vesicles, which leads to small accumulations of vesicles in the cell body (Fig. S6A and movie 1), while adding increasing amounts of BICDR-1 completely changes the balance towards retrograde transport and concentrates Rab6 vesicles in the cell body (Fig. 6A, Fig. S6B). We added new figures to clarify this in the revised manuscript (S6A, B).

This is confusing because I thought the message was that BicDR1 was sequestering the vesicles at the cell centre which should be released once BicDR1 level decreased. Here the authors suggest movement with them and this raises the question of the sequestration and how it is mediated. In this regard, I don't understand the sentence Page 9: "BICDR-1 was observed on mobile Rab6-positive vesicles and tubular structures which docked and fused with larger BICDR-1/Rab6 membrane domains in the cell body (Fig. 6D, Movie S3)."

We propose that BICDR-1 is a cargo-adaptor protein present on Rab6 vesicles not only at the centrosome but also throughout the young neuron (Fig. 5C). In our model BICDR-1 can be attracted to Rab6 vesicles at any location in the cell, but once a certain concentration of BICDR-1 is present on these Rab6 vesicles, the direction and distribution of secretory vesicle transport is immediately altered, setting the balance in the retrograde direction. This is now more clearly discussed in the revised manuscript.

Also, as I mentioned above, there is no convincing evidence in Figure 6A and B that Rab6 co-localises with BicDR1 at the cell centre (despite claims starting the paragraph page 9)

We now show in Figure 5C the colocalization of endogenous Rab6 and endogenous BICDR-1 on vesicles in the cell body.

In the same paragraph, I really do not understand the sentence "Consistently, the secretory vesicle markers NPY-GFP and BDNF-GFP are both decreased in the developing neurites and clustered with BICDR-1 and Rab6 in the cell body (Fig. 6A)". And I do not agree with the conclusion: "Together these data indicate that BICDR-1 concentrates secretory vesicles in the neuronal cell body and prevents anterograde transport into growing neurites." I don't see where is the data showing the prevention of anterograde transport. Perhaps a less compact, more restful manner of presentation would lead to less ambiguity in the reasoning.

We now quantify the NPY-GFP distribution in neurons following BICDR-1 expression (Fig. 6D). These data show that expression of BICDR-1 strongly accumulates NPY-GFP positive secretory vesicles in the soma and causes a marked decrease in the number of NPY-GFP vesicles in developing neurites, compared to control cells (Fig. 6C,D). Similar results were obtained with other neuronal secretory vesicle markers, such as GFP-Sema3A and BDNF-GFP (Fig. 6D and Fig. S7F,G). Together these data strongly suggest that BICDR-1 enhances retrograde transport, concentrates secretory vesicles in the neuronal cell body and further prevents anterograde transport into growing neurites.

12) The authors seem to propose that the only way to deliver membrane to the neurites are carriers coming for the cell body. However, it has been shown that at least dendrites have so called Golgi outposts that can supply part of the membrane needed for growth. This is not addressed at all in the introduction.

In young neurons at the time of BICDR-1 expression (DIV1-2) no Golgi outposts are present yet. A study by the Ehlers lab showed that Golgi outposts first appeared after 3 DIV and steadily increased up to 12 DIV, a time at which neurons are undergoing rapid synaptogenesis (Horton and Ehlers, J Neuroscience, 2003)
Fig. 1 for the referees.

GST-pull down assay with GST-Rab6A, GST-Rab11B and extracts of Cos7 cells expressing either Flag-BICDR-1 or Flag-BICDR-1-K512M. Flag-tagged proteins were detected by Western blotting with antibodies against Flag, GST-Rab6A was visualized using Amido Black.

Fig. 2 for the referees.

Hippocampal neuron transfected at DIV2 with mCherry-BICDR-1 (red) and GFP-VAMP7 (green) fixed and stained for Rab6A (blue) after 2 days. Scale bar, 10 µm. Note the lack of VAMP7 and Rab6/BICDR-1 colocalization.
Your revised manuscript has now been re-reviewed by some of the original referees. As you will see, one of them has a few remarks that I would like you to consider. Please provide us with possible modifications that in this case are fully up to your discretion as soon as you can to enable official acceptance and timely publication of your study.

Yours sincerely,

Editor
EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

The ms has been strongly improved and should be published. There are still some typos (e.g.: page 5, line 4: (... and Bicaudal-D related protein 1 (BICDR-2), respectively...); page 14, line 29 : (... and C. elagans...).

I have just some comments that do not require any experiments but that could be briefly discussed: Do authors observe anterograde movements of BDNF-GFP with mCherry-BICDR-1 as BDNF-GFP anterograde transport is dependent on kinesin-1? Then coordination antero/retrograde between BDNF and other secreted factors could be different. Could it be linked to control of post-Golgi secretion (constitutive versus activity dependent secretion)? On page 14, authors suggest that coordination could be mediated by other factors. One good example is certainly the huntingtin protein whose phosphorylation controls BDNF trafficking by recruiting kinesin-1 (Colin et al., EMBO J, 2008).

2nd Revision - Authors' Response

Thank you for your e-mail on February 26th, we are very pleased with the swift and positive response from the referees. We have modified the manuscript according to the additional remarks of referee #2 and hope that our study can now be accepted for publication.