THE EMBO JOURNAL

Manuscript EMBO-2011-78067

NYAP: a phosphoprotein family that links PI3K to WAVE1 signaling in neurons

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Review timeline:

Submission date:	03 May 2011
Editorial Decision:	06 June 2011
Revision received:	22 July 2011
Editorial Decision:	23 August 2011
Revision received:	25 August 2011
Accepted:	30 August 2011

Transaction Report:

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

1st Editorial Decision 06 June 2011

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

As you can see the referees find the analysis interesting. Both referees # 1 and 2 are supportive of publication here pending relative minor changes and I would like to ask you to address their concerns raised in a revised version. Referee #3 is more negative and raises many different issues with the manuscript. As far as I can see most of the requested controls and clarifications are already part of the manuscript. I therefore kindly ask you to go carefully through the points raised by referee #3. Most of the raised issues can be addressed in the point-by-point response and need no further experiments/changes, but if there are suggestions that you find will improve the already strong manuscript you can choose to incorporate that into the text and figures. Just to make it clear, the manuscript is close to being ready for publication here and it is just a matter of tying a few loose ends. If you have any further questions please do not hesitate to contact me.

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

Thank you for submitting your interesting study to the EMBO Journal. I am looking forward to seeing the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript by Yokoyama et al., the authors explore the function of a newly identified family of phosphoproteins, the NYAPs, in neurons. The authors demonstrate that NYAPs are phosphorylated by Fyn in response to stimulation by contactin5 (Tag-1), which results in an increased association of NYAPs with the p85 PI3K subunit. Loss of NYAP 1-3 in mice leads to decreased PI3K, as well as attenuation of pAkt and GTP-loading of the small GTPase Rac. Conversely, overexpression of NYAP1 or NYAP2 is sufficient in enhancing Akt activity in neurons, in dependence of phosphorylation of NYAP proteins. Mass spec analyses of protein complexes that associate with NYAP2 revealed the presence of 4 proteins, several of which represent constituents of the WAVE complex involved in Arp2/3 dependent actin regulation. The authors go on to explore the function of NYAP in the WAVE complex, and identify a region within NYAP essential for WAVE complex association. Co-immunoprecipitation experiments further revealed the presence of a ternary complex consisting of PI3K, NYAPs and WAVE1 in the brain of WT, but not in brain of triple NYAP KO mice or in tissues deficient in NYAP expression. In addition, reconstituting expression of NYAP in HEK293 cells induced the formation of this ternary complex. In general, the analyses of this new signalling complex are rather well done and impressive. I have more, but resolvable, concerns regarding the characterization of the functional significance (see below).

Main concerns:
Figure 8A: The effect of WAVE expression on stress fibers in Hela cells if not convincing as control, non-transfected cells are out of focus, hazy. Overall this image demonstrates the F-actin staining with less contrast than other images within figure. Did authors use similar settings during acquisition of images? NYAP 1-150 and NYAP 1-161 appears to accumulate to the nucleus, which might influence to interpretations of the results? The authors should comment in the text.

Figure 9: Some data would benefit from better and larger images. This concerns A, B, but also images demonstrating the morphology of neurons in E. Also, the authors did specify the neuronal cell type used in the experiments in the text, nor in figure legend.
The authors demonstrate a reduction in overall brain size in NYAP triple KO mice; however, I am not convinced by the presented data that this reduction excludes the possibility of increased apoptotic mechanisms (which was analysed by TUNEL, see supplementary figure) and/or decreases in cell size (which was analysed exactly how?). If the authors pursue an interpretation that connectivity in triple KO brains is affected (see text), a staining using neurofilament antibody (or something similar) should resolve this issue relatively quickly. Overall, the histological analyses of triple knock out brains seem extremely preliminary and far less convincing than the presented biochemistry. Therefore, in order to facilitate rapid publication of this largely impressive and convincing work I recommend omitting some (less convincing) data (analyses of cell death and cell size, nurturing phenotype) and altering the text accordingly. I recommend the authors should include better, high resolution image of the overall effect of loss of NYAPs in the triple KO mice (Nissl stain). Another possibility would be to dissect out whole brains and include images in figure.

Overall, the Material and Method section is rather superficial. For example, information on neuronal type and/or culture conditions should be included.

Others:
Page 10; bottom of page - 'expression of wild-type NYAP1 and NYAP2' should be changed to 'NYAP1 or NYAP2'

Figure 5F: quantification required; does loss of NYAP function in triple KO mice leads to a reduction in total Akt? Authors should include an additional loading control (i.e. GAPDH or else). Also, the authors should test other PI3K activating stimuli in WT and triple KO neurons. For

example, is BDNF (or any other growth factor) mediated activation of PI3K affected in the absence of NYAPs?

Introduction: the statement of 'although neuronal phenotypes of knockout mice of PI3K itself have not been observed' should be modified and reference to Tohda et al. 2006, 2009; Acosta-Martinez et al. 2009; Eickholt et al. 2007.

Referee #2 (Remarks to the Author):

Yokoyama and colleagues describe in the current manuscript the identification and characterisation of a novel protein family tentatively termed NYAP. These proteins are substrates for the Fyn-kinase and are predominantly expressed in the brain. The authors demonstrate convincingly and by using 4 new KO mice (single KO for each NYAP gene and triple KO), that NYAPs interact with the regulatory subunit p85 of Pi3Kinase and with Sra-1 and Nap1, subunits of the WAVE-complex. They show that NYAP functions upstream of Pi3Kinase and subsequent cytoskeletal remodelling but downstream of contactin and Fyn. Contactins are neuronal GPI anchored protein that serve as adhesion molecules, although the mode of signal transduction through the PM is poorly defined. For contactin 1 a cooperation with Notch and/or L1CAM was suggested. The current manuscript clearly places NYAPs between Fyn and Pi3K downstream of contactin5.

The authors first identified a novel Fyn substrate that they termed NYAP1. By database screens they identify homologies to two more gene products, termed NYAP2 and -3, also known as myo16. NYAP1 and 2 lack the myosin motor and F-actin binding domains but share all other features of the NYAP-family described here. The authors carefully determine the spatiotemporal expression pattern and then continue to map the pathway NYAPs are involved in. They demonstrate that contactin engagement leads to Pi3-K activation and downstream signalling such as Akt phosphorylation and Rac-activation. Moreover they show that this requires Tyr-phosphorylation of NYAPs and they even map and mutate the respective residues (2 per NYAP). The striking specificity of this pathway is demonstrated by the fact that RTKs like NGFR, EGFR or PDGFR do not induce NYAP phosphorylation, since they activate Pi3 kinase directly. Thus, this experiment confirms a very specific Pi3-kinase activation path. It remains open whether NYAPs are utilised for Pi3K activation also by other adhesion molecules or by other pathways that involve Fyn kinase activity, or if NYAPs are specific to contactins. The requirement of NYAPs for these processes is step by step demonstrated by using material from the individual and triple. KOs.

The authors further screened for NYAP interaction partners other that p85 and identified the WAVE-complex subunits Sra-1 and Nap as well as the Rho-GAP ARHGAP26 and a protein termed ACOT9. Interaction with the Sra-1 and Nap1 is again analysed in detail. Finally, interesting effects of NYAPs and fragments thereof upon ectopic expression in HeLa cells are described.
To make it a concise story I would concentrate in the discussion on the facts that NYAPs are required for Pi3K activation downstream of contactins, that NYAPs bind to further downstream effectors especially WAVE-complex and promote neuronal morphogenesis (see specific points). Nevertheless, these are a very intriguing findings presented in an impressive and almost complete piece of work.

Specific points
Page 10: The statement "The 150 kDa phosphoprotein was co-migrated with IRS1/2 on the SDS-PAGE (our unpublished observations)." is confusing and should be removed. There are dozens of potentially Tyr-phosphorylated proteins in the brain that have an apparent MW of 150 kDa. Anyway, this is not important for this work

Page 11: The statement "WAVE1, Abi2, HSP70, and several other proteins were detected in the NYAP2 proteome (our unpublished observations)." Should be reworded. It is of importance that these proteins were detected as well, because Sra1 (probably in conjunction with its intimate interactor Nap1) were hypothesized to have WAVE-complex independent functions/interactions in the brain (Napoli et al., 2008; Schenck et al., 2003; Schenck et al., 2001). However, the identification of WAVE and Abi proteins speak for WAVE-complex interaction as stated. This should be explicitly stated.

Page 12: when describing the mapping of the WAVE-complex interaction surface the text is confusing. The statement that "These data suggest that the region around NYAP2(150-161) is critically involved in its interaction with the WAVE complex (see Figure 1B)" does not fully hold true: The fact that all three NYAPS bind to WAVE-complex around this region calls for a conserved interaction surface which is likely that just N-terminal of the claimed motif. Construct NYAP2 1-150 may not bind because just two or three residues of this conserved site (namely KLS) are missing. In addition nothing can be said about the minimal length of this binding site, since N-terminal truncations in this region are not tested. Thus, the authors should replace this argument by the clear statement that the region required for WAVE-complex binding is (i) conserved between NYAPS and (ii) ends in all cases directly N-terminal of the SH2-docking side for p85.
Moreover, since NYAPs bind to the entire WAVE complex, which is a super stable protein assembly, they to date cannot define which complex subunit is responsible for this interaction. NYAPs may equally well bind to Sra-1 nap1, WAVE1,,2,or 3 or to Abi1 or Abi2. They may even bind to Brick that was implicated in similar processes, e.g. neuronal connectivity (Qurashi et al., 2007). This should be clearly stated.

Page 12 and Fig 7a: The sentence "Further, the association was absent in WT livers, HEK293T cells, and the CG4 oligodendrocyte cell line, none of which express the NYAPs (Figure 7A)." needs to be reworded since the liver experiment is not more that a negative control. There is no WAVE1 expressed in liver and so a specific WAVE1 IP can never co-IP anything. So even after transduction of a NYAP gene into liver cells a WAVE1 IP should be negative. If the authors wish, they could test a WAVE2 IP or alternatively reword the statement and change figure and legend accordingly.

Page 12 , Fig 7D and discussion page 17: expression of NYAP in HEK293 causes Pi3K-WAVE interaction. The authors don't know about the molecular basis of WAVE-complex / NYAP interaction thus they again look at WAVE exemplary for the WAVE-complex. This should be made clear. In the discussion they discuss WAVE-protein Tyr phosphorylation (based on Tyr151 in W3) but leave out a proper discussion on yet another important phosphor-tyrosine dependent P85 interaction with a WAVE-complex subunit, important downstream of receptor tyrosine kinases, namely on Abi (Abi1 Y407) (Innocenti et al., 2003). This should be included in the discussion.

Page 13, fig8, Fig S5: Over-expression of 'naked' WAVE1 was reported to suppress lamellipodia and sequester Arp2/3 complex (Machesky and Insall, 1998). This can be seen for instance in Fig S5a. In less frequent cases and dependent on the expression level WAVE1 can be incorporated in the endogenous WAVE-complex and then localise to lamellipodia (as seen in Fig 8A). While WAVE and the entire WAVE-complex are absolutely essential for lamellipodia, neither WAVE nor WAVE-complex induce tem, because they are not the limiting factors. The limiting factor is active Rac whicj locally activates WAVE-comlex.The effects of full length NYAPs on the actin system is striking but looks opposite to what is expected when Rac and WAVE-complex activity are high! The cells display numerous filopodia. Whether stress fibres are reduced or turned into peripheral rather than central stress fibres, is not clear from the images. The authors should seriously consider the possibility that NYAP binds and inhibits WAVE complex. The images provided do not support the notion that WAVE becomes activated. The constructs that only bind WAVE-complex but not Pi3K do not render the stress fiber pattern as stated correctly but again, the cells display more spike like extensions further indicating that there may be a negative rather that positive effect on lemallipodia/ruffle formation at play.

The authors should either state this more clearly as one possible interpretation of these results, or -if they are convinced on their positive mode of action- must provide evidence for WAVE-complex activity.

Minor points:
In Fig 8H the lower right arrow head must be black (non transfected). There are more arrow mix-ups in Fig S5 (D, E, H ect).

Page 18, discussion of GRAF. It may be worth mentioning that the presence of a GAP for RhoA is in line with NYAP-dependent Rac-activation. Local inhibition of RhoA may as such lead to increased rac1 since active RhoA is known to negatively impact on Rac1 via GAPs such as ARHGAP22 and ARHGAP24 (Ohta et al., 2006; Sanz-Moreno et al., 2008).

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Schenck, A., Bardoni, B., Langmann, C., Harden, N., Mandel, J.L., and Giangrande, A. (2003). CYFIP/Sra-1 Controls Neuronal Connectivity in Drosophila and Links the Rac1 GTPase Pathway to the Fragile X Protein. Neuron 38, 887-898.
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Referee #3 (Remarks to the Author):

Yokoyama and colleagues report the characterization of three neuron-specific phospho-proteins (NYAP1-3). The authors take advantage of knockout mice to show that NYAP-family proteins are both substrates of Fyn and the major binding partners of p85 in the brain. Genetic ablation of NYAPs perturbs PI3K signaling. NYAP triple KO mice show reduced brain size and shorter neurites, a phenotype consistent with neuronal hypotrophy. Since NYAPs bind to Sra1 and Nap1, Yamamoto and colleagues claim that NYAPs link PI3K to WAVE-based-complex-mediated actin remodeling to regulate neuronal morphogenesis.

This manuscript can be divided in two parts:
- The characterization of NYAPs as phospho-proteins (Figure 1-4) and the phenotype of the triple KO animals (Figure 9) are in general convincing. The observations that NYAPs are the major phospho-proteins in the mouse brain (i.e. they account for about 80% of the total phosphoproteins) and that PI3K signaling is affect in their absence are novel and might be of interested for people working in the field.

- The signaling part (Figure 5-8) is quite weak and most of the conclusions are either not sound or the result of gross overinterpretations: the data do not really show that NYAPs couple PI3K signals to the WAVE complex. It also does not demonstrate that a PI3K-NYAP-WAVE complex regulates neurite outgrowth.

Although I find the possible existence of an NYAP-mediated link between PI3K and WAVE1 potentially important for a general audience, this study is undermined by adventuring into the signaling field in a superficial manner. The manuscript shows only that NYAP is a phospho-protein implicated in PI3K signaling. All te other sections are preliminary and flawed. Its ambitious title " NYAP: a phospho-protein family that links PI3K to WAVE1 signaling in neurons" is not supported by experimental data.

Major points

In all Figures, total lysates and IPs are shown separately. This makes it impossible to evaluate how strong all these interactions are. Furthermore, internal negative controls are constantly missing (i.e. it is always presented a protein that co-precipitates with another one, but never one that does not).

Figure 1
When I looked at the alignment, my first impression was that these three proteins only shared 2-3 similar aminoacidic stretches. This feeling was further corroborated by the WAVE1-binding region, which actually shows no homology between NYAP1, 2 and 3. Some site-directed mutagenesis is required to claim that this is a genuine WAVE-binding site.
I am wondering if stating that these proteins form a family is justified. There is no information as to how these analyses were carried out.

Figure 3
C: It is impossible to conclude whether phosphorylation of NYAP1-3 is sustained without showing their expression levels. This information should be added.
F-I: Although 293T express Src-family kinases (SFKs), NYAPs require the co-expression of Fyn for phosphorylation to be detected. Since SFKs are supposed to be active in 293T cells, the authors should explain their counterintuitive observations.
Moreover, the Y1F-Y2F mutants still show some phosphorylation, indicating the existence of additional phosphorylation sites. The absence of pTyr signal in panel I might be simply due to epitope-tagged NYAPs having a lower expression in neurons than in 293T cells. Conclusions on this point should be moderated. Alternatively, more analysis is needed to convince the reader that NYAPs possess only to phospho-acceptor sites.

Figure 4
A-B: Y1F does not bind at all to p85, although NYAP1 and 2 have to pYXXMs. Conversely, both Y2F mutants display a reduced ability to interact with p85. The authors should comment on that.
E: The band of about 150kDa co-precipitates with p85 and is also detected by the anti-pYXXM antibodies. Does this suggest that there is a fourth NYAP protein?
F: Both a negative control and the anti-NYAP blots are missing. To formally claim that NYAPs account for the majority of p85-assocated pY, it is essential to show that the anti-p85 antibodies depleted p85 from the lysate.

Figure 5
A: Both negative and positive controls are absent. Most importantly, are NYAPs membrane-bound proteins? This is a critical point that needs to be addressed and represent a key point. If so, do membrane-associated NYAPs bind to PI3K?
B: the reduction in PI3K activity might be due to less PI3K being in the membrane fraction. What if the authors normalize PI3K activity for PI3K levels in wt and TKO membranes?
D: the drop in Rac activity is not convincing.
E: Although these blots have a strong background, NYAP1 overexpression results in a higher AKT activity than NYAP2. However, the latter is more expressed than the former. Knowing how the protein levels of NYAP1 and NYAP2 are increased with respect to the control is essential to understand whether this phenotype is linked to inherent functional properties of these two proteins or simply due to different overexpression levels.

Figure 6
D-F: In addition to being subunits of the WAVE-based complex, Sra1 and Nap1 can form other complexes. (Weiner OD et al., Plos Biology). Therefore, it is absolutely necessary to demonstrate that also Abi1 (or Abi2) and WAVE are pulled down by NYAPs.
In light of the crystal structure of the mini-WRC, it is also important to assess if NYAPs associate with Sra1 and/or Nap1. Next, does phosphorylation of NYAPs play a role in WAVE binding?

Figure 7
None of the anti-WAVE1 (or anti-p85) IPs is probed for NYAPs.
How could the authors come up with the model presented in Figure 7E?
Also, is there any competition between the WAVE complex and p85 for binding to NYAPs?
Why should NYAPs mediate the PI3K-WAVE interaction? It has been reported that Abi1 can directly associate with p85 (Innocenti et al. JCB).

Figure 8 (and S. Fig. 5)
It is not really clear what the authors try to demonstrate here. WAVE1 is poorly expressed in HeLa cells, which have high WAVE2 levels. It is established that WAVE proteins are involved in

membrane ruffling and this is the phenotype that should be investigated. Overall, this figure is obscure.

Figure 9
F-G: Since differences are very small, I dug into how statistical analysis was performed. A one-tail T-test is inappropriate and gives p values two times smaller than a two-tail test (the one to be used). This implies that, in some cases, differences might not be truly significant.
The effect of C5F is dubious (compare G with F): neurite length is about 50 um and upon C5F stimulation becomes about 58. In figure 9F, WT neurites are between 50 and 60. What is the explanation for these discrepancies?
To strengthen this point, it might be useful taking advantage of PI3K inhibitors.
Anyway, these data not show that NYAP simultaneously interact with PI3K and WVE to promote neurite elongation.

Minor points

Figure 2C: It would be nice to see the same analysis in the appropriate KO brain sections.
Figure 3D: the legend gives the reader no information useful to understand how quantification was carried out.
S. Fig 2: NYAP2 has a weak signal. Adding the KO will help assess whther the signal is indeed specific.

Text

The manuscript is clearly written and concise. The discussion is instead lengthy and full of overstatements (some of which are reported below).
The literature on WAVE and the WAVE complex should be cited in a fair manner.

Some suggestions
Pg 4: CDK5 phosphorylates only WAVE1.
Pg 8: The paragraph on Contactin 5 is only for specialist.
Pg 16: the data to do show that NYAPs account for most of the p85-mediated PI3K activation in neurons.
Pg 17: the data to do show that most of the PI3K-WAVE1 association is indirect. May be, WAVE1 simply need to be activated (and/or translocated to the PM) to bind to PI3K.
Pg 18: the data to do show that Contactin, Fyn, NYAPs and PI3K function in the same signaling pathway.

P.S.: I really liked that the authors used NYAP KO mice to generate good antibodies!

1st Revision - authors' response 22 July 2011

A point-by-point list of our responses to the referees' comments
Referee #1

1. *Figure 8A: The effect of WAVE expression on stress fibers in HeLa cells if not convincing as control, non-transfected cells are out of focus, hazy. Overall this image demonstrates the F-actin staining with less contrast than other images within figure. Did authors use similar settings during acquisition of images?*
   NYAP 1-150 and NYAP 1-161 appears to accumulate to the nucleus, which might influence to interpretations of the results? The authors should comment in the text.

As pointed-out by this referee, original Figure 8A which shows the actin phenotype in WAVE1-overexpressing cells looks slightly hazy, although the image acquisition setting was the same as that for other images. In the original manuscript, we showed other WAVE1-overexpressing cells in Supplementary Figure 5A and B, and we could replace these with Figure 8A. Nonetheless, we determined to remove images of WAVE1-overexpressing cells from Figure 8 and Supplementary Figure 10 (Supplementary Figure 5 in the original manuscript) as suggested by referee #2, because we could not discriminate 'naked' WAVE1 from WAVE1 incorporated in the endogenous complex.

Revised Figure 8 now shows that 1) NYAPs regulate remodeling of the actin cytoskeleton and that 2) both PI3K and WAVE complex-binding regions in NYAPs are required for this regulation. We did not state whether NYAPs activate or inhibit the activity of the WAVE complex in HeLa cells. Please see also our response to comment 6 of referee #2.
Because levels of cytoplasmic expression of NYAP2(1-150) and NYAP2(1-161) are similar to that of other mutants used in Figure 8, it is likely that NYAP2(1-150) and NYAP2(1-161) have no effect on actin remodeling. However, as this referee commented, the NYAPs accumulated in the nucleus might have negatively affected remodeling of the actin cytoskeleton. As we can't exclude this possibility at present, we commented on this point in the text (page 14, lines 14 to 20).

2. *Figure 9: Some data would benefit from better and larger images. This concerns A, B, but also images demonstrating the morphology of neurons in E.*
   Also, the authors did specify the neuronal cell type used in the experiments in the text, nor in figure legend.
   The authors demonstrate a reduction in overall brain size in NYAP triple KO mice; however, I am not convinced by the presented data that this reduction excludes the possibility of increased apoptotic mechanisms (which was analysed by TUNEL, see supplementary figure) and/or decreases in cell size (which was analysed exactly how?). If the authors pursue an interpretation that connectivity in triple KO brains is affected (see text), a staining using neurofilament antibody (or something similar) should resolve this issue relatively quickly. Overall, the histological analyses of triple knock out brains seem extremely preliminary and far less convincing than the presented biochemistry. Therefore, in order to facilitate rapid publication of this largely impressive and convincing work I recommend omitting some (less convincing) data (analyses of cell death and cell size, nurturing phenotype) and altering the text accordingly. I recommend the authors should include better, high resolution image of the overall effect of loss of NYAPs in the triple KO mice (Nissl stain). Another possibility would be to dissect out whole brains and include images in figure.

Appreciating the referee's constructive comments, we omitted the "less convincing" data, such as TUNEL, cell size, and nurturing phenotypes. In addition, as suggested by the referee, we included enlarged Nissl-staining and photographs of whole brains in Figure 9 of the revised manuscript and amended description accordingly (page 15, lines 9 to 11). Finally, I would like to add that we analyzed cortical neurons in this study, which is now clearly stated in the revised text and figure legend (page 15, line 18, page 23, line 10, and page 46, line 21).

3. *Overall, the Material and Method section is rather superficial. For example, information on neuronal type and/or culture conditions should be included.*

In the Materials and Methods section of the revised manuscript, we added detailed information such as neuronal cell type, cell density, and concentration of poly-L-lysine in neuronal culture.

4. *Page 10; bottom of page - 'expression of wild-type NYAP1 and NYAP2' should be changed to 'NYAP1 or NYAP2'*

We corrected the sentence as suggested (page 11, line 14).

5. *Figure 5F: quantification required; does loss of NYAP function in triple KO mice lead to a reduction in total Akt? Authors should include an additional loading control (i.e. GAPDH or else).*
   Also, the authors should test other PI3K activating stimuli in WT and triple KO neurons. For example, is BDNF (or any other growth factor) mediated activation of PI3K affected in the absence of NYAPs?

We quantified amount of total Akt in WT and TKO brains. The data are shown in Supplementary Figure 5 and added relevant description in the revised text (page 11, lines 11 to 12).
We also cultured WT and TKO neurons and added BDNF, and found that Akt was activated in TKO neurons as in WT. The data are shown in Supplementary Figure 6 and described in the revised text (page 11, lines 21 to 22).

6. *Introduction: the statement of 'although neuronal phenotypes of knockout mice of PI3K itself have not been observed' should be modified and reference to Tohda et al. 2006, 2009; Acosta-Martinez et al. 2009; Eickholt et al. 2007.*

As suggested, we modified the statement to include neuronal phenotypes of PI3K knockout mice (page 3, lines 12 to 16).

Referee #2

1. *Page 10: The statement "The 150 kDa phosphoprotein was co-migrated with IRS1/2 on the SDS-PAGE (our unpublished observations)." is confusing and should be removed. There are dozens of potentially Tyr-phosphorylated proteins in the brain that have an apparent MW of 150 kDa. Anyway, this is not important for this work*

As suggested, we removed the statement on IRS1/2 (page 10, lines 18 to 19).

2. *Page 11: The statement "WAVE1, Abi2, HSP70, and several other proteins were detected in the NYAP2 proteome (our unpublished observations)." should be reworded. It is of importance that these proteins were detected as well, because Sra1 (probably in conjunction with its intimate interactor Nap1) were hypothesized to have WAVE-complex independent functions/interactions in the brain (Napoli et al., 2008; Schenck et al., 2003; Schenck et al., 2001). However, the identification of WAVE and Abi proteins speak for WAVE-complex interaction as stated. This should be explicitly stated.*

As suggested, we reworded to state clearly that WAVE1 and Abi2, as well as Sra1 and Nap1, are components of the WAVE complex (page 12, lines 9 to 11). In addition, to further confirm the observation obtained from the proteome analysis, we newly added data showing the interaction between NYAPs and WAVE1 proteins in exogenous expression system (HEK293T cells; Supplementary Figure 8) and in the brain (Supplementary Figure 9). These data are described in the revised text (page 12, lines 14 to 17). We also carefully worded WAVE-complex independent functions/interactions of Sra1 and cited suggested references (page 12, lines 12 to 14).

3. *Page 12: when describing the mapping of the WAVE-complex interaction surface the text is confusing. The statement that "These data suggest that the region around NYAP2(150-161) is critically involved in its interaction with the WAVE complex (see Figure 1B)" does not fully hold true: The fact that all three NYAPS bind to WAVE-complex around this region calls for a conserved interaction surface which is likely that just N-terminal of the claimed motif. Construct NYAP2 1-150 may not bind because just two or three residues of this conserved site (namely KLS) are missing. In addition nothing can be said about the minimal length of this binding site, since N-terminal truncations in this region are not tested. Thus, the authors should replace this argument by the clear statement that the region required for WAVE-complex binding is (i) conserved between NYAPS and (ii) ends in all cases directly N-terminal of the SH2-docking site for p85.*

   Moreover, since NYAPs bind to the entire WAVE complex, which is a super stable protein assembly, they to date cannot define which complex subunit is responsible for this interaction. NYAPs may equally well bind to Sra-1, Nap1, WAVE1,2,or 3 or to Abi1 or Abi2. They may even bind to Brick that was implicated in similar processes, e.g. neuronal connectivity (Qurashi et al., 2007). This should be clearly stated.

We took seriously the referee's comment on the WAVE complex interaction surface and stated, as suggested, that the region required for the interaction with the WAVE complex is conserved between the NYAPs just before the NHMs (page 13, lines 2 to 10). We also marked this region with an orange box in Figure 1B of the revised manuscript.
The precise mode of interaction between NYAPs and the WAVE complex is not clear at this moment. Regarding Brick/HSPC300, we could not detect Brick/HSPC300 in the proteome analysis (Figure 6A), possibly due to its very small molecular weight. However, given the super stable nature of the WAVE complex, as this referee pointed, it is very likely that Brick/HSPC300 is also associated with the NYAPs. This point was briefly mentioned in the revised text (page 12, lines 11 to 12 and page 18, lines 7 to 8) and we cited the suggested reference (page 20, lines 18-19).

4. *Page 12 and Fig 7a: The sentence "Further, the association was absent in WT livers, HEK293T cells, and the CG4 oligodendrocyte cell line, none of which express the NYAPs (Figure 7A)." needs to be reworded since the liver experiment is not more than a negative control. There is no WAVE1 expressed in liver and so a specific WAVE1 IP can never co-IP anything. So even after transduction of a NYAP gene into liver cells a WAVE1 IP should be negative. If the authors wish, they could test a WAVE2 IP or alternatively reword the statement and change figure and legend accordingly.*

We reworded the description to clearly indicate that the liver experiment was carried out as a negative control (page 13, lines 17 to 19 of the revised manuscript). In addition, although we mentioned in the figure legend of the original manuscript that WT livers were examined as a negative control, this statement was moved to the text of the revised manuscript (page 13, lines 18 to 19).

5. *Page 12, Fig 7D and discussion page 17: expression of NYAP in HEK293 causes Pi3K-WAVE interaction. The authors don't know about the molecular basis of WAVE-complex / NYAP interaction thus they again look at WAVE exemplary for the WAVE-complex. This should be made clear.*
   In the discussion they discuss WAVE-protein Tyr phosphorylation (based on Tyr151 in W3) but leave out a proper discussion on yet another important phospho-tyrosine dependent p85 interaction with a WAVE-complex subunit, important downstream of receptor tyrosine kinases, namely on Abi (Abi1 Y407) (Innocenti et al., 2003). This should be included in the discussion.

As suggested, we explicitly described that we examined WAVE1 exemplary for the WAVE1-complex (page 13, lines 13 to 15). I would like to add that this is because the commercially obtained anti-Nap1 and anti-Sra1 antibodies were not applicable for immunoprecipitation.
Regarding Abi proteins, we commented on the Abi-p85 interaction in the discussion of the revised manuscript (page 18, line 10 to page 19, line 3). There we also cited the work by Dubielecka *et a*l (2010). However, tyrosine phosphorylation of Abi proteins in the WAVE complex (in the WAVE1 immunoprecipitate) was not visible in our hand (Supplementary Figure 9) and its association with p85, probed by anti-phosphotyrosine blotting (Figure 4E), was undetectable in the brain. Furthermore, WAVE-PI3K association was completely lost in TKO brains (Figure 7A). Thus, Abi-mediated association of PI3K with the WAVE complex is unlikely in the brain, although it would be relatively strong in MEF cells and the LNCaP prostate adenocarcinoma cell line where Abi-p85 association has been reported. We discussed these issues in the text properly (page 18, line 10 to page 19, line 3).

6. *Page 13, fig8, Fig S5: Over-expression of 'naked' WAVE1 was reported to suppress lamellipodia and sequester Arp2/3 complex (Machesky and Insall, 1998). This can be seen for instance in Fig S5a. In less frequent cases and dependent on the expression level WAVE1 can be incorporated in the endogenous WAVE-complex and then localise to lamellipodia (as seen in Fig 8A). While WAVE and the entire WAVE-complex are absolutely essential for lamellipodia, neither WAVE nor WAVE-complex induce them, because they are not the limiting factors. The limiting factor is active Rac which locally activates WAVE-complex. The effects of full length NYAPs on the actin system is striking but looks opposite to what is expected when Rac and WAVE-complex activity are high! The cells display numerous filopodia. Whether stress fibres are reduced or turned into peripheral rather than central stress fibres, is not clear from the images. The authors should seriously consider the possibility that NYAP binds and inhibits WAVE complex. The images provided do not support the notion that WAVE becomes activated. The constructs that only bind WAVE-complex but not Pi3K do not render the stress fiber pattern as stated correctly but again, the cells display more spike like extensions further indicating that there may be a negative rather that positive effect on lamellipodia/ruffle formation at play. The authors should either state this more clearly as one possible interpretation of these results, or -if they are convinced on their positive mode of action- must provide evidence for WAVE-complex activity.*

As the referee pointed, presence of many limiting factors (such as Rac) makes it difficult (or even inappropriate) to estimate the activity of the WAVE complex by comparing with the phenotype in ‘naked WAVE1’ expressing cells. So, we determined to remove WAVE1-overexpression from this figure, and clearly stated that “precise action of NYAPs on the WAVE1 complex remains to be

elucidated." (for example, page 17, lines 5 to 7 and page 18, lines 7 to 8). In the revised version, our conclusions of HeLa assays are 1) NYAPs regulate remodeling of the actin cytoskeleton, and 2) both PI3K and WAVE1 interacting surfaces are required, although 3) activity of the WAVE complex remains unclear. Accordingly, we carefully reworded the entire manuscript as follows: #1, "NYAPs-mediated PI3K-WAVE1 association allows remodeling of the actin cytoskeleton" instead of "allows efficient actin remodeling" (page 2, line 11); #2, "the NYAPs activate PI3K and concomitantly recruit the downstream effector WAVE protein to the close vicinity of PI3K and regulate neuronal morphogenesis" instead of "to regulate neuronal morphogenesis" (page 2, line 14; page 5, lines 1 to 2); #3, "contribute to remodeling of the actin cytoskeleton in this assay" instead of "contribute to the efficient activation of the WAVE complex" (page 15, line 5). Moreover, the result section on the effect of NYAPs on the actin cytoskeleton in HeLa cells were divided with a subheading "The NYAPs mediate remodeling of the actin cytoskeleton" from the section describing NYAPs-WAVE interaction for clarification (page 14, line 4).

7. *In Fig 8H the lower right arrowhead must be black (non transfected). There are more arrow mix-ups in Fig S5 (D, E, H etc).*

We believe that there are no arrow mix-ups. As was written in the figure legend of the original manuscript, black and white arrowheads indicated the state of the actin stress fibers, regardless of whether the cells were transfected or not. Nevertheless, to avoid confusion, we restated this in the text (page 14, lines 8 and 10).

8. *Page 18, discussion of GRAF. It may be worth mentioning that the presence of a GAP for RhoA is in line with NYAP-dependent Rac-activation. Local inhibition of RhoA may as such lead to increased rac1 since active RhoA is known to negatively impact on Rac1 via GAPs such as ARHGAP22 and ARHGAP24 (Ohta et al., 2006; Sanz-Moreno et al., 2008).*

Because RhoA activity is downregulated in the TKO brain (76% of WT; our unpublished observation), downregulation of Rac1 activity observed in the whole brain of TKO mice (Figure 5D) is not likely mediated by the well-established mutual antagonism between RhoA and Rac1. Therefore, participation of GRAF in Rac1 regulation may be only locally occurring. This point is included in the revised manuscript together with suggested references (page 19, lines 18 to 20).

Referee #3

All Figures: total lysates and IPs are shown separately. This makes it impossible to evaluate how strong all these interactions are. Furthermore, internal negative controls are constantly missing (i.e. it is always presented a protein that co-precipitates with another one, but never one that does not).

We demonstrated 'relative' strength of the interaction: for example, in Figure 4E, we showed that NYAPs are the predominant tyrosine-phosphorylated proteins found in the anti-p85a immunoprecipitates. No other tyrosine-phosphorylated proteins except the 150 kDa phosphoprotein were detected by the anti-phosphotyrosine antibodies. Therefore, we concluded that NYAPs-PI3K interaction is "relatively" stronger than the interaction between PI3K and any other tyrosine-phosphorylated proteins expressed in the brain. We agree with the referee in that the interaction strength (such as Kd) is important biochemical factors, but we believe that such information is beyond the focus of this manuscript. As for negative controls, we carefully included appropriate controls in the original manuscript: in NYAPs IP, we always included NYAPs TKO brains which ruled out the possibility of nonspecific-binding of the antibodies; in PI3K IP and WAVE IP, we examined with another antibodies. For example, we used anti-p85 from MBL (mouse monoclonal) and Millipore (rabbit polyclonal) and obtained the same results. For WAVE IP, we confirmed the results by reciprocal immunoprecipitation with anti-PI3K p85a antibodies. These were all described in the initial version of the manuscript.

Figure 1: When I looked at the alignment, my first impression was that these three proteins only shared 2-3 similar aminoacidic stretches. This feeling was further corroborated by the WAVE1-binding region, which actually shows no homology between NYAP1, 2 and 3. Some site-directed mutagenesis is required to claim that this is a genuine WAVE-binding site. I am wondering if

stating that these proteins form a family is justified. There is no information as to how these analyses were carried out.

Although only several stretches, such as the NHM, are shared by all three members of the NYAP family, there are long stretches shared by two of them; for example, the N-terminal regions of NYAP2 and 3, and the C-terminal regions of NYAP1 and 2.
As pointed by referee #2 (comment 3), only a part of NYAP2(150-161) (possibly, $K^{150}L^{151}S^{152}$) may directly participate in the interaction with the WAVE1 complex. This is why we described "NYAP2(150-161) is critically involved in the interaction" rather than "NYAP2(150-161) interacts with the complex." The augment by this referee and referee #2 was probably raised because we emphasized the importance of NYAP2(150-161). To clarify the concerns on the WAVE-binding sequence, we modified Figure 1B and the text (please see also our response to comment 3 of referee #2).
As for the justification of the family, we regarded NYAP1, 2, and 3 as a family based on the partial similarity in the NHM. This is clearly described in page 6, lines 15 to 16 of the revised manuscript. Although not mentioned in the text, their common ability of binding with the p85 subunit of PI3K and the ability to mediate WAVE1-PI3K association also help justify that they are members of the same family.

Figure 3: C: It is impossible to conclude whether phosphorylation of NYAP1-3 is sustained without showing their expression levels. This information should be added.
F-I: Although 293T express Src-family kinases (SFKs), NYAPs require the co-expression of Fyn for phosphorylation to be detected. Since SFKs are supposed to be active in 293T cells, the authors should explain their counterintuitive observations.
Moreover, the Y1F-Y2F mutants still show some phosphorylation, indicating the existence of additional phosphorylation sites. The absence of pTyr signal in panel I might be simply due to epitope-tagged NYAPs having a lower expression in neurons than in 293T cells. Conclusions on this point should be moderated. Alternatively, more analysis is needed to convince the reader that NYAPs possess only two phospho-acceptor sites.

C: Levels of NYAPs protein expression were shown in the original manuscript (Supplementary Figure 11 of the revised manuscript).
F-I: We believe that it is not counterintuitive that exogenous FynYF expression further enhances tyrosine kinase activity in cells. Indeed, as shown in the bottom panel of Figure 3F-H (anti-Fyn blotting), expression levels of FynYF are much higher than those of endogenous Fyn. These are described in the revised figure legend (page 42, lines 22 to 24).
As for the remaining phosphorylation faintly seen in Y1F-Y2F mutants, we moderated the conclusion as suggested (page 9, lines 16-18).

Figure 4: A-B: Y1F does not bind at all to p85, although NYAP1 and 2 have two pYXXMs. Conversely, both Y2F mutants display a reduced ability to interact with p85. The authors should comment on that.
E: The band of about 150kDa co-precipitates with p85 and is also detected by the anti-pYXXM antibodies. Does this suggest that there is a fourth NYAP protein?
F: Both a negative control and the anti-NYAP blots are missing.
To formally claim that NYAPs account for the majority of p85-assocated pY, it is essential to show that the anti-p85 antibodies depleted p85 from the lysate.

A-B: As for Y1F-PI3K binding (Figure 4A and B), we described possible mechanisms in the revised text (page 10, lines 3 to 8).
E: As for the 150 kDa phosphoprotein (Figure 4E), we could not find proteins with NYAP homology sequences in the database screening. This is described in page 6, lines 16 to 17 of the revised manuscript. In addition, WAVE1-PI3K association completely disappeared in NYAP1, 2, and 3 triple KO mice, suggesting that there are no more members in the NYAP family.
F: As for PI3K IP experiments (Figure 4E and F), we do not think that anti-NYAPs blots are necessary to draw the conclusion that NYAPs account for the majority of PI3K p85-associated phosphorylation. Here, we are dealing with phosphoproteins and performed anti-p85 IP to selectively precipitate phosphorylated forms of NYAPs. Anti-NYAPs blotting data may be informative, though not essential, in some degree for estimating biochemical properties of interaction between PI3K p85 and individual members of the NYAP family. But, unfortunately, it is

very difficult to detect co-precipitated NYAPs with our anti-NYAP antibodies, despite our attempt to generate good antibodies as this referee noticed (*P.S.: I really liked that the authors used NYAP KO mice to generate good antibodies!*). Again, we believe that such detailed biochemical information is beyond the focus of this manuscript. Presence of phosphorylated NYAPs indicates presence of NYAPs themselves in the p85a immunoprecipitates.
To ensure the specificity of the experiment (that is, to rule out the possibility that anti-p85 (from MBL) non-specifically precipitates unknown proteins which bind with the NYAPs or anti-p85 (MBL) reacts with NYAPs themselves), we used another anti-p85 antibody (from Millipore) and obtained virtually the same results. This is described in the original manuscript (correspond to the description in page 43, lines 17 to 19 of the revised manuscript).
Regarding efficiency of anti-p85a immunoprecipitation, we do not think it's necessary to completely precipitate p85a from the lysates to draw the conclusion, because this figure shows the "relative" abundance of the NYAPs among PI3K-associated tyrosine phosphoproteins. Moreover, as was evident in the anti-p85a blot in Figure 4F, the amount of precipitated p85a from the P56 brain lysate was much lower than that from the P0 brain lysates. This observation indicated that the amount of anti-p85 antibody we used was not saturated with p85a protein in the lysate.

Figure 5: A: Both negative and positive controls are absent.
Most importantly, are NYAPs membrane-bound proteins? This is a critical point that needs to be addressed and represent a key point. If so, do membrane-associated NYAPs bind to PI3K?
B: the reduction in PI3K activity might be due to less PI3K being in the membrane fraction. What if the authors normalize PI3K activity for PI3K levels in wt and TKO membranes?
D: the drop in Rac activity is not convincing.
E: Although these blots have a strong background, NYAP1 overexpression results in a higher AKT activity than NYAP2. However, the latter is more expressed than the former. Knowing how the protein levels of NYAP1 and NYAP2 are increased with respect to the control is essential to understand whether this phenotype is linked to inherent functional properties of these two proteins or simply due to different overexpression levels.

A: Membrane localization of NYAPs is newly demonstrated in Supplementary Figure 4. Detection of association between membrane-bound NYAPs and PI3K is technically difficult, because membrane fraction is densely packed at the bottom of the ultracentrifuge tube and unable to be lysed in immunoprecipitation buffer. We also analyzed membrane localization of WAVE1 protein as a control which does not change between WT and TKO.
B: As for PI3K activity assay (Figure 5B), we started from equal amount of total brain lysates, and the activity was not normalized to PI3K levels in the membrane (this is clearly stated in the Figure legend, page 44, lines 3 to 4). In the present work, the precise biochemical and structural mechanism of PI3K activation remains unknown: for example, we are not sure whether or not NYAPs induce conformational changes of the PI3K p85-p110 dimer which leads to activation of PI3K. But, again, we believe that analysis of such biochemical details belongs to the future study.
D: Rac1 activity (Figure 5D): As shown in the figure, we demonstrated the statistical significance, and indeed, in all cases Rac1 activity is lower in TKO than in WT.
E: Levels of adenovirus-mediated overexpression of NYAP1 and 2 (Figure 5E): Levels of endogenous NYAPs were very low in this small number of neuronal cells infected with the adenovirus. Therefore, most of NYAPs in these cells were likely expressed from the adenovirus vectors. Given that the level of NYAP2 overexpression was higher than that of NYAP1 overexpression, our data may suggest that NYAP1 activates PI3K/Akt stronger than NYAP2. In a similar vein, we noticed that association of NYAP1 with the WAVE1 complex was much weaker than those of NYAP2 and 3 (please compare NYAP1, 2, and 3 in Supplementary Figure 9). These observations suggest that biochemical characteristics of NYAP1, 2, and 3 are slightly distinct in a quantitative point of view. In the present study, we analyzed common features of NYAPs. Individual roles for NYAP1, 2, and 3 will be addressed in the future.

Figure 6: D-F: In addition to being subunits of the WAVE-based complex, Sra1 and Nap1 can form other complexes (Weiner OD et al., PLoS Biology). Therefore, it is absolutely necessary to demonstrate that also Abi1 (or Abi2) and WAVE are pulled down by NYAPs. In light of the crystal structure of the mini-WRC, it is also important to assess if NYAPs associate with Sra1 and/or Nap1.
Next, does phosphorylation of NYAPs play a role in WAVE binding?

D-F: Please see our response to comment 2 of referee #2 for the NYAP-WAVE interaction. As referee #2 pointed, the entire WAVE complex is a super stable protein assembly and we to date cannot define which subunit in the complex is responsible for this interaction.
As illustrated in Figure 1B, the WAVE binding region is distinct from the phosphotyrosine-containing NHM motifs and phosphorylation of NYAPs may have nothing to do with WAVE binding. These were described in the original manuscript (page 13, lines 10 to 11 of the revised manuscript).

Figure 7: None of the anti-WAVE1 (or anti-p85) IPs is probed for NYAPs. How could the authors come up with the model presented in Figure 7E?
Also, is there any competition between the WAVE complex and p85 for binding to NYAPs? Why should NYAPs mediate the PI3K-WAVE interaction? It has been reported that Abi1 can directly associate with p85 (Innocenti et al. JCB).

To address the first issue, we newly performed experiments and demonstrated the presence of NYAPs in the WAVE1 immunoprecipitates (shown in Supplementary Figure 9 of the revised manuscript).
There is no competition between the WAVE complex and p85 for binding to NYAPs, because p85 is present in the WAVE1 immunoprecipitate. Moreover, binding surfaces of the NYAPs to the WAVE complex and p85 are different, which was described in the original manuscript (page 13, lines 10 to 11 of the revised manuscript).
As for Abi-mediated interaction between the WAVE complex and p85, see our response to comment 5 of referee #2.

Figure 8 (and S. Fig. 5): It is not really clear what the authors try to demonstrate here. WAVE1 is poorly expressed in HeLa cells, which have high WAVE2 levels. It is established that WAVE proteins are involved in membrane ruffling and this is the phenotype that should be investigated. Overall, this figure is obscure.

The relevance of the data in these figures were constructively discussed in our responses to comment 6 of referee #2 who had also related (but not identical) concern. So, please see our response to referee #2.

Figure 9: F-G: Since differences are very small, I dug into how statistical analysis was performed. A one-tail T-test is inappropriate and gives p values two times smaller than a two-tail test (the one to be used). This implies that, in some cases, differences might not be truly significant.
The effect of C5F is dubious (compare G with F): neurite length is about 50 um and upon C5F stimulation becomes about 58. In figure 9F, WT neurites are between 50 and 60. What is the explanation for these discrepancies? To strengthen this point, it might be useful taking advantage of PI3K inhibitors.
Anyway, these data not show that NYAP simultaneously interact with PI3K and WAVE to promote neurite elongation.

F-G (J-K of the revised manuscript): As pointed-out here, we agree that the two-tail test is appropriate. Then, we analyzed again with the two-tail test and described actual p values in the text (page 15, lines 11, 13, 14, 20, and page 16, lines 1 and 2).
Figure 9F and G (Figure 9J and K in the revised version) are the data from different experiments: slight differences in the culture conditions (the lot of medium, B27 supplement, and dishes, or addition of mock medium) may have caused the discrepancies.
As for the causal relationship between the PI3K-NYAP-WAVE1 interaction and neurite elongation, we carefully reworded the discussion (page 17, lines 7 to 8 in the revised manuscript). Please see also our response to comment 6 of referee #2.

Minor points:
Figure 2C: It would be nice to see the same analysis in the appropriate KO brain sections.

We included KO sections in Figure 2C of the revised manuscript.

Figure 3D: the legend gives the reader no information useful to understand how quantification was carried out.

We included the marker lane, and described that "Protein concentration of C5Fc was estimated by comparing with SDS-PAGE Standards (high range, Bio-Rad)" (page 42, lines 8 to 9).

S. Fig 2: NYAP2 has a weak signal. Adding the KO will help assess whether the signal is indeed specific.

Indeed, Figure 2C and D and Supplementary Figure 2 were mounted on the same glass slide. We added description in the Supplementary figure legend of the revised manuscript that "WT sections were mounted on the same glass slides containing sections from corresponding *Nyap1*, *2*, or *3* KO mice as negative controls (see Figure 2C and D)."

Pg 4: CDK5 phosphorylates only WAVE1.

Appreciating the referee's comment, we amended description in page 4, line 15 of the revised manuscript.

Pg 8: The paragraph on Contactin 5 is only for specialist.

We agree with this comment and added more information on Contactins in the revised text (page 9, lines 1 to 4).

Pg 16: the data to do show that NYAPs account for most of the p85-mediated PI3K activation in neurons.

To be correct, we reworded the sentence to "NYAPs account for most of PI3K-associated tyrosine phosphorylation in neurons" (page 17, line 21).

Pg 17: the data to do show that most of the PI3K-WAVE1 association is indirect. May be, WAVE1 simply need to be activated (and/or translocated to the PM) to bind to PI3K.

We newly observed that WAVE1 membrane localization was unaffected in TKO mice. These data are shown in Figure 5A of the revised manuscript.

Pg 18: the data to do show that Contactin, Fyn, NYAPs and PI3K function in the same signaling pathway.

We demonstrated that Contactin5-induced activation of the PI3K-Akt pathway was dependent on the NYAPs (Figure 5F). The data indicate that Contactin, Fyn, NYAPs, and PI3K function in the same signaling pathway. This is described in page 20, lines 4 to 5 of the revised manuscript. But we are not concluding that these molecules are always functioning in this signaling pathway, which we don't think necessary to mention.

2nd Editorial Decision 23 August 2011

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

As you can see below, both referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore pleased to say that we can accept the paper for publication here. Before doing so, referee #2 has a few minor suggested text changes that I would like to incorporate in a revised manuscript. As soon as we receive the revised version we will proceed with the acceptance of the paper for publication here. Please include a point-by-point response when you submit the revised version.

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have been very responsive to the points raised by the reviewers. This is the first characterization of a novel regulator within the PI3K signaling pathway, and the current version is clearly worthy of publication in EMBO Journal.

Referee #2 (Remarks to the Author):

Yokoyama and colleagues describe in the revised manuscript the identification and characterisation of a protein family termed NYAP. These proteins are substrates for the Fyn-kinase and predominantly expressed in the brain. The authors demonstrate convincingly and by using 4 new KO mice (single KO for each NYAP gene and triple KO), that NYAPs interact with the regulatory subunit p85 of Pi3Kinase and with the WAVE-complex. They show that NYAP has a crucial function in the Rac-WAVE-complex signalling axis but the exact nature of this function remains unknown. However the authors aklso show that the signal path is downstream of contactin, an adhesion molecule) and involved Fyn-activity. The authors carefully determine the spatiotemporal expression pattern and then continue to map the pathway NYAPs are involved in. They demonstrate that NYAP engagement leads to Pi3K-activation and Rac-activation.
The authors moreover identify two important tyrosines, map and mutate these residues. The pathway is demonstrated to be very specific to Contactin since RTKs like NGFR, EGFR or PDGFR do involve NYAP phosphorylationa and activate Pi3 kinase directly.
The authors further screened for NYAP interaction partners other that p85 and identify the WAVE-complex in addition to others. In the following, the authors establish a causal relation between NYAPs, PI3K and WAVE-complex.

The authors have carefully addressed the reviewer's comments and amended the text. This mainly was toning down statements that were too strong to be supported by the data. I would have appreciated more restructuring of the manuscript. However, given that the authors here describe for the first time three independent knockouts and a corresponding triple knockout, and even reach quite some molecular detail in describing the pathways that involve NYAPS, I will not request further experimentation that may just come because the findings made me curious. I am convinced that publication of the current data is important to the field since this study will lead to improved understanding how PI3K-Rac-WAVE-signaling contributes to neuronal connectivity and probably pave the way to insights in genetic variations that affect the brain without being lethal, which are at the basis of disease rather than lethal ones.

minor comment:

the abstract still contains the sentence:

..., which we show here, allows remodelling of the actin cytoskeleton.

I would remove this, since the exact effect of WAVE-complex interaction remains unclear. This statement seems to promise something that is not answered in the ms.

Additionally, the text needs a bit of editing for typos and readability. I would remove statements to "own unpublished observations".
Example
NYAPs triple knockout (TKO) mice were apparently healthy and fertile, but showed a maternal nurturing defect and a mating defect (our unpublished observations).

suggestion
NYAPs triple knockout (TKO) mice were apparently healthy and fertile and are currently analyzed for behavioral abnormalities.

The model in Figure 7 contains F-?) actin. The presence of actin (or Arp2/3 complex in between!) and the status of WAVE-complex activity was not tested. Thus I would remove actin. I would

remove ACOT9 and GRAF from it since they were not further studies.

I would attempt to place the model to the end of the figures/story

2nd Revision - authors' response 25 August 2011

A point-by-point list of our responses to the referees' comments

Referee #2

1. *the abstract still contains the sentence:*
   ..., which we show here, allows remodelling of the actin cytoskeleton.
   I would remove this, since the exact effect of WAVE-complex interaction remains unclear. This statement seems to promise something that is not answered in the ms.

As suggested, we removed this phrase from the abstract (page 2, lines 9 to 11).

2. *Additionally, the text needs a bit of editing for typos and readability. I would remove statements to "own unpublished observations".*
   Example
   NYAPs triple knockout (TKO) mice were apparently healthy and fertile, but showed a maternal nurturing defect and a mating defect (our unpublished observations).
   Suggestion
   NYAPs triple knockout (TKO) mice were apparently healthy and fertile and are currently analyzed for behavioral abnormalities.

As suggested, we removed "our unpublished observations" as follows:
Page 6, lines 18-20: We reworded the sentence as suggested by the referee.
Page 9, line 6: We removed the sentence "Administration of the Contactin6-Fc protein and the anti-Contactin5 antibody also induced tyrosine phosphorylation of the NYAPs in neurons (our unpublished observations)."
Page 12, lines 7 and 8 "WAVE1, Abi2, HSP70, and several other proteins were detected in the NYAP2 proteome (our unpublished observations)": The referee pointed previously that detection of WAVE1 and Abi2 in the proteome is informative. So, we decided not to remove the whole sentence. We identified these proteins (WAVE1 and Abi2; score 422 and 203 in the Mascot analysis, respectively) in the excised gel slice of approximately 75kDa, in which GRAF was found to be most abundant (Figure 6A). Therefore, we decided not to include WAVE1 and Abi2 in Figure 6A but instead described their presence in the text as a fact, and simply removed the phrase "our unpublished observations" from this sentence.
Page 17, lines 17 and 18 "In contrast, the repertoires of PI3K p85-associated phosphoproteins vary by cell types" in the discussion: We believe that these data are peripheral to the major message of this manuscript but important to discuss this work in the broader context. So, we decided not to remove this sentence.

3. *The model in Figure 7 contains F-?) actin. The presence of actin (or Arp2/3 complex in between!) and the status of WAVE-complex activity was not tested. Thus I would remove actin. I would remove ACOT9 and GRAF from it since they were not further studies.*

As suggested, we removed actin, GRAF, and ACOT9 from the model. We also removed relevant description from the figure legend (that is, "MYO16/NYAP3 has a myosin motor domain and directly interacts with F-actin.").

4. *I would attempt to place the model to the end of the figures/story*

Because the number of figures is limited to 9, we decided not to place the model as Figure 10. We believe that the model, which represents the summary of the molecular analysis, would be suitable at the end of Figure 7 rather than at Figure 9 which shows brain and neuronal phenotypes.

