THE
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Pentraxin 3 regulates synaptic function by inducing AMPA receptor clustering via ECM remodeling and β1-integrin

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Review timeline:

Submission date:	29th Mar 2018
Editorial Decision:	14th May 2018
Revision received:	11th Aug 2018
Editorial Decision:	5th Sep 2018
Revision received:	12th Sep 2018
Accepted:	1st Oct 2018

Editor: Karin Dumstrei

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 14th May 2018

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

As you can see from the comments, both referees appreciate the findings reported. They raise a number of different issues that I anticipate that you should be able to address in a good manner. Given the input from good experts in the field, I would like to invite you to submit a revised manuscript. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

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://emboj.embopress.org/about#Transparent_Process

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

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #1:

This extensive work identifies pentraxin 3 (PTX3) as an important factor secreted from astrocytes in synapse maturation. More specifically, the authors show that recombinant PTX3 augments via its N-terminal portion the frequency and amplitude of mEPSCs of glutamatergic synapses. PTX3 also increases the number (density) of puncta of the presynaptic active zone protein bassoon that are positive for detection of AMPARs by their surface labeling with an antibody against their N-termini. It appears that PTX3 acts by recruiting AMPAR to so-called silent synapses, which had little to no AMPARs before PTX3 application. Their work implicates PTX3-induced changes in the ECM as colocalization of the chondroitin sulfate-containing protein aggrecan is increased by PTX3 and treatment with hyaluronidase removes aggrecan from the ECM surrounding neurons and prevents PTX3-induced postsynaptic AMPAR recruitment. They further implicate the ECM protein TSG6 and beta3 integrin in PTX3-induced AMPAR recruitment as it was abrogated in TSG6 KO neurons and by an antibody that impairs integrin beta1 function.

The authors find that PTX3 binds with its N-terminus to the C-terminal region of thrombospondin (TSP), another factor secreted from astrocytes that promotes synapse formation. Interestingly, whereas TSP augments the number of synapses as defined by the number of presynaptic bassoon puncta apparently likely increases synaptic strength of individual synapses, PTX3 increases specifically the number of active synapses (i.e. it unsilences silent synapses) without affecting bassoon puncta density. These data are supported by a reduction in density of sGluA-positive bassoon puncta and mEPSC frequency and, less so, amplitude in PTX3 KO mice.

I only have one somewhat major concern and one minor.

Major Concern

1. Fig. 5 F/G shows that TSP but not PTX3 increases bassoon puncta density and Fig. 5H/I that PTX3 increases the fraction of bassoon puncta that contain sGluA immunoreactivity in the absence but not presence of full length TSP. In the presence of the E123 fragment of TSP, which by itself increases bassoon puncta density to the same extend as full length TSP presumably by binding to the gabapentin/pregabalin receptor alpha2delta but by itself does not bind to PTX3, PTX3 can increase the fraction of bassoon puncta that show GluA surface labeling. The interpretation is that full length bassoon inhibits TSP by binding it with its C-terminal region. What is missing is a direct test of the effect of TSP1 on the density of glutamatergic synapses. The effect of TSP1 on the density of bassoon puncta that are positive for sGLuA is not shown but needs to be included to make sure findings from the earlier work, which also did not fully investigate this aspect of TPS action, apply to the current system. In addition, bassoon labels most synapses including glutamatergic and GABAergic ones. It would be desirable to analyze changes in the density of a synaptic marker specific for glutamatergic synapses such as VGlut1 and ideally in addition mEPSC frequency and amplitude under the various conditions in parallel (e.g., effect of TSP, PTX3, and TSP1+PTX3).

Minor Concern

2. The authors should more clearly describe the binding studies that define the exaxct region(s) of TSP that bind to PTX3. It is not immediately clear if E123 is required for PTX3 binding although, importantly, it is clear that E123 by itself doesn't bind PTX3.

Referee #2:

The manuscript (87833) entitled, "The innate immune molecule PTX3 regulates the synaptic content of AMPA receptors via extracellular matrix remodeling and beta 1 integrin" by Fossati et al. provides evidence for a molecular mechanism through which astrocyte-derived pentraxin3 (PTX3) induces neuronal AMPA receptor clustering via signaling through the perineuronal net. Authors utilized in vitro neuron and/or astrocyte cultures from wild-type, TSG6 knockout, or PTX3 knockout animals to determine the role of matrix/PTX3 interactions in synapse development in vitro. They show that miniature EPSC frequency and amplitude increase upon treatment of hippocampal neurons by full length PTX3 or N-terminal PTX3. Authors also demonstrated reduced

miniature EPSCs in vivo, using hippocampal slices obtained from PTX3 knockout animals. In addition, by using biochemical approaches, authors identified domains of Thrombospondin 1 (TSP1) that interact with PTX3 and provided evidence that TSP1 abolishes PTX3-induced AMPAR clustering which is dependent on integrin β1-signalling.
Overall, this study is potentially important and exciting, because the results show that a glial pentraxin (PTX3) can regulate GluA clustering and functional development of synapses. The authors also provide some mechanistic insights into how PTX3 controls functional synapse formation by exploring three different avenues (TSG6, integrin β1 and TSP1). However, these mechanistic aspects of the study remain superficial and do not provide a complete story that explains PTX3 function at the synapse. Furthermore, there are several major issues/concerns that the authors should address prior to be considered for publication;
1. As a general concern for the entire manuscript, the results should be better presented by including representative images that correspond to the quantitative data and by providing rigorous controls for the reagents used (such as antibodies and assays). For example, in Figure 1 we are presented by PTX3 ELISA and qPCR results; however, there are no controls that provide assurance that the PTX ELISA or qPCR methods used are accurate.
2. The current representative images and the results shown in bar graphs are often contradictory to each other. For example, Fig. 2J, N-terminal PTX3 treatment does not seem to induce additional GluA clustering compared to Ctr or C-term. However, the bar graph in Fig. 2K describes the N-term PTX3 treatment is as effective as TTX. The same issues are repeatedly found in many figures throughout the manuscript making it hard to trust the rigorousness of the data analyses which underlie the quantification presented in the graphs.
3. The authors use GluA&Bsn/Bsn as a measure to quantify GluA containing synaptic puncta. By the description they provide, the ratio between the # of Bsn that juxtapose GluA cannot be more than the total number of Bsn. So, the ratios should always be below 1. But in several of the graphs the ratios are often above 1. Please explain and clarify. As it is described currently, it does not make sense.
4. It is unusual that a factor that enhances synaptic AMPAR content, such as PTX3 as the authors indicate, is mostly increasing the frequency of synaptic events rather than increasing the amplitude. The authors explain this by a statement "In fact, exogenous addition of AMPARs to the post-synaptic density or upregulation of postsynaptic protein levels have been previously shown to result in elevation of mEPSC frequency but not amplitude (Sinnen et al, 2017; Sun & Turrigiano, 2011)." This is a confusing and inaccurate statement. An increase in frequency of synaptic events signify either an increase in presynaptic release probabilities or an increase in the number of functional synapses. Based on all the data that they provide; a better interpretation of their results would be that PTX3 converts silent postsynapses to AMPAR containing functional ones. Thus, PTX3 increases the numbers of functional synapses resulting in a net increase in the frequency of postsynaptic events.
5. Relative necessity of glial PTX3 for GluA clustering should be further supported by additional experiments. Previous studies found that astrocyte-secreted Glypican 4 induces surface clustering of GluA1 via neuronal pentraxin secretion from the presynapses (Allen at al., 2012 and Farhy-Tselnicker 2017). The manuscript does not provide any insight into the relationship between the effects of PTX3-mediated GluA clustering compared to Glypican4/NPTX-signaling. Are these two signals cooperative or opposing? Do they target different types of AMPARs? Also they most certainly should site Farhy-Tselnicker, Neuron, 2017.
6. In Fig. 3, the experiments utilizing neurons isolated from TSG6 KO lack proper negative and positive controls.
7. In Fig. 4, authors claim that beta 1 integrin is involved in PTX3-mediated synapse formation. These results, though interesting, are very preliminary and does not necessarily fit with the rest of the story line. These claims should be further supported by directly testing sufficiency and necessity of beta 1 integrin in neurons for the functions attributed to PTX3. Particularly, mEPSCs in Fig. 4E shows that addition of MEK1 inhibitor already exerts a strong reduction compared to control, hence, it is not clear if blocking downstream of beta 1 integrin causes reduced frequency in the mEPSC of PTX3 + PD treatment.
8. Fig. 5F and G should be accompanied by post-synaptic marker staining as shown in Fig. 3A to identify and clarify synaptogenic versus synaptic clustering functions of PTX3.

1st Revision - authors' response 11th Aug 2018

Point to Point rebuttal letter

Referee #1:

1. *Fig. 5 F/G shows that TSP but not PTX3 increases bassoon puncta density and Fig. 5H/I that PTX3 increases the fraction of bassoon puncta that contain sGluA immunoreactivity in the absence but not presence of full length TSP. In the presence of the E123 fragment of TSP, which by itself increases bassoon puncta density to the same extend as full length TSP presumably by binding to the gabapentin/pregabalin receptor alpha2delta but by itself does not bind to PTX3, PTX3 can increase the fraction of bassoon puncta that show GluA surface labeling. The interpretation is that full length bassoon inhibits TSP by binding it with its C-terminal region. What is missing is a direct test of the effect of TSP1 on the density of glutamatergic synapses. The effect of TSP1 on the density of bassoon puncta that are positive for sGLuA is not shown but needs to be included to make sure findings from the earlier work, which also did not fully investigate this aspect of TPS action, apply to the current system. In addition, bassoon labels most synapses including glutamatergic and GABAergic ones. It would be desirable to analyze changes in the density of a synaptic marker specific for glutamatergic synapses such as VGlut1 and ideally in addition mEPSC frequency and amplitude under the various conditions in parallel (e.g., effect of TSP, PTX3, and TSP1+PTX3).*
We thank the Reviewer for having raised these issues. This prompted us to carry more experiments and analyses, which -we believe- further increased the strength of our study. In particular:

i) What is missing is a direct test of the effect of TSP1 on the density of glutamatergic synapses: **The effect of TSP1 (or E123) on the density of bassoon puncta was already shown in fig. 5G. In addition, we have now analyzed the effect of TSP1 on synaptic GluA puncta (Fig 5I, see point ii), confirming that TSP1 increases bassoon puncta density but has no effect on GluA puncta.**

ii) The effect of TSP1 on the density of bassoon puncta that are positive for sGLuA is not shown but needs to be included to make sure findings from the earlier work, which also did not fully investigate this aspect of TPS action, apply to the current system: **To address the Reviewer's request, we analyzed the effect of TSP1 on the density of synapses containing surface GluA. While throughout the manuscript data were analyzed by measuring the colocalizing surface GluA and Bsn puncta normalized to total Bsn puncta (surface GluA&Bsn/Bsn), we have here performed a different type of analysis (quantification of the density of synaptic GluA puncta) because TSP1 induces an increase in synapse number and therefore Bsn puncta. The new figure (Fig. 5I) shows that first, TSP1 does not affect the density of GluA puncta and, second, that the ability of PTX3 to enhance synaptic GluA content is prevented by TSP1 co-incubation but not by co-incubation with the E123 fragment of TSP1.**

iii) It would be desirable to analyze .... mEPSC frequency and amplitude under the various conditions in parallel (e.g., effect of TSP, PTX3, and TSP1+PTX3): **As suggested by the Reviewer, we have analyzed mEPSC frequency and amplitude under the various conditions in parallel (e.g., effect of TSP1, PTX3, and TSP1+PTX3). The results, which**

fully confirm the data obtained by sGluA quantitation are now reported in new Suppl. Fig. 6.

iv) It would be desirable to analyze changes in the density of a synaptic marker specific for glutamatergic synapses such as VGlut1: **concerning this point, we wish to point out that we used Bsn as a reference marker for quantifying synaptic surface AMPARs throughout the paper because Bsn specifically labels the synaptic active zones, while a vesicular presynaptic marker, such as vGLUT1, may also label synaptic vesicle clusters which are detectable in neuronal cultures even in the absence of synaptic contacts (Matteoli et al. JCB 1992; Kraszewski et al. J Neurosci 1995). Furthermore, we have shown that PTX3 specifically enhances glutamatergic but not GABAergic neurotransmission by means of electrophysiological and confocal approaches (see supplemental figure 3 for a complete morphological and functional characterization of PTX3 action on GABAergic inhibitory synapses).**

Minor Concern

2. The authors should more clearly describe the binding studies that define the exact region(s) of TSP that bind to PTX3. It is not immediately clear if E123 is required for PTX3 binding although, importantly, it is clear that E123 by itself doesn't bind PTX3.

The reviewer raises an interesting issue. Our data indicate that a fragment of TSP1 comprising the E123, Ca and the C-terminal globular domains binds PTX3. The finding that E123 and Ca do not bind PTX3 suggests that these two domains are not directly involved in PTX3 recognition. However, the structure of the signature domain is complex, with intricate molecular interactions among residues belonging to different domains. Therefore, as suggested by the reviewer, we cannot exclude an indirect involvement of E123 or of the Ca domain in the binding of TSP1 to PTX3, through an effect on the overall structure of the signature domain. Results and Discussion have been modified to take into account this possibility (pages 8-9 and 14).

Referee #2:

1. As a general concern for the entire manuscript, the results should be better presented by including representative images that correspond to the quantitative data and by providing rigorous controls for the reagents used (such as antibodies and assays). For example, in Figure 1 we are presented by PTX3 ELISA and qPCR results; however, there are no controls that provide assurance that the PTX ELISA or qPCR methods used are accurate.

We thank the Reviewer for raising these issues, which have been now fully addressed. In particular:

i) As a general concern for the entire manuscript, the results should be better presented by including representative images that correspond to the quantitative data….: **See answer to point 2.**

ii) ….and by providing rigorous controls for the reagents used (such as antibodies and assays). For example, in Figure 1 we are presented by PTX3 ELISA and qPCR results;

however, there are no controls that provide assurance that the PTX ELISA or qPCR methods used are accurate.

ELISA used to quantify murine PTX3 expression has been developed from original reagents, i.e. monoclonal antibodies and recombinant proteins, produced by the Authors. Supplemental fig. 1A shows the results the screenings performed when we were developing monoclonal antibody (MoAb) 2C3 against murine PTX3. The supernatant of 2C3 hybridoma was tested against human and murine PTX3. Panel 1A reports the dose response result of 2C3 on immobilized murine or human PTX3, showing no interaction with human PTX3 while the antibody recognizes murine PTX3. This antibody is then used as capturing antibody in the ELISA assay for measuring murine PTX3 levels in the present study. In particular, the procedure was the following: purified recombinant murine and human PTX3 were immobilized in 96 well-ELISA plates (Nunc MaxiSorp 446612) in 15mM carbonate buffer pH 9.6 (4°C O/N). After blocking for 2 hrs with 5% dry milk in PBS 1X with Ca^{++} Mg^{++} + 0.05% Tween 20, pH 7.00. Then different dilutions of 2C3 were added.

The evaluation of the amplification efficiency of real-time RT-PCR assay designed for PTX3 expression was assessed by preparing a standard curve using 5-fold serial dilutions of the cDNA extracted from astrocyte primary cell cultures (from 420ng to 3,36ng). Supplemental Fig. 1D shows a base-10 semi-logarithmic graph that plots the threshold cycle (Ct) versus the concentration of the sample. Both PTX3 and GAPDH, as reference gene, fit the data into a straight line, which are parallel to each other (slope respectively -3.04 and -3.07, R2>0.9) The amplification specificity was confirmed by the melting curve profile, showing one single peak, produced at the end of the PCR, heating slowly the products from 60°C to 95°C the products and measuring the fluorescence (Suppl Fig 1B and C).

Concerning the PTX3 blocking antibody (MNB4): the specificity of this tool has been extensively characterized in previous publications (Scarchilli, 2007; Doni, 2015, see page 10), which were already quoted in the manuscript.

2. The current representative images and the results shown in bar graphs are often contradictory to each other. For example, Fig. 2J, N-terminal PTX3 treatment does not seem to induce additional GluA clustering compared to Ctr or C-term. However, the bar graph in Fig. 2K describes the N-term PTX3 treatment is as effective as TTX. The same issues are repeatedly found in many figures throughout the manuscript making it hard to trust the rigorousness of the data analyses which underlie the quantification presented in the graphs.

We apologize with the Reviewer for these inconsistencies. We have now substituted the following panels in order to provide more representative images: all panels in Figs. 2J and 3K; panels PTX3 and aCD29+PTX3 in Fig. 4A; panels Ctr and PTX3 in Fig. 5H; panel blocking Ab in Fig.6A; panel WT in Fig.6C. Also to facilitate the readers, arrowheads have been added to figure panels.

3. The authors use GluA&Bsn/Bsn as a measure to quantify GluA containing synaptic puncta. By the description they provide, the ratio between the # of Bsn that juxtapose GluA cannot be

more than the total number of Bsn. So, the ratios should always be below 1. But in several of the graphs the ratios are often above 1. Please explain and clarify. As it is described currently, it does not make sense.

The measurements of GluA-containing synaptic puncta were always shown as normalized values to the vehicle-treated conditions, to facilitate the readers in appreciating the change of this parameter upon treatment. This was already stated in the figure legends.

4. It is unusual that a factor that enhances synaptic AMPAR content, such as PTX3 as the authors indicate, is mostly increasing the frequency of synaptic events rather than increasing the amplitude. The authors explain this by a statement "In fact, exogenous addition of AMPARs to the post-synaptic density or upregulation of postsynaptic protein levels have been previously shown to result in elevation of mEPSC frequency but not amplitude (Sinnen et al, 2017; Sun & Turrigiano, 2011)." This is a confusing and inaccurate statement. An increase in frequency of synaptic events signify either an increase in presynaptic release probabilities or an increase in the number of functional synapses. Based on all the data that they provide; a better interpretation of their results would be that PTX3 converts silent postsynapses to AMPAR containing functional ones. Thus, PTX3 increases the numbers of functional synapses resulting in a net increase in the frequency of postsynaptic events.

We thank the Reviewer for her/his comment, which nicely summarizes the major finding of our study, i.e. that "PTX3 increases the numbers of functional synapses resulting in a net increase in the frequency of postsynaptic events". As suggested by the Reviewer, we have now removed the sentence "*In fact, exogenous addition of AMPARs to the post-synaptic density or upregulation of postsynaptic protein levels have been previously shown to result in elevation of mEPSC frequency but not amplitude (Sinnen et al, 2017; Sun & Turrigiano, 2011)*", which could be confusing, from the Results section. However, it is very clear that, following alterations of PTX3 levels, a consistent change in both mEPSC frequency and synaptic surface GluA content is always detectable, whereas mEPSC amplitude changes are less robust. This is in line with literature data, showing that mEPSC frequency is the first parameter to be modulated by moderate increases of synaptic strength. Indeed, it has been reported that the addition of AMPARs to the post-synaptic density through an optogenetic-based approach or the up-regulation of postsynaptic protein levels results in elevation of mEPSC frequency but not amplitude (Sinnen et al., 2017, Neuron 93, 646–660; Sun and Turrigiano, J Neurosci 2011; Saglietti et al. Neuron 2007; El-Husseini et al., 2000; Sala et al., 2001; Roussignol et al., 2005). These considerations have been added in the Discussion (page 12).

5. Relative necessity of glial PTX3 for GluA clustering should be further supported by additional experiments. Previous studies found that astrocyte-secreted Glypican 4 induces surface clustering of GluA1 via neuronal pentraxin secretion from the presynapses (Allen at al., 2012 and Farhy-Tselnicker 2017). The manuscript does not provide any insight into the relationship between the effects of PTX3-mediated GluA clustering compared to Glypican4/NPTX-signaling. Are these two signals cooperative or opposing? Do they target different types of AMPARs? Also they most certainly should site Farhy-Tselnicker, Neuron, 2017.

We thank the Reviewer for having raised this point. PTX3 and Glypican 4 appear to work through different mechanisms. In particular, glypican 4, which enhances active excitatory synapse formation (Allen et al. 2012), increases the expression and release of neuronal pentraxin 1 (NP1 or NPTX1), which is in turn responsible for recruiting dendritic GluA1-containing AMPARs (Farhy-Tselnicker, Neuron, 2017). These data have been quoted in the revised text (pages 6 and 13). We now provide evidence that, conversely, neuronal exposure to PTX3 does not affect the expression of NP1, NP2 or glypican 4 (new Suppl Fig. 5), indicating that PTX3-mediated GluA clustering and Glypican4/NPTX-signaling are independent processes, probably acting at different developmental stages of neuronal development. These data have been described and discussed in the revised text (pages 6 and 13).

6. In Fig. 3, the experiments utilizing neurons isolated from TSG6 KO lack proper negative and positive controls.

We thank the Reviewer for having raised this point, which was fully addressed in the revised manuscript. We now provide the results of a new set of experiments using TSG6 +/+ and -/- littermates, showing that PTX3 increases the number of functional excitatory synapses in WT, TSG6 +/+ but not in TSG6-/- neurons (pages 6-7). To assess whether the lack of effects upon PTX3 administration in TSG6 -/- cultures could result from possible defects in receptor trafficking, we tested the effect of 24hrs exposure to TTX, which induces GluA insertion into the plasma membrane. The results showed that TTX exposure enhances synaptic surface GluA content in both +/+ and -/- cultures (indicating that the AMPARs insertion process is not altered *per se* in TSG6-/- neurons), whereas PTX3 is effective only in +/+ neurons. Therefore, TSG6 is a necessary component of the extracellular matrix in this process.

7. In Fig. 4, authors claim that beta 1 integrin is involved in PTX3-mediated synapse formation. These results, though interesting, are very preliminary and does not necessarily fit with the rest of the story line. These claims should be further supported by directly testing sufficiency and necessity of beta 1 integrin in neurons for the functions attributed to PTX3. Particularly, mEPSCs in Fig. 4E shows that addition of MEK1 inhibitor already exerts a strong reduction compared to control, hence, it is not clear if blocking downstream of beta 1 integrin causes reduced frequency in the mEPSC of PTX3 + PD treatment.

We performed the experiment suggested by the Reviewer to investigate whether the involvement of integrin and ERK signaling in the functions attributed to PTX3 are directly linked or rather independent redundant pathways. To do this, we exploited the surface GluA assay that specifically assesses the postsynaptic GluA clustering. Indeed, mEPSC activity may be influenced by presynaptic effects of b1 integrin and ERK1/2 inhibition. Consistently, as noticed by the Reviewer, analysis of "mEPSCs in Fig. 4E shows that addition of MEK1 inhibitor already exerts a strong reduction compared to control", that does not appear when GluA clustering is examined (Fig 4H.) The results of the surface GluA assay, showed in new figure 4I, indicated that the concomitant blockade of b1 integrin and ERK1/2 signaling by co-incubation with αCD29 and PD does not induce additive effects relative to single αCD29 or PD applications. Of note, the inhibition of each of the two pathways individually produces a rather complete blockade

of the PTX3-induced effect (see also fig. 4B and H). These data support the hypothesis that, in this process, b1 integrin and ERK1/2 are not independent pathways, but they are instead linked. These considerations have been added to text (pages 7-8).

8. Fig. 5F and G should be accompanied by post-synaptic marker staining as shown in Fig. 3A to identify and clarify synaptogenic versus synaptic clustering functions of PTX3.

The experiment suggested by the Reviewer is not doable in our conditions. Indeed, we assessed the effects of TSP1 (or E123) in synapse formation, concomitantly with the effect of PTX3 on surface GluA clustering. We therefore quantified synapse density (bsn/µm in fig 5F-G) as well as the density of surface GluA (sGluA/µm in new fig 5H-I) in the same neuronal coverslips, stained for Bsn, surface GluA and tubulin. We did not have the possibility to add also a postsynaptic marker in this experimental setting. However, we have previously shown that Bsn staining in neuronal cultures is equivalent to, or even more stringent than, PSD-95 labeling (Supplemental fig 2A-B).

2nd Editorial Decision 5th Sep 2018

Thanks for sending me the revised version. Your study has now been re-reviewed by the referees and their comments are provided below. As you can see both referee appreciate the introduced changes and support publication here.

Before we can move ahead with the formal acceptance of the paper there are just a few things to sort out. You can send me the revised version using the link below

We need

- A running title

- An ORCID ID for Michela Matteoli

- 3-5 keywords

- An author contribution section

- MS in word format

- For the figures. We no longer have supplemental figures => you can have 5 expanded view figures - If you have more supplemental figures they will go into an appendix. Please see http://emboj.embopress.org/authorguide#expandedview. Note that EV figure legends should be added to the MS text and that the appendix needs to have a ToC.

- We need an author checklist - please see guide to authors

- We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Could you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels).

- Regarding the title - I think it would be nice to mention that PTX3 regulates synaptic function. Would something like this maybe work? Please feel free to modify and change

The innate immune molecule PTX3 regulates synaptic function by inducing AMPA receptor clustering via extracellular matrix remodeling and β1 integrin

Let me know if you have any further questions

REFEREE REPORTS:

Referee #1:

The authors thoroughly addressed the concerns.

Minor:

Please note that at times "synaptically" is misspelled as "sinaptically"

Referee #2:

The authors have thoroughly addressed all of my previous concerns. The conclusions the authors make are now well supported by the newly added data/analyses. Overall, this manuscript now provides potential mechanistic insights into how the astrocyte-driven PTX3 control functional synapse formation/AMPAR clustering via iTSG6, integrin β1, and TSP1.

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Corresponding Author Name: Elisabetta Menna, Michela Matteoli

Journal Submitted to: EMBO journal
Manuscript Number: EMBOJ-2018-99529R1

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

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

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

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http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	There is no sample size calculation stated in the text. However our sample sizes are similar or larger than similar studies in the field and all statistical tests were selected as appropriate to the sample sizes.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See above.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from analysis. For in vitro experiments, neuronal cultures showing some signs of poor health (such as fragmentation of neuronal processes) were not included in the experimental groups, so that they did not contribute to the data.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Confocal images were quantified in an automated unbiased method by using ImageJ or Fiji softwares, as indicated in the methods section. For in vivo experiments, littermates mice were assigned to the different experimental groups in a randomic way. For in vitro experiments, since single dishes of a same neuronal culture preparation were assumed to be equal each other, we did not conceive any relevant process of randomization and neuron dishes were randomly assigned to the different experimental groups.
For animal studies, include a statement about randomization even if no randomization was used.	For in vivo experiments, we used littermates; mice of the same genotype were randomly assigned to experimental groups to reduce procedural bias.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	To overcome subjective bias during result analysis, automated sotware-based methods of analysis were employed in the different experimental settings: confocal images were analyzed by ImageJ or Fiji; electrophysiology data by Clampfit-10.6 or Mini Analysis softwares as specifically reported in the methods section.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding procedures were applyed in analyzing the data. Analyses of slice electrophysiology experiments were carried out using Clampfit-10.6 software, which is a computer-based unbiased method.
5. For every figure, are statistical tests justified as appropriate?	Yes, to the best of our knowledge. Distribution of data was assessed by D'agostino-Pearson omnibus normality test or Shapiro-Wilk normality test by using GraphPad Prism software. Then parametric tests were used for normally distributed data and non-parametric tests were used in cases of not normally distributed data, as clearly stated in figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, which were tested in each case. In particular, we tested if data are normally distributed before applying any statistical test with the D'Agostino-Pearson omnibus normality test or Shapiro-Wilk test and choose the appropriate (parametric or not) statistical test accordingly.

Is there an estimate of variation within each group of data?	Yes we used the standard error of the mean, as specified in every figure legend.
Is the variance similar between the groups that are being statistically compared?	Yes, as you may appreciate by SEM given in the figures.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	This information is provided in the Methods section of the manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C57bl6 WT mice (Charles-River), PTX3 KO mice (129/Sv-C57Bl/6 mixed and 129/Sv inbred genetic background, born in house) and TSG6 KO mice (C.129S6-Tnfaip6tm1Cful/J, Strain 012903, Jackson labs). PTX3 KO and TSG6 KO mice come from a breeding het x het. Animals were housed and bred in the SPF animal facility in individually ventilated cages, with water and food ad libitum and following the cycle 12H light/dark.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Procedures involving animals handling and care were conformed to protocols approved by the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council directive 2010/63/EU, OJ L 276/33, 22-09-2010; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). All efforts were made to minimize the number of mice used and their suffering.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm it

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

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

Antibodypedia
1DegreeBio
ARRIVE Guidelines

NIH Guidelines in animal use
MRC Guidelines on animal use
Clinical Trial registration
CONSORT Flow Diagram
CONSORT Check List

REMARK Reporting Guidelines (marker prognostic studies)

Dryad

Figshare

dbGAP

EGA

Biomodels Database

MIRIAM Guidelines
JWS Online
Biosecurity Documents from NIH
List of Select Agents