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TLR3 downregulates expression of schizophrenia gene Disc1 via MYD88 to control neuronal morphology

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

1st Editorial Decision 25 April 2016

Thank you for the submission of your manuscript to EMBO reports. I have read your work with interest, and discussed it with my colleagues here. I regret to say that we all agree that as it stands, it is not well suited for us.

We note that your study reports that TLR3 inhibits Disc1 expression in a Myd88-dependent and cytokine-independent manner. You show that poly(I:C) treatment inhibits neurite growth of cultured cortical and hippocampal neurons in a TLR3- and Myd88-dependent manner, that this effect is independent of cytokine secretion, that TLR3 knockdown increases dendritic growth, that poly(I:C) treatment downregulates psychiatric disease related genes in cultured neurons and in vivo, that it reduces Disc1 protein levels in cultured neurons in a TLR3 and Myd88-dependent manner, and that Disc1 overexpression rescues neurite length of poly(I:C) treated neurons while the expression of a schizophrenia-associated Disc1 mutant does not.

We recognize that your findings that only wildtype but not mutant Disc1 can rescue neurite growth of poly(I:C) treated neurons are potentially interesting, as they suggest that viral infections could reduce Disc1 levels and thus neurite growth, with potential implications for the development of psychiatric diseases. However, we also note that the data are nearly exclusively based on cell culture experiments, and that both TLR3 and Disc1 are known to control neurite outgrowth. A link between immune activation and Disc1 and psychiatric diseases has also been reported before. We therefore think that in vivo data would be required to support your findings and for consideration of the manuscript for publication here. I am sorry to say that as it stands, we have decided not to proceed with in-depth review.

1st Revision - authors' response 21 June 2016

Two months ago, we submitted our manuscript entitled “TLR3 downregulates expression of schizophrenia gene *Disc1* via MYD88 to control neuronal morphology” by Chen et al. (corresponding author: Yi-Ping Hsueh) to *EMBO Reports*. You recommended an *in vivo* rescue study would be required for you to consider publication of our study.

We very much appreciate your constructive suggestion. We totally agree that the *in vivo* rescue experiment is important to support and strengthen our findings. Therefore, we applied *in utero* electroporation to investigate the *in vivo* effect of *Disc1* overexpression on poly(I:C) treatment. We now provide more evidence showing that overexpression of DISC1 in mouse brain results in resistance to the reduction of dendritic arborization caused by TLR3 activation. These *in vivo* rescue experiments suggest that DISC1 indeed mediates the downstream of TLR3 signaling in the regulation of neuronal morphology and also link innate immunity, neuronal development and psychiatric diseases. With this new *in vivo* study, we hope you can reconsider our submission.

Thank you again for your consideration and we look forward to hearing from you.

2nd Editorial Decision 14 July 2016

Thank you for the submission of your manuscript to our journal. We have now received the full set of referee reports that is copied below, as well as cross-comments from referee 2.

As you will see, while referee 1 is positive, referee 3 thinks that the study is not sufficiently developed. This is a borderline decision that could go either way. I sent referee 3's concerns to referee 2 and s/he comments that identifying the signals downstream of TLR3 that mediate the effects on gene expression and neuron morphology is out of the scope of this study. However, referee 2 agrees that it would be interesting to investigate effects of TLR3 on the synapse, as this would provide a link to neuron function. We therefore decided that all referee concerns and suggestions should be addressed except for the signaling mechanisms downstream of TLR3 that impact on gene expression and neuron morphology. Referee 2 also remarks that one novel finding of your study is that TLR3 interacts with Myd88 in neurons, and that these data should be strengthened along the lines suggested.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 7 main figures, I suggest that you layout your manuscript as a full article. For a normal article there are no length limitations, but it should have more than 5 main figures, the results and discussion sections must be separate, and the entire materials and methods included in the main manuscript file. Please change the reference style to the numbered EMBO reports style that can be found in EndNote. Supplementary data at EMBO press are called expanded view now, eg figures, tables and movies. Please change the names accordingly.

REFEREE REPORTS

Referee #1:

Chen and colleagues present results of an interesting and elegant study that assesses the impact of neuronal TLR3 activation on axonal and dendritic development. They highlight a particular role for Disc1 in regulating the TLR3-mediated effects, which has relevance to schizophrenia-associated pathology. Until quite recently, the expression of TLRs on neuronal cells has been a contentious issue. This study offers a functional role for TLR3 in neurons, independent of the impact of glial activation. In doing so, the authors also describe a non-canonical pathway through which TLR3 likely influences neuronal morphogenesis.

This is an extremely well presented manuscript. The experiments appear to have been performed rigorously and thoroughly, with evidence that strongly supports the conclusions and interpretation of the authors. Figures are clear and adequately display all relevant information. I am slightly confused however by the rationale for the experiments described on page 9 and results displayed in Figure 5 (and Supplementary figure 1). The authors state that the efficiency of their TLR3 knockdown was less than 1%. Is it the case therefore that only those 1% of cells in which knockdown was confirmed were subjected to analysis of dendritic growth? It is unclear how this experiment provides additional information to the conditioned media experiment on Tlr3-/- cells described in figure 4. Perhaps the text relating to this data could be revised to clarify the rationale and conclusion drawn.

It is my opinion that this study is of significant interest in the areas of Neuroscience and Immunology, and subject to minor revision to the text, is of sufficient novelty and quality to merit publication in EMBO Reports.

Referee #2:

In this study, Chen et al report that polyI:C (pIC) induced activation of TLR3 signaling causes aberrant neuronal morphogenesis in a cell autonomous fashion. The propose that MYD88, and not TRIF, is the major signaling adaptor that regulates neuronal morphology in response to TLR3 activation. Finally, they implicate a role for DISC1 downstream of this novel TLR3-MYD88 signaling pathway in TLR3-mediated dysregulation of neuronal morphogenesis. Overall, the findings presented in this manuscript are interesting. However, there a few key areas that need to be addressed to solidify their major findings and conclusions.

(1) Proposing that MYD88-dependent, and not TRIF3, signaling is responsible for TLR3-induced negative regulation of neuronal morphogenesis is quite provocative. Especially considering that TRIF is the prototypical signal adaptor that coordinates TLR3-mediated signaling in the majority of all other cell types. Although compelling, some additional lines of evidence and controls are needed to substantiate their conclusions from these experiments. For one, does the development of neurons in the TLR3 mutant or MYD88 null environments influence their behavior following pIC treatment. It appears that there are some baseline differences in total dendrites and dendritic tip numbers between their WT controls neurons and those neurons that have been genetically targeted. This implicates that the environment that the neurons are generated in during development may potentially influence responsiveness to signals later on. To address this, it would be informative to evaluate whether pharmacological inhibitor or genetic knockdown of MYD88 or TRIF in WT neurons also regulates neuronal morphology in a similar manner as to what they report for TRIF mutant and MYD88-deficient neurons.

(2) Control data confirming that TRIF and MYD88 are both functionally ablated in neurons derived from mutant and knockout strains are not provided.

(3) Do the authors believe that the interaction between TLR3 and MYD88 is unique to neurons and their overexpression system in HEK293T cells? The interaction data presented in Figure 3 only suggests that TLR3 and MYD88 interact when these proteins are highly overexpressed. To verify that this interaction does occur during physiological settings it should be confirmed in primary cells without transfection and/or overexpression. It would be most convincing if this was accomplished in the primary neurons, such as the ones that they used throughout the manuscript in their in vitro

system.

(4) Critical control experiments are also needed to solidify the data presented in Figure 4. The authors should show that the WT conditioned medium that was used to treat the TLR3-deficient neurons in Figure 4D is bioactive and can induce upregulation of cytokines, signaling, or any other biological readout when it is applied to TLR3-deficient neurons.

(5) Transfection of cortical neurons with shTLR3-plamids in Figure 5 markedly influences dendritic length in comparison to the other control treatment (shCtrl). These starting differences in their control treatment groups maybe confounding the interpretation of their experimental results. Therefore the authors should clarify what contributes to this difference in dendrite length between the saline control groups and why they believe that this does not affect their experimental results.

(6) The experimental design for the Figure 5 experiments is somewhat confusing. In the text the authors state that their transfection efficiency is less than 1% and that the overwhelming majority of neurons in culture are WT and are not characterized by TLR3 knockdown. It appears from reading the text that they evaluated dendrite length and tip number in all of the cultured neurons. If this is the case, the signal from the WT neurons would far outweigh any effect that TLR3 knockdown could have in this system. Therefore, the most appropriate evaluation would only involve assessment of the transfected neurons.

(7) The same set of loading controls were used for all four of the experimental immunoblots presented in Figure 6. It would be most appropriate to provide loading control data from each of the membranes. This should be relatively easy considering that their proteins of interest are all larger in size (100-60 kDa) than the most commonly used loading controls (beta-actin and GAPDH), and it would be feasible to blot for their protein of interest and the loading control on the same membrane.

(8) The authors do not provide sufficient background information on DISC1 biology. Providing more details regarding what is known about DISC1 in relationship to their findings would help the reader better understand the importance and scope of this discovery.

Referee #3:

Perinatal risks, including viral infection and brain injury, are implicated in the etiology of schizophrenia. However, the molecular pathology underlying the perinatal risks remains to be understood. The study with Toll-like receptor 3 (Tlr3)-knockout mice indicated that TLR3 activation regulated the dendritic growth in an independent manner of the secreted proteins including cytokines. To address the physiological relevance of the TLR3-mediated signaling with neuronal morphogenesis, authors examined the effect of TLR3 activation on the expression of the disease-related genes, which modulated neuronal morphology. The TLR3 activation acted through myeloid differentiation primary response gene 88 (MYD88) to negatively control Disrupted in schizophrenia 1 (Disc1) expression. Exogenous DISC1 suppressed the effects of TLR3 activation on dendritic arborization. In this manuscript, authors have mentioned a novel mechanism of TLR3 underlying neuronal morphogenesis. However, a paper has already reported the involvement of TLR3-mediated signaling in axonal outgrowth (Cameron et al., J Neurosci., 27(47), 2007). Although the relationship of TLR3 activation with the DISC1 down-regulation is entire novel and of substantial interest, it remains unknown how the TLR3 signaling regulates the expression of the susceptibility genes including Disc1. A major shortcoming is that this study has not addressed the cell signaling regarding the TLR3/MYD88 complex for the neuronal morphology and the gene expression. For instance, MyD88 is involved in the PI3K/AKT and the MAPK cascade as an upstream signaling component. What is TLR3/MYD88-mediated downstream signaling for the gene expression and the neuronal morphology? Authors can employ several inhibitors in your culture system to evaluate the cell signaling regarding TLR3/MYD88. In addition, authors showed an atypical interaction between TLR3 and MYD88, because TLR3 interacted with MYD88 at N-terminus but not C-terminus. To evaluate the MYD88 region responsible for the dendritic growth, authors can perform the rescue experiment in MYD88-KO neuron using the MYD88 fragments. The data are potentially interesting, but there are some issues that need to be clarified before the manuscript is ready for publication.

Specific comments:

1. Does the overexpression of MYD88 affect the expression of DISC1 as well as the dendritic growth?

2. Synaptic disconnectivity is implicated in the pathology of psychiatric disorders including schizophrenia (Penzes et al, Nat Neurosci, 14, 2011). An intriguing question is whether the TLR3-mediated signaling is involved in the spinogenesis. Understanding of the association of the innate immune cell signaling with synaptic pathology would strengthen the impact of this manuscript.

3. A previous report (Ibi et al., Neurosci Res, 64(3), 2009) showed that Poly I:C stimulation had no effect on the expressions of the psychiatric genes including DISC1 and NRG1. Authors should explain the discrepancy between the gene expression studies.

4. Authors should confirm the description of a Disc1-disease allele, Disc1-L604F, because several reports have described Disc1-L607F (Singh et al., Neuron, 72(4),2011; Malavasi et al, Hum Mol Genet, 21(12), 2012).

2nd Revision - authors' response 14 October 2016

Thank you very much for your email of 15 July 2016 in which you forwarded the comments of three referees and invited us to submit a revised manuscript back to EMBO Reports. Based on the referees' constructive suggestions and comments, we have extensively modified our manuscript by including more experimental results, discussion and explanations about the rationale of our experimental design. Consequently, we believe that the revised manuscript has been significantly improved. We thank you and all three referees for the constructive suggestions, which have further strengthened our points.

Referee #1's comments:

This is an extremely well presented manuscript. The experiments appear to have been performed rigorously and thoroughly, with evidence that strongly supports the conclusions and interpretation of the authors. Figures are clear and adequately display all relevant information. I am slightly confused however by the rationale for the experiments described on page 9 and results displayed in Figure 5 (and Supplementary figure 1). The authors state that the efficiency of their TLR3 knockdown was less than 1%. Is it the case therefore that only those 1% of cells in which knockdown was confirmed were subjected to analysis of dendritic growth? It is unclear how this experiment provides additional information to the conditioned media experiment on Tlr3-/- cells described in figure 4. Perhaps the text relating to this data could be revised to clarify the rationale and conclusion drawn. It is my opinion that this study is of significant interest in the areas of Neuroscience and Immunology, and subject to minor revision to the text, is of sufficient novelty and quality to merit publication in EMBO Reports.

We thank Referee #1 for her/his strong support. Regarding TLR3 knockdown shown in Figure 5, we have revised our statement to clarify our point (p. 9). The results in Figure 4 suggest that secreted factors are not involved in the function of TLR3 in restricting dendritic growth because conditioned medium collected from neuronal culture treated with poly(I:C) did not restrict dendritic growth of $Tlr3^{-/-}$ *neurons. To further support that poly(I:C) directly acts on neurons to control dendritic morphology in a cell-autonomous manner, but not other mechanisms such as direct cell-cell contact, we considered a knockdown experiment. Our knockdown constructs coexpress GFP, which serves two functions: one is to outline cell morphology; the other is to act as a marker to indicate*

transfected cells. We analyzed transfected cells, namely knockdown neurons or neurons transfected with control vector, based on the signals of GFP. In my lab, we routinely use calcium phosphate precipitation to deliver plasmid DNA into neurons for morphological study. Because less than 1% of neurons can be transfected by calcium phosphate precipitation, transfected neurons are always surround by non-transfected cells. This system is perfect to investigate whether TLR3 acts cell-autonomously to downregulate neuronal morphology. When poly(I:C) is applied to transfected neuronal cultures, it acts on both the transfected neurons and non-transfected neurons contained in those cultures. If the effect of poly(I:C) is non-cell-autonomous, the non-transfected neurons can be expected to influence (whether via secreted factors, direct contact or other means) transfected neurons and result in shorter dendrites of transfected neurons. In contrast, if the effect of poly(I:C) is cell-autonomous, the treatment cannot restrict dendritic growth of TLR3 knockdown neurons, even if the knockdown neurons are surrounded by normal non-transfected neurons that still respond to poly(I:C). We have now integrated these detailed explanations into the revised manuscript to clarify our point (p.9).

Referee #2's comments:

2-1. Proposing that MYD88-dependent, and not TRIF3, signaling is responsible for TLR3-induced negative regulation of neuronal morphogenesis is quite provocative. Especially considering that TRIF is the prototypical signal adaptor that coordinates TLR3-mediated signaling in the majority of all other cell types. Although compelling, some additional lines of evidence and controls are needed to substantiate their conclusions from these experiments. For one, does the development of neurons in the TLR3 mutant or MYD88 null environments influence their behavior following pIC treatment. It appears that there are some baseline differences in total dendrites and dendritic tip numbers between their WT controls neurons and those neurons that have been genetically targeted. This implicates that the environment that the neurons are generated in during development may potentially influence responsiveness to signals later on. To address this, it would be informative to evaluate whether pharmacological inhibitor or genetic knockdown of MYD88 or TRIF in WT neurons also regulates neuronal morphology in a similar manner as to what they report for TRIF mutant and MYD88-deficient neurons.

Based on Referee #2's suggestion, TRIF and MYD88 were knocked down in wild-type cultured neurons. As we mentioned in our response to Referee #1, calcium phosphate precipitation is routinely used in my laboratory to deliver plasmids into cultured neurons. Due to the low transfection efficiency (< 1%), we expect that transfected neurons are surrounded by non-transfected cells. Thus, the environmental effect can be minimized. Because the knockdown constructs coexpress GFP, we can use GFP to label transfected cells and outline neuronal morphology. Consistent with our model, MYD88 knockdown increased the total dendritic length. In contrast, TRIF knockdown did not influence dendritic growth. Moreover, similar to the results using poly(I:C) to treat the Trif mutant and Myd88$^{-/-}$ neurons, MYD88 knockdown neurons did not respond to poly(I:C) even though poly(I:C) still shortened the dendritic lengths of TRIF knockdown neurons. These new results strengthen the role of MYD88 in controlling dendritic growth and have been included in the revised manuscript as new Figure 2D and Expended View Figure EV2. These data also strengthen the cell-autonomous effect of the TLR3-MYD88 pathway in regulating neuronal morphology related to the conclusion of Figure 5 (see our response to Referee #1).

2-2. Control data confirming that TRIF and MYD88 are both functionally ablated in neurons derived from mutant and knockout strains are not provided.

Both Trif mutant and Myd88$^{-/-}$ mice were established by previous studies (Hoebe et al., 2003, Nature 424:743; Hou et al., 2008, Immunity 29:272). In those studies, the mutant or knockout mice were shown to be functionally ablated. In our previous study, we also confirmed that Myd88$^{-/-}$ neurons failed to respond to TLR7 activation (Liu et al., 2013, JN 33:11479). In the revised manuscript, we further provide evidence that, in contrast to wild-type neurons, Trif mutant neurons did not produce Tnfa and Il-1b in response to poly(I:C) stimulation. For Ifnb, the expression levels in response to poly(I:C) decreased dramatically from 100-fold to 4-fold relative to Hprt transcripts when compared wild-type and Trif mutant neurons (Figure 4 vs. Expended View Figure EV1). These results support that Trif mutant neurons indeed failed to properly respond to poly(I:C) stimulation. These new data also imply that TLR3 may use two distinct pathways to control the responses to poly(I:C): 1) via TRIF, TLR3 activation is able to induce cytokine production for inflammatory and antiviral responses; 2) through MYD88, TLR3 may deliver the signal to cell-autonomously regulate dendritic morphology. These new data about the effects of Trif mutation on cytokine expression are included in the revised manuscript as Expended View Figure EV1 and are now discussed in the Discussion section.

2-3. Do the authors believe that the interaction between TLR3 and MYD88 is unique to neurons and their overexpression system in HEK293T cells? The interaction data presented in Figure 3 only suggests that TLR3 and MYD88 interact when these proteins are highly overexpressed. To verify that this interaction does occur during physiological settings it should be confirmed in primary cells without transfection and/or overexpression. It would be most convincing if this was accomplished in the primary neurons, such as the ones that they used throughout the manuscript in their in vitro system.

We would certainly love to investigate the interaction between endogenous TLR3 and MYD88 in neurons and the cell-type specificity of this interaction. Since lacking of TLRs expression, the HEK293T cell line has been widely used in the field of innate immunity to study the function, signaling and protein-protein interaction of TLRs by transfection of specific TLR. We therefore also followed previous experimental designs to explore the interaction between TLR3 and MYD88 in HEK293T cells. The results were shown in the original manuscript as Figure 3A-3C. To investigate the interaction of endogenous proteins, excellent antibodies for immunoprecipitation and immunoblotting are required. We screened several commercially-available TLR3 antibodies and even tried to generate antibodies ourselves. Combining TLR3-transfected HEK293T cells, wild-type and Tlr3$^{-/-}$ mice, we looked for specific antibody recognizing TLR3. Unfortunately, after screening a total of 35 different commercially-available antibodies and home-made antisera, we still could not obtain specific antibody for endogenous TLR3. For MYD88, the most popular commercially-available antibody recognizes MYD88, as well as a non-specific protein species very close to MYD88. Due to the cross-reactivity of commercially-available MYD88 antibody (abcam, ab2064), we also generated our own MYD88 antibody. We obtained several excellent MYD88 antisera from mice. Our MYD88 antisera are able to specifically recognize MYD88 in immunoblottings as well as immunoprecipitations. However, because we do not have specific antibody for TLR3, we are unable to test whether endogenous MYD88 and TLR3 interact with each other in neurons. It is unclear why it is so difficult to generate TLR3-specific antibody. Perhaps TLR3 is a critical regulator in innate

immunity. B lymphocytes that produce TLR3 antibody are likely to be eliminated or anergized to minimize the negative effect of TLR3 antibody in animals. We have to consider other approaches to detect endogenous TLR3 in order to address the questions regarding TLR3 and MYD88 interactions. For Referee #2's information, some data about the screening of TLR3 and MYD88 antibodies are shown here.

(Data not included in the Peer Review Process File)

2-4. Critical control experiments are also needed to solidify the data presented in Figure 4. The authors should show that the WT conditioned medium that was used to treat the TLR3-deficient neurons in Figure 4D is bioactive and can induce upregulation of cytokines, signaling, or any other biological readout when it is applied to TLR3-deficient neurons.

Our previous study demonstrated that neuronal TLR7 activation induces IL-6 production and secretion from cultured neurons to the medium. IL-6 in the conditioned medium collected from TLR7-activated neuronal cultures can then restrict dendritic growth of receiving $Tlr7^{-/-}$ neurons (Liu et l., 2013, JN 33:11479). The study of TLR7 showed that the conditioned medium collected from neuronal cultures is bioactive, at least for cytokines. We thus applied the same experimental design of conditioned medium to our TLR3 study. Unexpectedly, the conditioned medium of poly(I:C)-treated neurons did not influence the growth of $Tlr3^{-/-}$ neurons. Based on our experience regarding TLR7/IL-6, we thus speculated that secreted factors are not involved in the function of TLR3 in regulating neuronal morphology. We would like to emphasize here that our study, as described in the current manuscript, indicated that the detailed regulation and pathways of neuronal innate immunity (at least for the TLR3-mediated response) could be different from other types of cells. Therefore, to test whether the conditioned medium "induces upregulation of cytokines, signaling, or any other biological readout when it is applied to TLR3-deficient neurons" might not be feasible, because we do not actually know how neurons respond to the factors secreted from TLR3-activated neurons. However, to further confirm the cell-autonomous mechanism for TLR3 to regulate neuronal morphology, we included a TLR3 knockdown experiment (Figure 5) in our study (see our response to Referee #1 and points 2-6 of our response to Referee #2). Although the TLR3 knockdown experiment does not directly address whether the conditioned medium is bioactive, it provides another way of demonstrating that TLR3 regulates neuronal morphology via a cell-autonomous mechanism. As described in point 2-1 of our response to Referee #2, we also found that knockdown of Myd88 in neurons promotes dendritic growth, which strengthens the evidence of a cell-autonomous effect of TLR3 and MYD88 in neurons.

2-5. Transfection of cortical neurons with shTLR3-plamids in Figure 5 markedly influences dendritic length in comparison to the other control treatment (shCtrl). These starting differences in their control treatment groups maybe confounding the interpretation of their experimental results. Therefore the authors should clarify what contributes to this difference in dendrite length between the saline control groups and why they believe that this does not affect their experimental results.

Endosomal TLRs, including TLR3, TLR7, TLR8 and TLR9, recognize either RNA or DNA derived from foreign pathogens as well as endogenous sources, such as death cells, exosomes and autophagosomes. In neuronal cultures, many dead cells are present that provide endogenous ligands to activate TLRs. Our previous studies showed that TLR7 detects endogenous single-

stranded RNA in the cultures to restrict dendritic growth (Liu et l., 2013, JN 33:11479; Liu et al., 2015, Experimental Neurology 269:202), providing direct evidence to support neuronal TLR activation by endogenous ligands. For TLR3, both mRNAs and the microtubule-binding protein, stathmin, have been shown to serve as intrinsic ligands (Kariko et al., 2004, JBC 279:12542; Bsibsi et al., 2010, JI 184:6929). Thus, TLR3 is expected to recognize intrinsic ligands in neuronal cultures. Because TLR3 activation restricts dendritic growth, knockdown of TLR3 is predicted to make neurons unresponsive to intrinsic TLR3 ligands, thereby resulting in longer dendrites. The results in Figure 5 are consistent with this speculation. We have integrated the above explanation into the Results of the revised manuscript (p. 9).

2-6. The experimental design for the Figure 5 experiments is somewhat confusing. In the text the authors state that their transfection efficiency is less than 1% and that the overwhelming majority of neurons in culture are WT and are not characterized by TLR3 knockdown. It appears from reading the text that they evaluated dendrite length and tip number in all of the cultured neurons. If this is the case, the signal from the WT neurons would far outweigh any effect that TLR3 knockdown could have in this system. Therefore, the most appropriate evaluation would only involve assessment of the transfected neurons.

We are sorry for the confusion. In Figure 5, we evaluated only transfected neurons, not all of the cultured neurons. Our knockdown constructs and control vector coexpress GFP, which has dual functions in our experiments; one is to serve as a marker to label transfected cells, the other is to outline neuronal morphology for quantification. Thus, based on the GFP signal, it is easy for us to identify those 1% of transfected neurons in our cultures. We have clarified this point and added a more detailed description about Figure 5 in the revised manuscript (p. 9).

2-7. The same set of loading controls were used for all four of the experimental immunoblots presented in Figure 6. It would be most appropriate to provide loading control data from each of the membranes. This should be relatively easy considering that their proteins of interest are all larger in size (100-60 kDa) than the most commonly used loading controls (beta-actin and GAPDH), and it would be feasible to blot for their protein of interest and the loading control on the same membrane.

We actually did blot the protein of interest and the loading control on the same membrane. To save space, we only showed a set of internal controls in Figure 6 of the original manuscript. To respond to Referee #2's suggestion, we now show all of the internal controls for each blot in new Figure 6E in the revised manuscript.

2-8. The authors do not provide sufficient background information on DISC1 biology. Providing more details regarding what is known about DISC1 in relationship to their findings would help the reader better understand the importance and scope of this discovery.

In the revised manuscript, we have provided more information about DISC1 in the Discussion (p. 14). We thank referee #2 for all of her/his comments and suggestions.

Referee #3's comments:

General Comments: A major shortcoming is that this study has not addressed the cell signaling regarding the TLR3/MYD88 complex for the neuronal morphology and the gene expression. For instance, MyD88 is involved in the PI3K/AKT and the MAPK cascade as an upstream signaling component. What is TLR3/MYD88-mediated downstream signaling for the gene expression and the neuronal morphology? Authors can employ several inhibitors in your culture system to evaluate the cell signaling regarding TLR3/MYD88. In addition, authors showed an atypical interaction between TLR3 and MYD88, because TLR3 interacted with MYD88 at N-terminus but not C-terminus. To evaluate the MYD88 region responsible for the dendritic growth, authors can perform the rescue experiment in MYD88-KO neuron using the MYD88 fragments. The data are potentially interesting, but there are some issues that need to be clarified before the manuscript is ready for publication.

We thank Referee #3 for all of her/his comments. It would certainly be interesting to further elucidate the downstream signaling pathway of TLR3 in controlling neuronal morphology. In the manuscript decision letter, the Editor Dr. Schnapp informed us that both she and Referee #2 thought that identification of the TLR3 downstream signaling pathway was beyond the scope of the current study. Therefore, we will leave this for future study.

Regarding the second suggestion, we cotransfected GFP and full length HA-tagged MYD88, the N- and C-terminal MYD88 and vector control into Myd88$^{-/-}$ neurons at 4 DIV and investigated the dendritic phenotype at 6 DIV (new Figure 3D). We noticed that overexpressed full-length MYD88 tended to form aggregates in neurons (new Figure 3E). It has been demonstrated that oligomerization via the N-terminal death domain is required for MYD88 to activate the downstream signal pathway (Lin et al., 2010, Nature 465:885; Into et al., 2010, JBC 285:35759). Aggregation of overexpressed MYD88 in neurons suggests that MYD88 overexpression is likely sufficient to activate downstream signaling. Indeed, we found that overexpression of full-length MYD88 reduced total dendritic length as well as total dendritic tips. Poly(I:C) treatment did not further enhance the effect of MYD88 overexpression (new Figure 3D). These findings echo our data shown in the original manuscript that MYD88 mediates the effect of poly(I:C) on dendritic growth (Figure 2). Importantly, the N-terminal region of MYD88 also tended to form aggregates in neurons and exhibited a similar effect on dendritic growth (new Figure 3E), supporting the model that the N-terminal region of MYD88 is critical for TLR3 signaling. In contrast to full-length and the N-terminal region of MYD88, the C-terminal region of MYD88 was evenly distributed in neurons and did not influence dendritic growth of Myd88$^{-/-}$ neurons no matter whether poly(I:C) was present or absent (new Figure 3D, 3E). These data strengthen the important role of the N-terminal region of MYD88 in response to TLR3 activation.

3-1. Does the overexpression of MYD88 affect the expression of DISC1 as well as the dendritic growth?

As shown in previous Figure 2C and new Figure 3D (see our response to Referee #3's general comments), Myd88 overexpression shortened total dendritic length of both wild-type and Myd88$^{-/-}$ neurons. To examine whether MYD88 overexpression influences DISC1 expression, we co-transfected control vector or HA-tagged MYD88 with a GFP construct into neurons and performed immunostaining using DISC1 antibodies. Although the DISC1 signal is pretty weak, we still

observed the reduction of DISC1 protein levels in poly(I:C)-treated neurons, as well as in MYD88-overexpressed neurons (Expended View Figure EV5). These data strength our model that MYD88 activation may regulate DISC1 expression and influence dendritic arborization in neurons.

3-2. Synaptic disconnectivity is implicated in the pathology of psychiatric disorders including schizophrenia (Penzes et al, Nat Neurosci, 14, 2011). An intriguing question is whether the TLR3-mediated signaling is involved in the spinogenesis. Understanding of the association of the innate immune cell signaling with synaptic pathology would strengthen the impact of this manuscript.

To address whether TLR3 activation influences dendritic spinogenesis, we have performed both in vitro and in vivo studies for the revised manuscript. For in vitro study (new Figure 8A), cultured neurons were transfected with GFP-actin to outline neuronal morphology at 12 DIV. Poly(I:C) was added into cultures at 17 DIV. Dendritic spine density and morphology were then analyzed at 18 DIV. We found that poly(I:C) treatment noticeably increased the spine density but reduced the width of spine heads, suggesting an impact of poly(I:C) treatment on dendritic spine formation. To demonstrate the in vivo effect of poly(I:C) treatment, we intraperitoneally injected poly(I:C) into Thy1-Yfp and Tlr3$^{-/-}$;Thy1-Yfp mice at P4 and P5 (one injection per day) and monitored dendritic spines at P21 (new Figure 8B-8D). In Thy1-Yfp mouse brains, similar to cultured neurons, poly(I:C) treated mice had more dendritic spines, but many of them were either thin spines or filopodia with smaller spine heads. The effect of poly(I:C) on dendritic spine density and morphology was specifically mediated by TLR3, because Tlr3$^{-/-}$;Thy1-Yfp neurons were not influenced by poly(I:C) treatment. These data suggest that TLR3 activation by poly(I:C) treatment at P4 and P5 also influences dendritic spine density and morphology at P21. As Referee #3 pointed out, synaptopathy is highly relevant to psychiatric disorders, so our new data further strengthen the role of TLR3 signaling in neuronal morphology and cognitive function. These new data are included in the revised manuscript as new Figure 8.

3-3. A previous report (Ibi et al., Neurosci Res, 64(3), 2009) showed that Poly I:C stimulation had no effect on the expressions of the psychiatric genes including DISC1 and NRG1. Authors should explain the discrepancy between the gene expression studies.

There are at least two differences between our study and the previous work. First, an outbred strain of ICR mice was used in the previous paper, whereas mice in the C57BL/6 background were used in our study. Different genetic backgrounds may result in different responses to poly(I:C) stimulation. In particular, ICR mice, but not C57BL/6, have been shown to carry a 25-bp deletion in the Disc1 gene and cannot express full-length DISC1 proteins (Kuroda et al., 2011, Human Molecular Genetics 20:4666). Second, the experimental procedures are different between the previous work and our study. In our study, a single shot of poly(I:C) was i.p. injected into neonatal mice at P5. Six hours later, the cortex and hippocampus were dissected for Q-PCR. In Ibi's work, poly(I:C) was injected once per day from P2 to P6. Two and 24 hours after the last injection, only hippocampus was harvested for Q-PCR. These differences may also account for the discrepancies. These explanations have been included in the Discussion section of the revised manuscript (p. 16).

3-4. Authors should confirm the description of a Disc1-disease allele, Disc1-L604F, because several reports have described Disc1-L607F (Singh et al., Neuron, 72(4),2011; Malavasi et al, Hum Mol Genet, 21(12), 2012).

The human DISC1 L607 residue corresponds to mouse Disc1 L604. This information has been included in the revised manuscript.

3rd Editorial Decision 07 November 2016

Thank you for the submission of your revised manuscript to our journal. It was sent back to all 3 referees, and all of them are very satisfied with the revised study and support its publication. We can therefore in principle accept it.

Only a few minor changes are required. Figure EV5 states n=2 but also calculates statistics. Statistics can only be calculated if n>2. Please either include one more experiment in the analysis, or remove the error bars and show the single data points of both experiments instead, along with their mean.

4th Revision - authors' response 08 November 2016

Thank you very much for your email of 7 November 2016 in which you informed us to slightly modify our revised manuscript for publications in *EMBO Reports*. We very much appreciate your suggestion and Referees' strong supports. We have modified Figure EV5 and corresponding legend to clarify the point. The files of 5 expanded view figures and 3 expanded view tables have been uploaded individually. A short summary, 3 bullet points and a synopsis image have also been uploaded. We noticed that we forgot to acknowledge Dr. Constance L. Cepko for the plasmids pCAGEN and pCAG-GFP. We thus take this opportunity to modify the Acknowledgement in the revised manuscript. Finally, considering copyright, we remove an image showing mouse pups in Figure 8B. It does not influence the data at all. For Figure 3D, we also changed "Myd88-/-" from Italic style to regular style to make it consistent with other figures.

4th Editorial Decision 09 November 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Yi-Ping Hsueh
Journal Submitted to:	EMBO reports
Manuscript Number:	EMBOR-2016-42586V2-Q

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 $\chi 2$ 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to estimate sample size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The used animal number is described in the each figure legend.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample was excluded from the analysis.
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.	The data were randomly collected and in a blinded manner
For animal studies, include a statement about randomization even if no randomization was used.	For animal studies, all available mice were subjected for analysis and no randomization was performed. This was described on p. 25.
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.	The data of neuronal images were randomly collected and in a blinded manner. It has been described in the paragraph of "Neuronal morphometry".
4.b. For animal studies, include a statement about blinding even if no blinding was done	Investigators were blinded to the experimental groups during the experiment and data analysis. This is stated in Methods.
5. For every figure, are statistical tests justified as appropriate?	Yes, the statistical tests are described in each figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The majority of data met the assumption (normal distribution) of these tests, except figure 2A Tip/pIC, 4A Tnfa/Sal and Ifnb/Sal, 4B Tip/Sal, 4C Tip/pIC, 7C Tip/pIC, 8A width and length, 8C width and length and 8D width and length
Is there an estimate of variation within each group of data?	Our data are presented as mean+/-SEM as described in Figure legends. But no further analyses were performed for variance estimation.
Is the variance similar between the groups that are being statistically compared?	NA

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).	All the informations of used antibodies are list in Expanded View Table EV2.
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.	A statement is reported in the Material and Method.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	A statement is included in the Materials and Methods.
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.	Yes, we have comfirmed that we have followed the guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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
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21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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

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