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Neuregulin 3 promotes excitatory synapse formation on hippocampal interneurons

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

1st Editorial Decision 7th Feb 2018

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the slight delay in getting back to you with a decision but I have now received the available input.

As you can see from the comments below, both referees find the analysis interesting but they also indicate that further analysis would be needed for consideration here. The concerns raised are clearly outlined below and I anticipate that you should be able to resolve with additional experimental data. Should that be the case then I would like to invite you to submit a revised version that addresses the concerns raised. Let me know if we need to discuss any of the specific points further

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:

General summary and opinion of significance and findings:

The manuscript by Muller et al. begins by studying how lentiviral over-expression of NRG3 in cultured hippocampal neurons from wt and NRG3 mutant mice results in an increase of glutamatergic synapses, defined by vGlut-positive presynaptic terminals apposed by GluA receptor puncta, selectively in parvalbumin-expressing GABAergic interneurons. Overexpressed NRG3 was observed to accumulate with ErbB4 in an EGF-like domain (i.e., ErbB receptor binding) - dependent fashion. Interestingly, similar to an earlier report about the role of NRG-ErbB4 at inhibitory synapses on GABAergic interneurons, Muller et al. observe that ErbB4 tyrosine kinase activity is not necessary for NRG3 effects on excitatory synapses. Either the connectivity or failure rates (see below) between CA1 principal neurons and PV interneurons, analyzed by cell-attached/whole-cell paired-recordings, are decreased in NRG3 -/- mice. This finding is consistent with reduced GluA4 puncta. Comparison of in vivo LFP recordings in CA1 from adolescent wt and NRG3 -/- mice during "quite" and "move" epochs show an increased frequency (~ 0.05 Hz) of population spikes in mutants, whereas the rate, power (~ between 125-175 Hz) and frequency of ripples were dramatically reduced. The authors find that gamma band power is augmented in NRG3 mutant mice, an observation that merits further analysis and discussion given the authors' findings of reduced excitatory drive onto PV GABAergic interneurons- a neuronal subtype that contributes to gamma oscillation frequency and power.

The manuscript by Muller et al. use a number of sophisticated approaches to study the functions of overexpressed NRG3 in vitro and lack of NRG3 in vivo. There are several novel findings that extend our present knowledge of this ErbB receptor ligand, and it's the first study to convincingly report NRG3 protein using KOs as controls. It is unclear, however, why the authors never do the simple experiment of comparing excitatory synapse formation onto inhibitory neurons in wt vs. mutant cultures without resorting to NRG3 overexpression. Furthermore , the exclusive focus on inputs onto PV-expressing interneurons (when in fact NRG3 is widely expressed in brain and ErbB4 is also present on most other interneuron types) introduces potential bias into the interpretation of experiments performed in acute slices or in vivo (Figs. 4 and 5) where circuits are intact. There are some instances where the writing lacks rigor by overstating the interpretation of experiments (i.e. equating all forms of plasticity and their role in behaviors) or by understating the current state of knowledge about NRG3 properties and functions. In this regard, published work that is relevant to authors' observations should be acknowledged more properly in the Introduction and other parts of the manuscript.

Specific major concerns essential to be addressed:

- An important conceptual point that affects the interpretation of much of this work is that little-to-no information is provided to educate the reader that NRG3 is widely expressed in most CNS neurons. This needs to be made clearer both in the writing and by addional data and/or panels, because in its present form the manuscript suffers from PV interneuron-centric 'tunnel vision' that influences the interpretion of the data, especially the in vivo findings. What is the extent of NRG3 expression in the hippocampus (ISH could be used)? Do GABAergic neurons also make NRG3? Do PV-negative GABAergic interneurons also co-cluster NRG3 and ErbB4?

- The authors should compare uninfected cultures from wt and NRG3 -/- mice to directly determine the extent of endogenous NRG3 co-localization with ErbB4 and its role in the formation or stabilization of glutamatergic synapses. What is the extent of endogenous NRG3 clustering with ErbB4 in cultures and in vivo? How many ErbB4 clusters on uninfected interneurons colocalize with endogenous NRG3? What are the number of vGlut1/2-positive puncta on uninfected PV neurons of wt vs. NRG3 mutant cultures? The authors should also compare directly by IFC the levels of endogenous vs. exogenous NRG3; the reference to a two-fold over-expression by Western blot is not very helpful because only a fraction of neurons were transduced (i.e., NRG3 overexpression per transduced neuron is likely much higher).

- Why did the authors not directly compare the frequency and amplitude of mEPSCs and mIPSCs in PV+ and PV- neurons from wild-type vs. NRG3 knockout mice? This type of experiment, which assesses endogenous NRG3, could yield a more complete picture of how synaptic inputs are altered in absence of NRG3.

- What was the rationale for focusing exclusively on glutamatergic innervation of PV interneurons and not to measure potential changes in GABAergic innervation (i.e., IPSCs)? NRG/ErbB4 effects on inhibitory synapses have been reported and the effects of NRG3 on these synapses could be important to understand the results in Figs. 4/5 and, significantly, a former study also reported that ErbB4 effects on GABAergic transmission onto interneurons is independent of its tyrosine kinase activity.

- Even though it is reasonable to speculate that NRG3-ErbB4 interactions affect synapse formation, have the authors excluded the possibility that NRG1-ErbB4 interactions are involved in synapse stabilization? Also, the authors suggest that "... NRG3 presentation clusters ErbB4 in apposing postsynaptic densities" or that NRG3 process drive synaptogenesis, but have they excluded the possibility that it was postsynaptic ErbB4 clusters that stabilized NRG3-expressing processes or that NRG3 and ErbB4 simply co-cluster through their juxtacrine interactions (i.e., that they are co-dependent)?

- While its correct that NRG3 -/- show slight differences in PPR (a specific type of short-term presynaptic plasticity), the general use of the word "plasticity" in the Abstract & Introduction is vague and potentially misleading. Most references to plasticity, whose role in spatial memory is tested in the Morris water maze, refer to distinct types of plasticity at either SC-CA1 or DG-CA3 synapses.

- Gamma band power is augmented in NRG3 mutant mice, an unexpected finding if considering reduced glutamatergic inputs onto PV interneurons and the fact that increased PV activity augments gamma oscillation frequency and power (Cardin et al. Nature 2009). The authors should discuss this point and consider a possible contribution of other synapses (see above).

- As explained earlier, the Abstract should be written to more accurately report the contents of the manuscript (i.e., what type of plasticity). The Introduction could also be strenghthened by covering recent findings and more properly recognizing publications that have reported observations pertinent to this work and the interpretation of its findings. For example, NRG3/ErbB4 interactions for cortical interneuron migration (Bartolini et al., 2017), importance of ErbB4 signaling and/or recruitment by NRG1/NRG3 for glutamatergic synapse development or stabilization (Krivosheya et al., 2008; Ting et al., 2011; Vullhorst et al., 2017), and ErbB4 kinase-dependent and independent functions on GABAergic synapses (Krivoyesha et al., 2008; Mitchell et al., 2013; Geng et al., 2017).

Other concerns/suggestions to be addressed:
- Comments about data in Figures:
Figure 1: Panel b: y-axis label is missing; what are the NRG expression levels normalized against? What was loaded, equal protein or equivalents?
Figure 2: Quantitative statements lack statistical data for panels c-c'; is the statement 'demonstrated that [...] and out-competed noninfected neurons during synaptogenesis' sufficiently supported by the data? Statements indicating that NRG3 "causes" increased synapse formation are over-interpreted.
Figure 3: Panels l-m are a bit of a diversion and should probably be moved to supplement.
Figure 4: How did the authors separate failures from connectivity between pairs? The sample traces shown in panel d do not seem to be representative of mean peak amplitudes in panel e.
Figure 5: Panel b and c not very informative (just mention frequency of spikes in text?)

Referee #2:

Neuregulins and signaling through their tyrosine kinase receptor ErbB4 have been implicated in early brain development, synaptic plasticity and GABAergic circuit function, as well as in neuropsychiatric disease. The investigation by Muller et al. focuses on the role of Neuregulin-3 (NRG3) in excitatory synapse formation in hippocampal PV interneurons and also addresses how

NRG3 genetic removal affects PV cell synaptic physiology and hippocampal network activity. While there are a few published studies including a recent study that examined NRG3 subcellular localization in hippocampal neuron cultures, NRG3 is much less studied compared to NRG1/2. Overall, the present study by Muller et al. is interesting and significant. In particular, their data support a novel proposal that NRG3 is a functional interaction partner of ErbB4 for promoting excitatory synapses in PV / fast spiking inhibitory neurons in the hippocampus. NRG3 my act as a synaptic adhesive element despite its low affinity to ErbB4. Their work used multiple approaches and consisted of a series of relatively extensive experiments. Generally it is felt that experiments were performed well and data are of good quality. However, some aspects of experiments can be enhanced and expanded to strengthen the current study. I think the authors need to consider the discussion of technical caveats of using conventional Nrg3 mutant mice for in vivo physiological recordings and behavior studies.

In addition, I would suggest the follow specific points:
1. It would be good to add "excitatory" to the title, as excitatory synapses are the focus of this work. "Neuregulin 3 promotes excitatory synapse formation....
2. The general writing of the manuscript can be improved with attention to grammar and wording. For instance, in the abstract, "a little investigated low-affinity ligand" should be corrected as "relatively little investigated...". The term of "ectopic Nrg1" can be problematic. Perhaps replace this with "exogenous", or "recombinant", or "virus mediated expression of Nrg1" throughout the text.
3. The first section of the Results should be expanded to look at NRG3 expression in different cell types, say PV vs. excitatory neurons. The NRG3 antibody looks working very well in Figure 1. Zhang et al. (1997) showed strong mRNA expression in the neocortex and hippocampus, and in situ signaling in the pyramidal or granule cell layer of the hippocampus is very strong. In contrast, as shown in NRG3 immunostaining data of Figure 1 and Supplementary Figure 1, the NRG3 protein likely is accumulated in axons or dendrites of excitatory neurons. Axonal accumulation is evident in mossy fiber staining in Supplementary Figure 1 a'. Further examination and analysis of the stained sections would be important for this work. Because PV neurons in visual cortex are known to express both NRG1 and ErbB4, I wondered if this could occur for NRG3. Larger high resolution image panels can be added to Figure1. One further relevant question is that if PV cells themselves express NRG3, how does their NRG3 subcelluar localization differ from that of excitatory pyramidal neurons in CA1? In addition, besides PV neurons, do NRG3 play a role in formation of excitatory synapses in excitatory neurons?
4. It would be important to provide sufficient details for the Results. For instance, I think the authors need to describe the use of GAD-EGFP mouse to identify inhibitory interneurons in Figure 2 and the relevant result section on page 7. It would also be good to report cell counts in addition to percentages of specific puncta.
5. Lentivirus-mediated gene replacement analysis using NRG3-/- mutant cells is elegant. In comparison, electrophysiological analysis is relatively weak, and should be expanded to study inhibitory synaptic connections between NRG3-/- PV neurons and excitatory neurons. This will allow for direct testing of their proposal that reduced excitatory synaptic inputs to PV interneurons in Nrg3 mutant result in a disinhibition of pyramidal cells.
6. It would be recommended for the authors to look at the potential changes of NR1/2 expression in Nrg3 mutant mice. As it is discussed on page 21, and given the known role of NRG1 in synaptogenesis, this analysis would either further pinpoint NRG3 specificity in PV synapse formation or ask for alternative mechanisms.
7. While adult mice were mentioned in some experiments, please define the ages for adult mice in the Methods.
8. It is noted that "anti-ErbB4 antibodies and Nrg3 mutant mice are available from the corresponding author on reasonable request." I think it would be good to the author to share anti-Nrg3 antibodies as well.

1st Revision - authors' response 13th May 2018

We thank both reviewers for their constructive comments. We changed our manuscript accordingly, and in doing so have substantially strengthened it. We also included new experiments suggested by the reviewers in order to address their specific questions. Our point-by-point responses to the reviewer's comments are indicated in blue below. In addition, we introduced edits that aim to

improve the clarity of the text. The parts that were re-written and that describe new data are indicated in blue in the revised version of the manuscript.

Referee #1:

General summary and opinion of significance and findings:

The manuscript by Muller et al. begins by studying how lentiviral over-expression of NRG3 in cultured hippocampal neurons from wt and NRG3 mutant mice results in an increase of glutamatergic synapses, defined by vGlut-positive presynaptic terminals apposed by GluA receptor puncta, selectively in parvalbumin-expressing GABAergic interneurons. Overexpressed NRG3 was observed to accumulate with ErbB4 in an EGF-like domain (i.e., ErbB receptor binding) - dependent fashion. Interestingly, similar to an earlier report about the role of NRG-ErbB4 at inhibitory synapses on GABAergic interneurons, Muller et al. observe that ErbB4 tyrosine kinase activity is not necessary for NRG3 effects on excitatory synapses. Either the connectivity or failure rates (see below) between CA1 principal neurons and PV interneurons, analyzed by cell-attached/whole-cell paired-recordings, are decreased in NRG3 -/- mice. This finding is consistent with reduced GluA4 puncta. Comparison of in vivo LFP recordings in CA1 from adolescent wt and NRG3 -/- mice during "quite" and "move" epochs show an increased frequency (~ 0.05 Hz) of population spikes in mutants, whereas the rate, power (~ between 125-175 Hz) and frequency of ripples were dramatically reduced. The authors find that gamma band power is augmented in NRG3 mutant mice, an observation that merits further analysis and discussion given the authors' findings of reduced excitatory drive onto PV GABAergic interneurons- a neuronal subtype that contributes to gamma oscillation frequency and power.

The manuscript by Muller et al. use a number of sophisticated approaches to study the functions of overexpressed NRG3 in vitro and lack of NRG3 in vivo. There are several novel findings that extend our present knowledge of this ErbB receptor ligand, and it's the first study to convincingly report NRG3 protein using KOs as controls. It is unclear, however, why the authors never do the simple experiment of comparing excitatory synapse formation onto inhibitory neurons in wt vs. mutant cultures without resorting to NRG3 overexpression. Furthermore, the exclusive focus on inputs onto PV-expressing interneurons (when in fact NRG3 is widely expressed in brain and ErbB4 is also present on most other interneuron types) introduces potential bias into the interpretation of experiments performed in acute slices or in vivo (Figs. 4 and 5) where circuits are intact. There are some instances where the writing lacks rigor by overstating the interpretation of experiments (i.e. equating all forms of plasticity and their role in behaviors) or by understating the current state of knowledge about NRG3 properties and functions. In this regard, published work that is relevant to authors' observations should be acknowledged more properly in the Introduction and other parts of the manuscript.

We thank the reviewer for supporting our work, and for pointing out the novel and convincing aspects of our data. We think that demonstrating the function of Nrg3 in synapse formation/stabilization onto ErbB4+ interneurons significantly advances our understanding of Nrg3 and ErbB4 functions in the brain. We also realize that the reviewer has valid concerns that we will address in more detail in our point-by-point response below. The major critique is that we overemphasize the role of Nrg3 in excitatory synapses on PV interneurons. We have toned down the introduction, and discuss other possible roles of Nrg3 to take this concern into account.

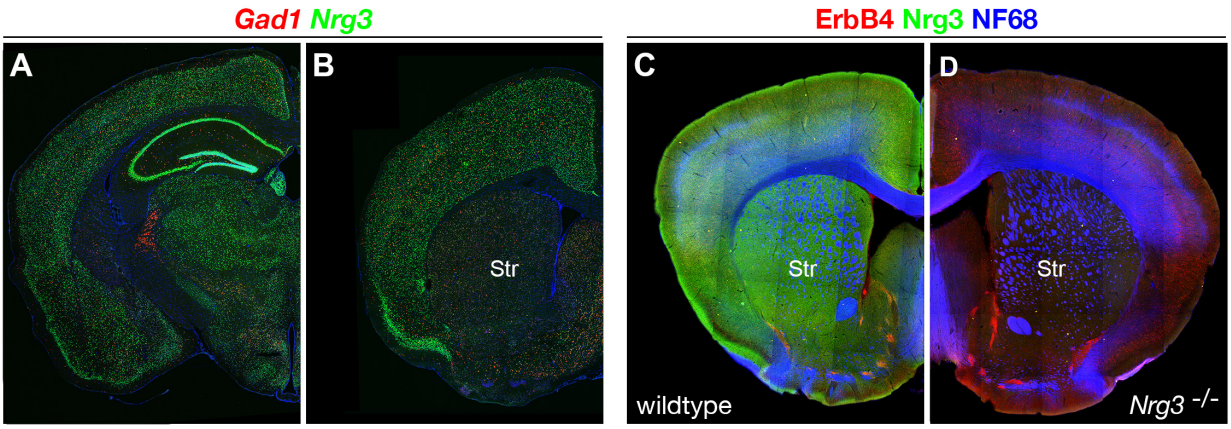
A re-occurring theme of the reviewer is about the lack of attention given to other synapse/neuron types. We therefore explain in the revised manuscript our rationale for focusing on glutamatergic innervation of PV interneurons (page 5, last paragraph of the introduction; pg. 8, last paragraph in the result section 'Nrg3 is enriched in excitatory synapses on inhibitory neurons in vivo' of the revised manuscript). Our first reason for doing so is the strong and reliable co-clustering of endogenous Nrg3 and ErbB4 in such synapses in the hippocampus and cortex in vivo. The second reason is that the phenotype of ErbB4 loss of function was previously analyzed in depth in interneurons (del Pino et al., 2013), and the physiological changes were assigned to a reduced excitatory input into interneurons. This was confirmed by specific (conditional) mutation of ErbB4 in PV+ interneurons (Chen et al., PNAS 2010), indicating that PV interneurons are a key player for many physiological functions assigned to ErbB4.

This immediately raised the following question: to what extent does the function of ErbB4 in glutamatergic innervation of PV interneurons depend on Nrg3. One of the interesting outcomes of our analyses is the extensive overlap between the phenotypes of Nrg3 and ErbB4 mutants, i.e. similar loss of excitatory input on PV interneurons, similarly increased sEPSCs in pyramidal neurons, similar spikes of hyper-synchronous activity, and similarly increased power of gamma-oscillation. Together, the similarity between mutant phenotypes makes a strong point for Nrg3 being a functionally important interaction partner of ErbB4.

It is possible and even likely that Nrg3 also functions in other neurons/synapses, which we discuss in the revised version, where we also included a limited set of cell biological and electrophysiolgical data on other synapse types. We discuss potential roles for Nrg3 in other synapse types in the last paragraph in the section 'Synapse specificity' in the revised manuscript. To analyze additional neurons/synapses to a similar depth as we do for glutamatergic innervation of PV interneurons would go beyond the scope of this paper. In line with the point raised by the reviewer, a report from the Mei laboratory on Nrg3 was published during the revision of our manuscript (Wang et al., PNAS 2018). In this paper, a role of Nrg3 on glutamatergic transmission in pyramidal neurons was reported. The authors suggest a cell autonomous and ErbB4-independent mechanism for this Nrg3 function.

The reviewer argues that the 'exclusive focus on inputs onto PV-expressing interneurons is astonishing (when in fact NRG3 is widely expressed in brain and ErbB4 is also present on most other interneuron types)……..'

We thank the reviewer for raising this point. Indeed, Nrg3 is broadly expressed and this is shown now in detail in the revised manuscript were we provide data obtained by in-situ hybridization, qPCR and immunohistology (Fig. 1 and Fig EV 1). Many but not all excitatory and inhibitory neurons in the cortex and hippocampus contain Nrg3 transcripts. mRNA levels of Nrg3 are comparatively low in the striatum, see figure below:



Wide-spread expression of Nrg3 in the forebrain.
A,B) Double fluorescence in situ hybridization for *Nrg3* (green) and *Gad1* (red) at two axial levels of the forebrain. The *Nrg3* mRNA level is markedly reduced in the striatum (Str).
C,D) Immunohistochemistry using antibodies against ErbB4 (red), Nrg3 (green) and neurofilament 68 (NF68, blue) on slices from wildtype (C) and *Nrg3*-/- animals (D). Remarkably, the Nrg3 protein level is as high in the striatum as in the cortex (C). Nrg3 staining is absent on the *Nrg3* mutant slice (D) proving the antigen specificity of the Nrg3 antibody.

In regards to ErbB4, most publications agree that in the neocortex and hippocampus of rodents, monkeys, and humans, ErbB4 expression is restricted to GABAergic interneurons, and its expression is particularly high in PV interneurons (Mitchell et al., PNAS 2013; Vullhorst D, et al. J. Neurosci. 2009; Neddens et al., Biol Psychiatry 2011, del Pino, Neuron 2013). This is also what we observe in the hippocampus of adult mice. In the cortex, PV-negative GABAergic neurons are more heterogeneous and co-express calbindin, calretinin, and other markers, but we observed that only a few of these cell types co-express ErbB4 at similarily high levels to the PV-positive interneurons of the hippocampus using immunohistochemistry. Thus, the statement that ErbB4 is also present in most other interneuron types is, at least in the cortex/hippocampus of 3-month old mice, not fully

supported by our data nor by data from other laboratories.

The reviewer also states that 'the exclusive focus on inputs onto PV-expressing interneurons introduces potential bias into the interpretation of experiments performed **in acute slices** or in vivo (Figs. 4 and 5) where circuits are intact.

*We thank the reviewer for raising this point. The experiments on acute slices presented in the original manuscript were paired recordings, which are technically very demanding but have the advantage that they unambiguously analyze monosynaptic connectivity, i.e. the excitatory input of pyramidal neurons onto inhibitory PV+ neurons. This type of experiment excludes indirect effects that can arise when miniature or spontaneous synaptic potentials are analyzed (see also below). Thus, the criticism of the reviewer does not hold for our paired recording experiments. The reviewer is right when he/she states that the **in vivo** recordings might reflect changes in the activity of other neuron/synapse types, and we discuss this in the section 'Nrg3, synaptic excitation of inhibitory neurons and hippocampal network activity' of the revised manuscript.*

The reviewer also states: There are some instances where the writing lacks rigor by overstating the interpretation of experiments (i.e. equating all forms of plasticity and their role in behaviors) or by understating the current state of knowledge about NRG3 properties and functions.

We find this criticism unduly harsh. We did not intend to equate all forms of plasticity but used it as short-hand for describing the paired pulse ratio, a form of short-term plasticity. Plasticity is no longer used in the revised version and is replaced by short-term plasticity. The reviewer requests in the specific comments below to cite and mention other papers that he/she states reflect the current state of our knowledge about NRG3. The majority of these papers that are subsequently mentioned by the reviewer do not refer to Nrg3, but to ErbB4 or Nrg2. We want to point out that the literature on ErbB4 has become extensive, which precludes citing all papers. We cite the papers that the reviewer mentions below in the revised version of the manuscript.

Specific major concerns essential to be addressed:

An important conceptual point that affects the interpretation of much of this work is that little-to-no information is provided to educate the reader that NRG3 is widely expressed in most CNS neurons. This needs to be made clearer both in the writing and by addional data and/or panels, because in its present form the manuscript suffers from PV interneuron-centric 'tunnel vision' that influences the interpretion of the data, especially the in vivo findings.

What is the extent of NRG3 expression in the hippocampus (ISH could be used)? Do GABAergic neurons also make NRG3?

We have now included additional ISH data (Fig. 1A of the revised manuscript). Our data show that Nrg3 is expressed broadly in the hippocampus and in the cortex, including by GABAeric neurons, but that not all neurons appear to express Nrg3.

Do PV-negative GABAergic interneurons also co-cluster NRG3 and ErbB4? What is the extent of endogenous NRG3 clustering with ErbB4 in cultures and in vivo? How many ErbB4 clusters on uninfected interneurons colocalize with endogenous NRG3?

We thank the reviewer for raising this point and have now included more detail on the neurons that co-cluster Nrg3/ErbB4 in the revised manuscript. Most publications agree that in the neocortex and hippocampus ErbB4 expression is particularly high in PV interneurons (Mitchell et al., PNAS 2013; Vullhorst D, et al. J. Neurosci. 2009; Neddens et al., Biol Psychiatry 2011, del Pino, Neuron 2013). This is also what we observe. Co-clustering in vivo is very clear on PV interneurons, but not on other interneuron types in the hippocampus. For hippocampal PV interneurons in vivo, we determined that 95.8 +/- 3.4% of Nrg3 puncta overlap with ErbB4 clusters and that 93.1 +/- 6.4% of ErbB4+ clusters contact Nrg3+ puncta, which is now mentioned on pg. 8 of the revised manuscript. Nrg3/ErbB4 co-clustering is also observed on cortical PV interneurons and in a small subset of cortical interneurons that is PV-negative and was not further defined (Fig. EV1 F). Our impression is that detectable co-clustering depends on ErbB4 expression levels. Future work using high-resolution microscopy would be needed to reliably test other neuron/synapse types. We have

not analyzed Nrg3/ErbB4 co-clustering in other brain regions (e.g. nucleus accumbens, amygdala) in which ErbB4 is expressed. In conclusion, other neuron types might co-cluster Nrg3 and ErbB4, but our in vivo data indicate that PV interneurons are the major cell type that co-clusters these two molecules at synapses.

In cultures of hippocampal neurons, co-clustering of endogenous Nrg3 and ErbB4 is observed on all ErbB4+ neurons (PV-positive and negative). Moreover, Nrg3 in culture is detected in both excitatory and inhibitory synapses on ErbB4+ neurons. However, the proportion of inhibitory synapses on ErbB4+ neurons that contains Nrg3 is low (around 20%), and these synapses contain less Nrg3 than excitatory synapses (Fig. 2B;C of the revised manuscript). This might explain why we do not observe consistent Nrg3 staining in inhibitory synapses in vivo.

The authors should compare uninfected cultures from wt and NRG3 -/- mice to directly determine the extent of endogenous NRG3 co-localization with ErbB4 and its role in the formation or stabilization of glutamatergic synapses.

We thank the reviewer for raising this point and have now included more detail on the neurons that co-cluster Nrg3/ErbB4 in the revised manuscript. Co-clustering of endogenous Nrg3 with ErbB4 in culture is extensive, i.e. around 80% of ErbB4 clusters are Nrg3-positive, and similarly, around 80% of Nrg3 clusters are ErbB4-positive (quantification is reported on pg. 9 of the revised manuscript).

What are the number of vGlut1/2-positive puncta on uninfected PV neurons of wt vs. NRG3 mutant cultures?

We have now included the requested experiment. We quantified uninfected cultures from wt and Nrg3-/- mice in Fig. 2D of the revised manuscript which demonstrated reduced excitatory innervation of ErbB4+ interneurons.

We shortly want to explain our rationale for using a mix of transduced/non-transduced neuron cultures instead of utilizing a simpler comparison between wildtype and Nrg3 mutant cultures. Synaptogenesis in culture depends on variables like neuronal density that can vary in different areas of the cover slip/dish or between different experiments. In the reconstituted cultures that we use, we compare synapses of transduced/non-transduced cells in the same area and even on the same ErbB4+ or PV+ dendrite. In our quantifications, we determine the ratios of various parameters (ErbB4 enrichment, synapse preference, synaptophysin enrichment, Nrg3 enrichment) between synapses from infected/noninfected cells. These ratios vary little between experiments and are thus more reliable than comparisons of different cultures.

The authors should also compare directly by IFC the levels of endogenous vs. exogenous NRG3; the reference to a two-fold over-expression by Western blot is not very helpful because only a fraction of neurons were transduced (i.e., NRG3 overexpression per transduced neuron is likely much higher).

We thank the reviewer for raising this point and have now included this quantification. We compared levels of endogenous and lentivirally-expressed Nrg3 in synapses. Lentiviral Nrg3 is present at a 2.2 times higher level than endogenous Nrg3 (Fig. EV3), and we therefore conclude that overexpression is mild.

Why did the authors not directly compare the frequency and amplitude of mEPSCs and mIPSCs in PV+ and PV- neurons from wild-type vs. NRG3 knockout mice? This type of experiment, which assesses endogenous NRG3, could yield a more complete picture of how synaptic inputs are altered in absence of NRG3.

We did analyze mEPSCs on interneurons from wild-type and Nrg3 mutant mice. We noted a relatively large variance in Nrg3 mutant slices and a trend towards lower mEPSCs in the mutants that did not reach statistical significance. During the discussion of the data, our electrophysiological experts pointed out that a number of complicating factors exist when interpreting connectivity rates from mini recordings.

1) Cut axons from neurons with their cell soma located outside the slice of interest could continue to release glutamate and function for hours following slicing.
2) The amplitude and frequency of spontaneously released minis is typically heterogeneous and does not necessarily reflect the sum of all inputs. This issue has recently been directly described in a study using electrophysiology and calcium imaging (Walker et al, PNAS, 2017). The study showed that mEPSCs originate from only a relatively small amount of very active synapses (50% of somatic events resulted from activity of 15% of the spines).
3) The properties and machinery of spontaneous (mEPSC, action potential independent, recorded in TTX) and evoked release (paired recording) are likely to be quite different.

Therefore, we chose to use paired recordings, i.e. dual cell recordings from monosynaptically connected neurons. While time consuming and technically demanding, paired recordings allow for the stimulation of a presynaptic neuron and the recording of a postsynaptic response. They allow a conclusive demonstration of a monosynaptic connection and the characterization of the properties of the connection.

What was the rationale for focusing exclusively on glutamatergic innervation of PV interneurons and not to measure potential changes in GABAergic innervation (i.e., IPSCs)?

We now mention our rationale for focusing on glutamatergic innervation of PV interneurons on page 5 (introduction, last paragraph), pg. 8 (results, end of section 'Nrg3 is enriched in excitatory synapses on inhibitory neurons in vivo') of the revised manuscript. We focused on glutamatergic innervation of PV interneurons in vitro because: 1) endogenous Nrg3 and ErbB4 co-clustering in such synapses was observed in vivo and in vitro, while co-clustering in inhibitory synapses was not clear in vivo and observed at a low frequency in vitro, and 2) the strong phenotype of ErbB4 loss of function in PV interneurons was previously assigned to a change of excitatory innervation.

In conclusion, we decided to concentrate on one brain region and on one synapse type (excitatory synapses onto PV neurons) in order to provide in depth cell biological analysis. Complementary electrophysiological data were generated in culture, in slices and in vivo. To analyze other neuron and synapse types in similar depth would go beyond the scope of a single paper. However, in the revised version we included sEPSC and sIPSC measurements in PV and pyramidal cells, and characterize Nrg3/ErbB4 co-localization in vitro in other synapse types.

NRG/ErbB4 effects on inhibitory synapses have been reported and the effects of NRG3 on these synapses could be important to understand the results in Figs. 4/5 and significantly, a former study also reported that ErbB4 effects on GABAergic transmission onto interneurons is independent of its tyrosine kinase activity.

We thank the reviewer for raising this point and have now induced more detail on Nrg3 and inhibitory innervation. As already mentioned above, Nrg3/ErbB4 co-clustering was not clear at inhibitory synapses in vivo but can be detected in vitro at low frequency. Nrg3 is less strongly recruited to inhibitory than to excitatory synapses. The reviewer is right to point out that several groups reported ErbB4 effects in inhibitory neurons that were ErbB4-kinase independent. We would like to make it clear that none of these papers mentions Nrg3. Nevertheless, in the discussion of the revised manuscript we cite papers that report ErbB4 effects on GABAergic transmission.

As we already mentioned above, to analyze other neuron types in similar depth would go beyond the scope of a single paper. However, in the revised version we included some sEPSC and sIPSC measurements in PV and pyramidal cells, and characterize Nrg3/ErbB4 co-localization in vitro in other synapse types.

Even though it is reasonable to speculate that NRG3-ErbB4 interactions affect synapse formation, have the authors excluded the possibility that NRG1-ErbB4 interactions are involved in synapse stabilization?

No, we cannot exclude that Nrg1-ErbB4 interactions are involved, because we have not investigated Nrg1-ErbB4 interactions in synapse formation/stabilization. Moreover, also Nrg3 might play a role in stabilization. The increased recruitment of SypCFP to presynaptic boutons was also ErbB4-kinase independent, an indication for synapse maturation, supports such a role. We observe Nrg3

effects on synapse numbers, to which formation and stabilization can both contribute. We discuss formation and stabilization as potential mechanisms on pg. 19 in the first paragraph of the discussion.

Also, the authors suggest that "... NRG3 presentation clusters ErbB4 in apposing postsynaptic densities" or that NRG3 process drive synaptogenesis, but have they excluded the possibility that it was postsynaptic ErbB4 clusters that stabilized NRG3-expressing processes or that NRG3 and ErbB4 simply co-cluster through their juxtacrine interactions (i.e., that they are co-dependent)?

We thank the reviewer for raising this point and have now included additional data that demonstrate that the clustering is co-dependent (Fig. 3G,N of the revised manuscript).

While its correct that NRG3 -/- show slight differences in PPR (a specific type of short-term presynaptic plasticity), the general use of the word "plasticity" in the Abstract & Introduction is vague and potentially misleading. Most references to plasticity, whose role in spatial memory is tested in the Morris water maze, refer to distinct types of plasticity at either SC-CA1 or DG-CA3 synapses.

We changed the wording to take the concern of the reviewer into account. In particular, we replaced plasticity with short-term plasticity throughout the revised manuscript.

Gamma band power is augmented in NRG3 mutant mice, an unexpected finding if considering reduced glutamatergic inputs onto PV interneurons and the fact that increased PV activity augments gamma oscillation frequency and power (Cardin et al. Nature 2009). The authors should discuss this point and consider a possible contribution of other synapses (see above).

We thank the reviewer for raising this point and agree that at first sight our finding: reduced excitatory input to PV neurons leads to an increased gamma band power, appears counterintuitive. However, other studies have linked a reduced excitatory drive to PV neurons with an increase in gamma activity. Higher levels of gamma, for example, are seen in the interneuron specific ablation of ErbB4 (del Pino et al., Neuron, 2013) and following ablation of the NR1-/- NMDA receptor subunit in PV neurons (Korotkova et al., Neuron, 2010; Carlén et al., Molecular Psychiatry, 2012). However, because the full cellular and network mechanisms generating gamma activity are not fully understood it is unclear why this occurs.

It is difficult to fully understand the mechanisms of gamma activity, in part because gamma is a network phenomenon that involves distant inputs to hippocampus (like the prefrontal cortex), but also because the generation of gamma oscillations does not only depend on pyramidal and PV neurons but also on additional local cell types (Veit et al., Nature Neuroscience, 2017). Additional complexity arises because the generation of brain rhythms also depends on the timing of synaptic input, not only on the number/density of synapses.

We now discuss the complexity of the origins of gamma oscillations in the revised manuscript where we also mention that other Nrg3-dependent neuron/synapse types might contribute to the change in gamma oscillation (Section 'Nrg3, synaptic excitation of inhibitory neurons and hippocampal network activity' of the discussion).

As explained earlier, the Abstract should be written to more accurately report the contents of the manuscript (i.e., what type of plasticity).

The abstract was changed to more accurately reflect the contents of the manuscript.

The Introduction could also be strengthened by covering recent findings and more properly recognizing publications that have reported observations pertinent to this work and the interpretation of its findings. For example, NRG3/ErbB4 interactions for cortical interneuron migration (Bartolini et al., 2017), importance of ErbB4 signaling and/or recruitment by NRG1/NRG3 for glutamatergic synapse development or stabilization (Krivosheya et al., 2008; Ting et al., 2011; Vullhorst et al., 2017), and ErbB4 kinase-dependent and independent functions on GABAergic synapses (Krivoyesha et al., 2008; Mitchell et al., 2013; Geng et al., 2017).

We now mention Bartolini et al., 2017, Krivoyesha et al., 2008, Mitchell et al., 2013; Geng et al., 2017, Ting et al. in the introduction or discussion of the revised manuscript. Vullhorst et al., 2017 was mentioned twice in the original manuscript and is again mentioned twice in the revised version.

Other concerns/suggestions to be addressed:
Comments about data in Figures:
Figure 1: Panel b: y-axis label is missing; what are the NRG expression levels normalized against? What was loaded, equal protein or equivalents?

The y-axis label was erroneously not included in the original manuscript, and should have stated that transcript levels that were obtained by qPCR analysis. We apologize for this, and corrected the figure in the revised manuscript. The levels were normalized to the level of Ube2l3 mRNA.

Figure 2: Quantitative statements lack statistical data for panels c-c';

This was erroneously not included in the original manuscript. Now we include quantitative data details for all our statistical analyses in the main text or figure legends, for instance pg. 8, pg. 9, pg. 11.

is the statement 'demonstrated that [...] and out-competed noninfected neurons during synaptogenesis' sufficiently supported by the data? Statements indicating that NRG3 "causes" increased synapse formation are over-interpreted.

We have reworded the text to take the concern into account. We now write: Nrg3-mutant neurons are poor donors of presynaptic terminals in the presence of competing neurons that produce recombinant Nrg3. Thus Nrg3+ cells outcompeted Nrg3-mutant cells. We toned the text down and now write that Nrg3 promotes, stimulates or enhances synapse formation and increases the number of synapses.

Figure 4: How did the authors separate failures from connectivity between pairs? The sample traces shown in panel d do not seem to be representative of mean peak amplitudes in panel e.

We now mention the method used to assess connectivity between neurons in the methods (pg. 35 in the section 'Paired recordings from cultured neurons'). Briefly, after averaging 50 trials, a pair was defined as connected when the average of the putative EPSC peak current (4 to 5ms after the stimulation) crossed the threshold of 1.5 standard deviations from the baseline mean (mean +1.5 standard deviations from the baseline mean).

Figure 3: Panels l-m are a bit of a diversion and should probably be moved to supplement.

We politely disagree with the reviewer. These panels show the effect of the strong overexpression on the formation of extensive contacts between neurons, which are indicative of adhesive Nrg3/ErbB4 function (Fig. 4H of the revised manuscript). Moreover, they show that Nrg3 is sufficient to induce postsynaptic specializations in ErbB4 neurons (Fig. 4I of the revised manuscript). Such assays have been used for the analysis of other synaptogenic proteins (neuroligins, neurexins, SynCAM), i.e. proteins that have adhesive functions in synaptogenesis. Thus, the panel shows for the first time that a Neuregulin suffices for synaptic specialization, which is in our opinion an observation that deserves to be presented in the main text.

Figure 5: Panel b and c not very informative (just mention frequency of spikes in text?)

We removed panels b and c in Fig. 5 and instead mention the spike frequency in the text.

Referee #2:

Neuregulins and signaling through their tyrosine kinase receptor ErbB4 have been implicated in early brain development, synaptic plasticity and GABAergic circuit function, as well as in neuropsychiatric disease. The investigation by Muller et al. focuses on the role of Neuregulin-3 (NRG3) in excitatory synapse formation in hippocampal PV interneurons and also addresses how

NRG3 genetic removal affects PV cell synaptic physiology and hippocampal network activity. While there are a few published studies including a recent study that examined NRG3 subcellular localization in hippocampal neuron cultures, NRG3 is much less studied compared to NRG1/2. Overall, the present study by Muller et al. is interesting and significant. In particular, their data support a novel proposal that NRG3 is a functional interaction partner of ErbB4 for promoting excitatory synapses in PV / fast spiking inhibitory neurons in the hippocampus. NRG3 my act as a synaptic adhesive element despite its low affinity to ErbB4. Their work used multiple approaches and consisted of a series of relatively extensive experiments. Generally it is felt that experiments were performed well and data are of good quality. However, some aspects of experiments can be enhanced and expanded to strengthen the current study.

We thank the reviewer for the constructive comments, for finding our work interesting and significant, and for calling it well performed and of good quality. We carried out the experiments suggested by the reviewer, and introduced changes into the text to address the general and specific points raised.

I think the authors need to consider the discussion of technical caveats of using conventional Nrg3 mutant mice for in vivo physiological recordings and behavior studies.

We now discuss this point, and point out that cell type specific Nrg3 mutations will be required to find out to what extent physiological and behavioral phenotypes are due to the excitatory synapses on hippocampal PV interneurons. In addition, Nrg3 was recently assigned an ErbB4-independent cell-autonomous role in glutamatergic transmission (Wang et al., Proc Natl Acad Sci U S A. 2018). Thus, strategies that distinguish cell autonomous and paracrine functions of Nrg3 will also be needed in the long term.

In addition, I would suggest the follow specific points:
1. It would be good to add "excitatory" to the title, as excitatory synapses are the focus of this work. "Neuregulin 3 promotes excitatory synapse formation....

We introduced the word excitatory into the title. The new title is now: Neuregulin 3 promotes excitatory synapse formation on hippocampal.

2. The general writing of the manuscript can be improved with attention to grammar and wording. For instance, in the abstract, "a little investigated low-affinity ligand" should be corrected as "relatively little investigated...".

We reworded the abstract and now use 'relatively little investigated' instead of 'little investigated' in the abstract.

The term of "ectopic Nrg1" can be problematic. Perhaps replace this with "exogenous", or "recombinant", or "virus mediated expression of Nrg1" throughout the text.

We removed the word 'ectopic' and now instead use recombinant, transfected or virally expressed Nrg1 (introduction and discussion).

3. The first section of the Results should be expanded to look at NRG3 expression in different cell types, say PV vs. excitatory neurons. The NRG3 antibody looks working very well in Figure 1. Zhang et al. (1997) showed strong mRNA expression in the neocortex and hippocampus, and in situ signaling in the pyramidal or granule cell layer of the hippocampus is very strong. In contrast, as shown in NRG3 immunostaining data of Figure 1 and Supplementary Figure 1, the NRG3 protein likely is accumulated in axons or dendrites of excitatory neurons. Axonal accumulation is evident in mossy fiber staining in Supplementary Figure 1 a'. Further examination and analysis of the stained sections would be important for this work.

We now include additional in situ data that demonstrate that Nrg3 is broadly expressed in excitatory and inhibitory neurons in the cortex and hippocampus, although not all neurons seem to express Nrg3 (Fig. 1A-A'').

Because PV neurons in visual cortex are known to express both NRG1 and ErbB4, I wondered if this could occur for NRG3. Larger high resolution image panels can be added to Figure1. One further relevant question is that if PV cells themselves express NRG3, how does their NRG3 subcelluar localization differ from that of excitatory pyramidal neurons in CA1?

We have now included data in Fig. 2F,F' of the revised manuscript that demonstrate that synaptically clustered Nrg3 is provided in trans.

In addition, besides PV neurons, do NRG3 play a role in formation of excitatory synapses in excitatory neurons?

In culture, we did not observe an enrichment of Nrg3 in excitatory synapses on excitatory neurons. Due to the fact that we did not observe any enrichment, we did not examine this possibility. Since ErbB4 is not expressed in excitatory neurons in the hippocampus/cortex, such a function of Nrg3 would need to be ErbB4 independent.

However, while were revising our manuscript, the laboratory of Lin Mei published such a function of Nrg3, and proposed a role of Nrg3 in the assembly of SNARE complexes in excitatory synapses. We refer the reviewer to this paper, and added a sentence to the discussion about the new findings in 'Controlling of glutamate release by neuregulin3 via inhibiting the assembly of the SNARE complex' (Wang et al., Proc Natl Acad Sci U S A. 2018).

4. It would be important to provide sufficient details for the Results. For instance, I think the authors need to describe the use of GAD-EGFP mouse to identify inhibitory interneurons in Figure 2 and the relevant result section on page 7.

The Gad1/Gad67-GFP allele was used in the new Figure 2 E,F for the mixed culture of neurons (mixed cultures from mice with two distinct genotypes). This allowed us to identify the interneurons from one genotype by GFP. In addition, we used the Gad1/Gad67-GFP allele in the paired recordings of cultured neurons (shown now in the revised Fig 4J-M). In all other figures/panels, we identify inhibitory neurons/dendrites by ErbB4 or parvalbumin antibodies, and inhibitory synapses by the use of vGAT antibodies. We now make it clear where we use the Gad1/Gad67-GFP allele in the result section of the revised manuscript.

It would also be good to report cell counts in addition to percentages of specific puncta.

This was erroneously not included in the original manuscript. Now we include quantitative data details for all our statistical analyses in the main text or figure legends, for instance pg. 8, pg. 9, pg. 11.

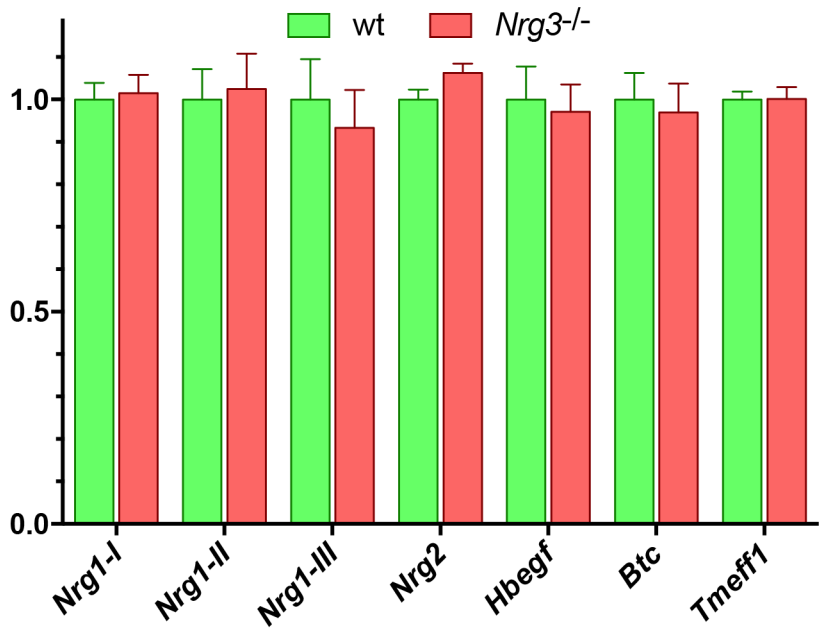
5. Lentivirus-mediated gene replacement analysis using NRG3-/- mutant cells is elegant. In comparison, electrophysiological analysis is relatively weak, and should be expanded to study inhibitory synaptic connections between NRG3-/- PV neurons and excitatory neurons. This will allow for direct testing of their proposal that reduced excitatory synaptic inputs to PV interneurons in Nrg3 mutant result in a disinhibition of pyramidal cells.

We now include data on sEPSCs and sIPSC measurements in PV and pyramidal neurons in the revised manuscript (Fig. 5 and Fig EV5). sEPSCs in pyramidal cells were increased, which demonstrates again the disinhibition of pyramidal cells. sIPSC performed in pharmacological isolation were unchanged, suggesting that the observed disinhibition is based largely upon a reduction in glutamatergic recruitment of interneurons (Fig. EV5).

6. It would be recommended for the authors to look at the potential changes of NR1/2 expression in Nrg3 mutant mice. As it is discussed on page 21, and given the known role of NRG1 in synaptogenesis, this analysis would either further pinpoint NRG3 specificity in PV synapse formation or ask for alternative mechanisms.

We assume that the reviewer refers to Nrg1 and Nrg2. We now compared transcript levels of all ErbB4 ligands (i.e. Nrg1 Types I-III, Nrg2, HB-EGF (Hbegf), betacellulin (Btc), Tomoregulin (Tmeff1). None of the expression levels of these transcripts is significantly changed (n=5 animals for

each genotype, age 4-5 months). Epiregulin could not be detected in hippocampal RNA, and Nrg4 gave very variable results between animals, independent from their genotype, and was therefore not included.



7. While adult mice were mentioned in some experiments, please define the ages for adult mice in the Methods.

The ages are now defined in the text and/or figure legends. Nrg3/ErbB4 co-clustering was analyzed in brains of 3-4-month old mice. Electrophysiology of acute slices was performed using brains of 3-4 week old mice, and network recordings on live animals were done using 6-week old mice.

8. It is noted that "anti-ErbB4 antibodies and Nrg3 mutant mice are available from the corresponding author on reasonable request." I think it would be good to the author to share anti-Nrg3 antibodies as well.

We used commercially available anti-Nrg3 antibodies as indicated in Table 2 where the source of all antibodies is described.

2nd Editorial Decision 12th Jun 2018

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been reviewed by the original referees and their comments are provided below. As you can see both referees appreciate the introduced changes and support publication here. Referee #1 has some good minor suggestions that I would like to ask you to address - shouldn't involve too much additional work.

I also agree with referee #1 that it would be good to cite the Vullhorst et al. 2017 paper when you first mention the topology and processing of NRG3 (Figure 1) - given the similarities it makes sense. For the same reason I also think it would be good to cite the same paper (results or discussion) when you discuss the role of the NRG3 EGF like domain in ErbB4 recruitment.

That should be all - once I get the revised version in then I will formally accept the manuscript. When you submit the revised version would you also take care of the following things:

- I have asked our publisher to do their prepublication check on the paper. I should receive the comments back tomorrow and will send you the file so that you can incorporate their suggestions.

- Callout for Supplementary Fig. 4 on page 36 should be updated to Fig. EV... I presume if is EV5, but please double check.

- We encourage the publication of source data, particularly for electrophoretic gels and blots. 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.

REFEREE REPORTS

Referee #1:

The revised manuscript by Muller et al. entitled "Neuregulin 3 promotes excitatory synapse formation on hippocampal interneurons" by Dr. Birchmeier and colleagues is significantly improved for numerous reasons. In their revised manuscript the authors have: included new experiments to address endogenous NRG3 expression, reorganized presentation of some of their results, improved their abstract so it more closely reports the major findings and rewritten parts of the text that more clearly state their rationale to focus on NRG3-expressing excitatory projections to Pv-positive neurons. The Discussion could be further enhanced by placing these findings in direct context of prior work on NRG3, including the recent PNAS paper.

In the opinion of this reviewer the revised manuscript is very strong and elegant, and it represents an important contribution to the field. Other minor comments/suggestions intended to make it easier for readers to appreciate the data shown or to facilitate reading are below.
- The new experiments in Fig. 2E,F are elegant and a great addition to the paper. Because the GFP signal is so strong, in Fig 2F it is difficult to determine that the neuron is also positive for ErbB4. Adding a panel to show green (GFP) and red (ErbB4) separately will help readers appreciate more easily this important experiment.
- As presently written, it is difficult to understand the origin and interpretation of Figures 1C and 1D without a statement in the Intro or Results about the topology and processing of NRG3.
- The sEPSC amplitudes and PPR in Fig. 4L,M are expressed differently than in Fig. 5 D,E. Also, it could be helpful to include the mean +/- SD in Fig. 5 where the amplitude differences are more modest.

Referee #2:

The authors have responded well to my previous comments and suggestions. Their study has been strengthened by newly added experimental data, re-writing of the text, new literature and broadened interpretation. I fully support its publication in the EMBO Journal.

2nd Revision - authors' response enter date

Reviewer #1
The revised manuscript by Muller et al. entitled "Neuregulin 3 promotes excitatory synapse formation on hippocampal interneurons" by Dr. Birchmeier and colleagues is significantly improved for numerous reasons. In their revised manuscript the authors have: included new experiments to address endogenous NRG3 expression, reorganized presentation of some of their results, improved their abstract so it more closely reports the major findings and rewritten parts of the text that more clearly state their rationale to focus on NRG3-expressing excitatory projections to Pv-positive neurons. The Discussion could be further enhanced by placing these findings in direct context of prior work on NRG3, including the recent PNAS paper.

In the opinion of this reviewer the revised manuscript is very strong an elegant, and it represents an important contribution to the field. Other minor comments/suggestions intended to make it easier for readers to appreciate the data shown or to facilitate reading are below.

We thank the reviewer for these friendly general comments.

- The new experiments in Fig. 2E,F are elegant and a great addition to the paper. Because the GFP signal is so strong, in Fig 2F it is difficult to determine that the neuron is also positive for ErbB4. Adding a panel to show green (GFP) and red (ErbB4) separately will help readers appreciate more easily this important experiment. The figure legend was changed accordingly.

We added an additional panel into Fig. 2F to separately show GFP and ErbB4 fluorescence.

- As presently written, it is difficult to understand the origin and interpretation of Figures 1C and 1D without a statement in the Intro or Results about the topology and processing of NRG3.

Several papers suggested Nrg3 to be similar to Nrg1 type III in topology; an in depth analysis was provided by Vullhorst et al 2017. We now changed the text describing Fig 1 C in the Result section (pg. 7). The changed sentence reads:
Nrg3 encodes a transmembrane protein whose domain structure was previously determined (Vullhorst et al, 2017; see Fig. 1C for a scheme of the Nrg3 structure).

- The sEPSC amplitudes and PPR in Fig. 4L,M are expressed differently than in Fig. 5 D,E. Also, it could be helpful to include the mean +/- SD in Fig. 5 where the amplitude differences are more modest.

The sEPSC and PPR in Fig. 4 and 5 are now displayed in the same manner; means are now indicated in the figures as '+' symbols; the figure displays the quantitative data as box plots with Tukey's whiskers and outlier. In addition, we included the numerical values for the mean and standard deviation in the legend to Figure 5. The data are highly variable but reach statistical significance.

Accepted 21 Jun 2018

Thank you for submitting the revised version to The EMBO Journal. I have now had a chance to take a look at everything and all looks good.

I am therefore very happy to accept the manuscript for publication here!

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Carmen Birchmeier
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-98858R

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 $\chi 2$ 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.

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	There is no sample size calculation stated in the text. However our sample sizes are similar or greater than similar studies in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We tried to use as few animals as possible, but a minimum of 4.
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.
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.	n.a.
For animal studies, include a statement about randomization even if no randomization was used.	Animal studies including GluA4 puncta count (Fig. 1H), slice electrophysiology (Fig. 5) and in vivo electrophysiology (Fig. 6) were performed randomly.
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.	GluA4 puncta were counted blind to genotype (Fig. 1H). Paired recordings in slices (Fig. 5) and in vivo electrophysiology (Fig. 6) were performed blind to genotype. Paired recordings in neuron culture were performed blind to the lentivirus used (Fig. 4J-M). Immunocytochemistry images were quantified using an automated routine to exclude subjective bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	GluA4 puncta were counted blind to genotype (Fig. 1H). Paired recordings in slices (Fig. 5) and in vivo electrophysiology (Fig. 6) were performed blind to genotype.
5. For every figure, are statistical tests justified as appropriate?	Yes, parametric tests were used for normally distributed data and non-parametric tests were used in cases of not normlly distributed data.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was tested using Shapiro-Wilk test.
Is there an estimate of variation within each group of data?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

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http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studi
http://datadryad.org	Dryad
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http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

Is the variance similar between the groups that are being statistically compared?	Data variance was compared in Graph Pad Prism before comparing data samples using t-test. Welch correction was applied if required.

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 primary antibodies used in this study are listed in Table 2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK 293T were treated with puromycin and were mycoplasm free.

* 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.	Nrg3, Nrg3<tm1.1Cbm> (Loos et al., 2014); heart-rescued ErbB4, ErbB4<tm1Grl>;Tg(Myh6-ERBB4)HT2Gass (Tidcombe, Jackson-Fisher et al., 2003); ErbB4flox, ErbB4<tm1Fej> (Long, Wagner et al., 2003); Parv-Cre, Pvalb<tm1(cre)Arbr> (Hippenmeyer, Vrieseling et al., 2005); Gad67-GFP, Gad1<tm1.1Tama> (Tamamaki et al., 2003); Ai14, (Gt(ROSA)26Sor<tm14(CAG-tdTomato)>Hze (Madisen, Zwingman et al., 2010); vGAT-Cre, Tg(Slc32a1-cre)2.1Hzo (Chao, Chen et al., 2010). All strains were maintained on a C57BL/6 background. Gender was not distinguished except for in vivo electropysiology (fig. 6) in which only males were used.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were done in accordance with the guidelines and policies of the European Union and the Max Delbrueck Center for Molecular Medicine and the Charité Berlin, Germany, and approved by the Berlin animal ethic committee.
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

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	No data were deposited in a public database.
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.	No computational models were generated or used.

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