Peer Review File

Manuscript Title: GluD1: A signal transduction machine masquerading as an ionotropic receptor

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The present MS describes a logical set of molecular and functional experiments that address the role of neurexin-cerebellin-GluD trans-synaptic signaling complex in regulating AMPAR and NMDARs at hippocampal excitatory synapses. The authors demonstrate the critical role of GluD1 and Cbln2 isoforms in hippocampal CA1 PC to subicalar PC synapses to mediate this effect. They also demonstrate that Nrxn1 increases NMDARs and Nrxn3 decreases AMPAR function though the same Cbln2-GluD1 pathway. Although the authors identified two extracellular sequences of GluD1 that interact with Cbln2 in a Nrxn type-dependent manner, however, it remains a mystery how the identity of Nrxns is transduced to the effectors through the same pathway.
The authors elegantly demonstrate that cerebellin2 (Cbln2) is expressed in the subiculum, but not in CA1 and CA3 pyramidal cells (PCs). They, therefore, performed their experiments in subicular PCs. This expression pattern cries for the performance of the following control: what is the effect of GluD1 and Cbln2 KO in CA1 and CA3 PCs?
Nrxns are known to be present in the presynaptic AZs and Cbln is a secreted molecule, implying that it localizes where its binding partners are. The subcellular distribution of GluD2 in the cerebellum is known, but the subcellular distribution of the GluD1 is not known. The reviewer would have liked to see data regarding the location of this key molecule of this signaling pathway. The presence of GluD1 within molecular distances from Nrxns would provide a very strong support of this model.

Minor points:
'The increase in mEPSC frequency in GluD1-deficient neurons may be secondary to the increased mEPSC amplitude because mEPSCs are detected more sensitively when the mEPSC amplitude is increased.'
In theory, the authors are right that an increase in amplitude could result in an apparent increase in frequency. However, a 20% increase in amplitude by no means could result in a 3-fold 'apparent' increase in frequency. That would require a baseline mEPSC amplitude distribution in which 75% of mEPSCs have amplitudes just below detection threshold plus the remaining 25% show a well-known skewed highly variable amplitude distribution. It is even more unlikely when one looks at the data recorded from subicular neurons (only 10% increase in amplitudes doubles the frequency).
In fig1 the authors claim that GluD1 KO decreases the NMDA EPSC, whereas they do not find this in Fig3e. Why?
'Wild-type GluD1 fully rescued the decreased NMDAR EPSCs and the increased AMPAR EPSCs observed in GluD1-deficient neurons (Fig. 4b-4e).'
The reviewer cannot see the Wt GluD1 rescue data!
In the abstract the authors state that 'we find that Neurexin-1 and Neurexin-3,
acting in a complex with Cerebellin-2, differentially control postsynaptic NMDA- or postsynaptic AMPA-receptors by activating distinct GluD1 and GluD2 effector pathways.'
The reviewer does not think this statement is supported by the data.

Referee #2 (Remarks to the Author):

Synapse adhesion proteins bridges the pre- and the postsynaptic compartments and play a crucial role in defining connectivity and functional properties of synapses. Neurexins (Nrxns) are a family of presynaptic adhesion proteins that undergo extensive splicing. Nrxn1 and Nrxn3 that contain splice site #4 (SS4+), are known to modulate postsynaptic AMPA and NMDA receptors although the underlying mechanisms have remained unclear. Here the authors take advantage of the fact that secreted cerebellins (Cblns) bind only to the SS4+ variants of Nrxns, and they test the hypothesis that presynaptic Nrxn1beta SS4+ and Nrxn3beta SS4+ regulate AMPA and NMDA receptors via interacting with Cbln-GluD complex. Surprisingly, they find that a chimeric transmembrane protein containing GluD1 or GluD2 extracellular and intracellular sequences are sufficient to transduce Nrxn-Cbln interaction signal without requiring the transmembrane domain specific to GluDs. Moreover, the same GluD molecule is capable of differentially transducing the activities of Nrxn1beta SS4+ and Nrxn3beta SS4+ to regulate NMDA receptors and AMPA receptors, respectively. Whereas these key findings are interesting, the study stops short of providing mechanistic insights beyond identifying two separate cytoplasmic sequences required for controlling NMDA and AMPA currents. Are there co-receptors involved? What are the interaction partners of the cytoplasmic sequences? The experiments are performed to a high standard, and the manuscript is written clearly. However, without further insights into how exactly the complex of Nrxn1beta SS4+ or Nrxn3beta SS4+ with Cbln2 engages NMDA or AMPA receptor regulation via GluDs, the study raises more questions than answers.

Specific points:

(1) Figure 1. While there is no change in VGlut1 puncta density, what about the density of VGlut1 puncta associated with excitatory postsynaptic marker, such as Homer? Quantification of synapse density based on double labelling with pre and postsynaptic markers would be desirable to support the claim for a lack of change in synapse density. Also, are the changes in mEPSC amplitude and AMPA and NMDA EPSC amplitudes reflected in the intensity of labelling for AMPA and NMDA receptor subunits at individual synapses?

(2) ED Figure 1f. The profile of PPR shown is unexpected. In controls, why would PPR be near unity at short inter-stimulus intervals but display depression at a longer interval? It may be that there is a run-down of responses especially if no recovery is observed at longer inter-stimulus intervals. Given that neurexins influence various presynaptic parameters, it is possible that postsynaptic ablation of GluD1 could affect some aspect of presynaptic terminals. To conclude that there are no changes in presynaptic properties, presynaptic release should be monitored by alternative means to support such a lack of effect.

(3) Line 143: As commented above for GluD1 loss, the observed lack of change in synapse density warrants a careful analysis.

(4) Upon knocking out GluD1 and/or Cbln2, were observed changes in AMPA-EPSCs and NMDA-EPSCs accompanied by changes in their waveform (as suggested by some of the example traces in many of the figures – e.g. Fig 1h,p, Fig 2l, Fig 3b, Fig 4b,f,i)? If so, then this could indicate a change in receptor subunit composition or synaptic cleft geometry. This requires further consideration.

(5) Figure 3. In order to address whether Cbln2-GluD1 can transduce the differential effects of Nrxn1betaSS4+ and Nrxn3betaSS4+ on NMDAR and AMPAR, respectively, an analysis at the level of individual synapses would greatly help in interpreting the results. It is not clear if the differential effects on NMDAR and AMPAR are observed at the same synapses or the EPSC changes reflect the

sum of differential effects across a synapse population of the neurons from which the recordings are made. Such differences have implications on the underlying mechanisms.

(6) Figure 3e. Upon GluD1 KO, overexpression of both Nrxn1betaSS4+ and Nrxn3betaSS4+ appears to be effective in rescuing the reduced NMDA EPSC. This is unlike for AMPAR EPSCs, in which overexpression of neither Nrxn1betaSS4+ nor Nrxn3betaSS4+ is able to reverse the increase in AMPA EPSC caused by the loss of GluD1. This suggests that there may be GluD1-independent mechanism by which Nrxn3betaSS4+ regulates NMDAR. This point needs to be further clarified, as it is not fully consistent with the present conclusions.

Author Rebuttals to Initial Comments:

We thank the editors and reviewers for their interest in, and careful assessment of, our work. To address the reviewers' comments, we have performed additional experiments as described in the detailed response to the reviewers' comments below.

Our originally submitted paper showed that activation of GluD1 by Nrxn1-Cbln2 complexes at hippocampal synapses increases NMDA-receptors, whereas activation of GluD1 by Nrxn3-Cbln2 complexes suppresses AMPA-receptors. We concluded that GluD1 (and GluD2, see Fig. 4) is a signaling nanomachine that regulates NMDA- and AMPA-receptors via distinct cytoplasmic effector sequences, and thereby transduces different presynaptic neurexin signals (Nrxn1 vs. Nrxn3) into non-overlapping postsynaptic responses (AMPA- vs. NMDA-receptors). Although GluDs are structurally homologous to ionotropic glutamate receptors like the AMPA- and NMDA-receptors (which they regulate), we found that, surprisingly, the ionotropic transmembrane architecture of GluD1 and GluD2 was notfunctionally required. Specifically, 'mini-GluD1' and 'mini-GluD2' proteins composed of onlytheir N-terminal cerebellin-binding domains and their C-terminal cytoplasmic sequences fully regulated AMPA- and NMDA-receptors as a function of neurexin-cerebellin binding. The major insight produced by our work thus was that GluDs function in manner completely different from what was envisioned: Not as derivatives of ionotropic glutamate receptors, but as signaling nanomachines that transduce a presynaptic neurexin-cerebellinsignal into a postsynaptic receptor response via a simple mechanism. This mechanism requires only the N-terminal cerebellin-binding and C-terminal effector sequences of GluDs, connected to each other by an otherwise irrelevant transmembrane region. These results, we believe, change the way we think about the mechanisms that shape the glutamate receptor composition of a synapse and reveal a new mechanism of action for GluD function.

In response to the reviewers' comments, we have now validated these conclusions with additional experiments that provide further controls for our conclusions, and that significantly expand our findings:

- Using minimal stimulation, we now demonstrate that individual synapses exhibit Cbln2- dependent changes in both AMPA- and NMDA-receptors, suggesting that their separate control by Nrxn1- and Nrxn3-Cbln2 complexes occurs at the same synapses (new Fig. 2).
- We show that the signaling specificity of Nrxn1-Cbln2 and Nrxn3-Cbln2 complexes is solely dependent on the alternatively spliced SS4 insert sequences (new Fig. 3).Strikingly, simply exchanging these sequences between Nrxn1 and Nrxn3 switchestheir specificity. This result in essence excludes the possible role of co-receptors, and suggests that specificity is

achieved via differential cerebellin-binding affinities that were previously documented.

We now identified short cytoplasmic sequence motifs of GluD1 that, when embedded inan otherwise unrelated cytoplasmic tail, are sufficient to mediate the cerebellin- dependent regulation of AMPA- and NMDA-receptors (new Fig. 5). This finding extendsthe notion of a simple transduction mechanism involving engagement of PSD targets.

- We demonstrate that GluD1 is indeed synaptic (new ED Fig. 1b, 1c), firming up a basic premise of our experiments.
- As an additional control suggested by the reviewers, we used double-labeling for pre- and postsynaptic markers to ensure that our quantification of synaptic puncta correctly identifies synapses (new ED Figs. 1h and 3d).
- We performed extensive additional experimental analyses of paired-pulse ratios, coefficients of variations, and EPSCs kinetics to establish that the various manipulations we apply do not cause a major change in presynaptic properties, suchas the release probability (multiple new ED Figures).
- We confirmed that CA1 region Schaffer collaterals that lack cerebellin expression are not altered by the Cbln2 deletion (new ED Fig. 2).

We hope that with these additions and changes, and with the corrections we instituted, the paper can be considered for publication in *Nature*. In the following, we will cite thereviewers' comments in full in *italic* typeface, and provide our response in regular blue typeface.

Referee #1:

The present MS describes a logical set of molecular and functional experiments that address the role of neurexin-cerebellin-GluD trans-synaptic signaling complex in regulatingAMPAR and NMDARs at hippocampal excitatory synapses. The authors demonstrate the critical role of GluD1 and Cbln2 isoforms in hippocampal CA1 PC to subicalar PC synapses to mediate this effect. They also demonstrate that Nrxn1 increases NMDARs and Nrxn3 decreases AMPAR function though the same Cbln2-GluD1 pathway. Although the authors identified two extracellular sequences of GluD1 that interact with Cbln2 in a Nrxn type-dependent manner, however, it remains a mystery how the identity of Nrxns is transduced to the effectors through the same pathway.

We appreciate the reviewer's very positive assessment of our study. We agree that it is an intriguing "*mystery how the identity of Nrxns is transduced to the effectors through the same pathway*". Motivated by the reviewer's comment raising this important question, we examined which neurexin sequences are responsible for their signaling specificity. Unexpectedly, we found that the alternatively spliced SS4 sequences of Nrxn1 and Nrxn3 alone are sufficient to confer specificity to Nrxn1 and Nrxn3 in regulating AMPA- vs. NMDA-receptors (new Fig. 3). Since the short alternatively spliced SS4 sequences constitute the binding site for cerebellins, this surprising finding implies that differential cerebellin binding by Nrxn1 and Nrxn3 encodes specificity, and that there is no co-receptorinvolved. Indeed, classical studies from the Mishina lab (Joo et al., 2011) demonstratedthat Nrxn1-SS4+ and Nrxn3-SS4+ exhibit distinct binding affinities for cerebellins.

The authors elegantly demonstrate that cerebellin2 (Cbln2) is expressed in the subiculum, but not in CA1 and CA3 pyramidal cells (PCs). They, therefore, performed their experiments in subicular PCs. This expression pattern cries for the performance of the following control: what is the effect of GluD1 and Cbln2 KO in CA1 and CA3 PCs?

We also agree that this is an important control to address. We have now performed this experiment. We show that the Cbln2 KO that causes the subiculum phenotype has no effect on CA3→CA1 Schaffer collateral synapses (new ED Fig. 2k), confirming our hypothesis consistent with the in situ hybridization data.

Nrxns are known to be present in the presynaptic AZs and Cbln is a secreted molecule, implying that it localizes where its binding partners are. The subcellular distribution of GluD2 in the cerebellum is known, but the subcellular distribution of the GluD1 is not known. The reviewer would have liked to see data regarding the location of this key molecule of this signaling pathway. The presence of GluD1 within molecular distancesfrom Nrxns would provide a very strong support of this model.

Again, we agree that this is an important question. We have used immunocytochemistry to analyze the localization of GluD1 in hippocampal neurons, and demonstrate that GluD1 is largely synaptic (new ED Fig. 1b, 1c).

Minor points:

'The increase in mEPSC frequency in GluD1-deficient neurons may be secondary to the increased mEPSC amplitude because mEPSCs are detected more sensitively when the mEPSC amplitude is increased.' In theory, the authors are right that an increase inamplitude could result in an apparent increase in frequency. However, a 20% increase in amplitude by no means could result in a 3-fold 'apparent' increase in frequency. Thatwould require a baseline mEPSC amplitude distribution in which 75% of mEPSCs have amplitudes just below detection threshold plus the remaining 25% show a well-known skewed highly variable amplitude distribution. It is even more unlikely when one looks at the data recorded from subicular neurons (only 10% increase in amplitudes doubles the frequency).

We concur that the increased amplitude is probably only one of the reasons resulting in a higher mEPSC frequency, and that we simplified this in the original manuscript. We have now rephrased this section. The increased mEPSC frequency is important because it provides additional evidence that the ablation of neurexin-cerebellin-GluD signaling does not decrease synapse numbers, as also shown by immunocytochemistry. As now stated inthe paper, an increased release probability could also elevate the mEPSC frequency, although our paired-pulse ratio measurements and determinations of the coefficient of variation suggest that there is no large increase in release probability. Another possibility isthat a homeostatic adjustment, possibly as a compensatory response, may be responsible.Since our

paper deals primarily with the discovery of GluD1 function as signaling devices, we have no investigated this question further.

In fig1 the authors claim that GluD1 KO decreases the NMDA EPSC, whereas they do not find this in Fig3e. Why?

In Fig. 3e of the original manuscript, the GluD1 KO significantly decreased NMDA-receptor-mediated EPSC amplitudes by ~40%. The reviewer in essence asks why this decrease is no longer significant after we co-express presynaptic Nrxn1β^{SS4+} or Nrxn3β^{SS4+} in the presence of the postsynaptic GluD1 KO. However, when compared to the activation of NMDA-receptor responses by presynaptic Nrxn1β^{SS4+} without the GluD1 KO, the suppression of NMDA-receptor responses by the GluD1 KO is still highly significant (Fig. 3). The lack of significance when compared to the control thus may reflect the fact that a complex biological experiment involves more variation.

To address this concern, we decided not to simply add 'n's', but instead to expand the scope of the experiment. In new experiments we repeated this overall experiment using Nrxn1 and Nrxn3 constructs in which the SS4+ splice sequences were swapped, confirming the suppression of NMDA-receptor responses by the GluD1 KO (new Fig. 3).

'Wild-type GluD1 fully rescued the decreased NMDAR EPSCs and the increased AMPAR EPSCs observed in GluD1-deficient neurons (Fig. 4b-4e).' The reviewer cannot see the Wt GluD1 rescue data!

The reviewer is correct, and we apologize for this mistake. The cited figure only demonstrates that a subset of the GluD1 mutants fully rescue, but does not include the WTrescue. The WT rescue is included in the next panel together with key mutants that do not rescue, and is also included in other rescue experiments. The wording of the text has now been corrected.

In the abstract the authors state that 'we find that Neurexin-1 and Neurexin-3, acting in a complex with Cerebellin-2, differentially control postsynaptic NMDA- or postsynaptic AMPA-receptors by activating distinct GluD1 and GluD2 effector pathways.' The reviewer does not think this statement is supported by the data.

Again the reviewer is correct in that we do not show directly that GluD2 acts by the same effector pathways as GluD1. However, we do show that full-length GluD2 and the minimal GluD2 construct fully rescue the GluD1 KO phenotype (now in the revised Fig. 4), and that the sequences involved in activating the AMPA- and NMDA-receptor pathways are completely conserved between GluD1 and GluD2 (ED Fig. 5). We changed the text to make this clearer, but feel the GluD2 inference is justified by the data.

Referee #2:

Synapse adhesion proteins bridges the pre- and the postsynaptic compartments and play a crucial role in defining connectivity and functional properties of synapses. Neurexins (Nrxns) are a family of presynaptic adhesion proteins that undergo extensive splicing. Nrxn1 and Nrxn3 that contain splice site #4 (SS4+), are known to modulate postsynaptic AMPA and NMDA receptors although the underlying mechanisms have remained unclear. Here the authors take advantage of the fact that secreted cerebellins (Cblns) bind only to the SS4+ variants of Nrxns, and they test the hypothesis that presynaptic Nrxn1beta SS4+ and Nrxn3beta SS4+ regulate AMPA and NMDA receptors via interacting with Cbln-GluD complex. Surprisingly, they find that a chimeric transmembrane protein containing GluD1 or GluD2 extracellular and intracellular sequences are sufficient to transduce Nrxn-Cbln interaction signal without requiring the transmembrane domain specific to GluDs. Moreover, the same GluD molecule is capable of differentially transducing the activities of Nrxn1beta SS4+ and Nrxn3beta SS4+ to regulate NMDA receptors and AMPA receptors, respectively. Whereas these key findings are interesting, the study stops short of providing mechanistic insights beyond identifying two separate cytoplasmic sequences required for controlling NMDA and AMPA currents. Are there co-receptors involved? What are the interaction partners of the cytoplasmic sequences? The experiments are performed to a high standard, and the manuscript is written clearly. However, without further insights into how exactly the complex of Nrxn1beta SS4+ or Nrxn3beta SS4+ with Cbln2 engages NMDA or AMPA receptor regulation via GluDs, the study raises more questions than answers.

We thank the reviewer for the positive and astute comments. The reviewer express the general criticism that "*… the study stops short of providing mechanistic insights beyond identifying two separate cytoplasmic sequences required for controlling NMDA and AMPA currents*". The reviewer then expands this criticism into two questions that we addressed with new data.

First, "*Are there co-receptors involved?*" In the revised manuscript, we have addressed thisquestion directly by exchanging the 30 residue insert sequence of SS4 between Nrxn1 and Nrxn3. When we place the Nrxn3 SS4-sequence into Nrxn1, Nrxn1 behaves like Nrxn3 (new Fig. 3). Conversely, when we place the Nrxn1 SS4-sequence into Nrxn3, Nrxn3 now behaves like Nrxn1 (new Fig. 3). Thus, the short cerebellin-binding sequence of neurexins that dictates their differential affinities for cerebellins also determines their signaling properties, ruling out co-receptors. We therefore conclude that differences in cerebellin- binding affinities are responsible for the differential signaling functions of Nrxn1 and Nrxn3.

Second, "*What are the interaction partners of the cytoplasmic sequences?*" To addressthis question, we further deconstructed GluD1 signaling beyond the insights reported in ourinitial submission. Our initial work described short sequences that are required for differential AMPA- or NMDA-receptor signaling. Surprisingly, we now identified very short sequence motifs of 8-12 residues that, when placed into an otherwise unrelated cytoplasmic sequence, confer regulation of either AMPA- or NMDA-receptors. Thus, very short cytoplasmic sequence motifs connected via a transmembrane region to extracellular cerebellin-binding domains are sufficient to regulate AMPA- or NMDA-receptors.

Given that GluDs have been studied for decades and that little was known of their functional mechanisms, these observations change our view by showing that GluDs function as nanomachines that operate independent of their ionotropic receptor architecture, and differentially transduce neurexin signals into a receptor response. It is quite amazing to us that a mini-GluD molecule composed only of a small extracellular cerebellin-binding domain and an 8-12 residue cytoplasmic sequence is able to performthe regulatory functions of GluD1!

Specific points:

(1) Figure 1. While there is no change in VGlut1 puncta density, what about the density of VGlut1 puncta associated with excitatory postsynaptic marker, such as Homer?Quantification of synapse density based on double labelling with pre and postsynaptic markers would be desirable to support the claim for a lack of change in synapse density.

Agreed. We have performed such measurements for both Figure 1 and Figure 2. Theseare now included in the Extended Data, confirming the previous conclusions (ED Figs. 1h and 3d).

Also, are the changes in mEPSC amplitude and AMPA and NMDA EPSC amplitudes reflected in the intensity of labelling for AMPA and NMDA receptor subunits at individualsynapses?

We have not performed quantitative immunolocalization of NMDA- and AMPA-receptors as a function of various genetic manipulations, which would require immuno-EM to be done properly. However, we believe this experiment is not necessary since we performed AMPAand NMDA puffing experiments (Fig. 4f-4k). These experiments monitor the total surface levels of AMPA- and NMDA-receptors. The results clearly show a massive change as a function of our genetic manipulations, demonstrating that GluD1 activation differentially controls AMPA- and NMDA-receptor surface levels. Moreover, these results agree with previous data on the effects of neurexin manipulations (Aoto et al., 2013; Dai et al., 2019).

(2) ED Figure 1f. The profile of PPR shown is unexpected. In controls, why would PPR be near unity at short inter-stimulus intervals but display depression at a longer interval? It may be that there is a run-down of responses especially if no recovery is observed at longer inter-stimulus intervals. Given that neurexins influence various presynaptic parameters, it is possible that postsynaptic ablation of GluD1 could affect some aspect of presynaptic terminals. To conclude that there are no changes in presynaptic properties, presynaptic release should be monitored by alternative means to support such a lack of effect.

We thank the reviewer for raising this issue, and agree that additional evidence for a lackof a presynaptic change would further support our conclusions. To achieve this, we now show that the coefficient of variation of evoked responses, an independent measure of the release probability, is unchanged by the various genetic manipulations (new ED Figs. 1j, 2e, 2j, and 3h). We also demonstrate that the kinetics of EPSCs are unchanged, suggesting that presynaptic parameters are normal (new ED Figs. 1i, 2d, 2i, 3e-3g, 4d-4f, 4l-4n, 7a-7i, and 9d-9f). Moreover,

although the reviewer is correct that a rundown of responses is possible during patch-clamp experiments, this would affect controls and mutants equally, whereas we saw no difference between controls and mutants.

(3) Line 143: As commented above for GluD1 loss, the observed lack of change in synapse density warrants a careful analysis.

Agreed. We performed extensive additional analyses as recommended. Specifically, we performed double-labeling experiments for pre- and postsynaptic markers to demonstrate that indeed there is no change in synapse numbers (new ED Figs. 1h and 3d). Moreover, please note that a synapse loss should lead to a decrease in mEPSC frequency but we observed an increase, as discussed above (see first minor comment by reviewer #1).

(4) Upon knocking out GluD1 and/or Cbln2, were observed changes in AMPA-EPSCs and NMDA-EPSCs accompanied by changes in their waveform (as suggested by some of the example traces in many of the figures – e.g. Fig 1h,p, Fig 2l, Fig 3b, Fig 4b,f,i)? If so, then this could indicate a change in receptor subunit composition or synaptic cleft geometry. This requires further consideration.

Agreed - this is an excellent suggestion. We now present extensive analyses of AMPA-and NMDA-EPSC kinetics demonstrating that there is no change (new ED Figs. 1i, 2d, 2i, 3e-3g, 4d-4f, 4l-4n, 7a-7i, and 9d-9f). Thus, there is no evidence for a change in receptor subunit composition, presynaptic properties, or synaptic cleft geometry.

(5) Figure 3. In order to address whether Cbln2-GluD1 can transduce the differential effects of Nrxn1betaSS4+ and Nrxn3betaSS4+ on NMDAR and AMPAR, respectively, an analysis at the level of individual synapses would greatly help in interpreting the results. It is not clear if the differential effects on NMDAR and AMPAR are observed at the same synapses or the EPSC changes reflect the sum of differential effects across a synapse population of the neurons from which the recordings are made. Such differences have implications on the underlying mechanisms.

Again, we thank the reviewer for an excellent suggestion. To examine relative NMDA- and AMPA-receptor responses at individual synapses, we performed minimal stimulation experiments. Comparing precisely matching control and Cbln2 cKO conditions, we find that the Cbln2 KO induces the same changes in individual synaptic responses as in bulk responses recorded from multiple synapses in the various genetic manipulations (new Fig. 2f-j and ED Fig. 2l). Specifically, we detected the same change in AMPA/NMDA ratio and AMPA- and NMDA-EPSC amplitudes as in bulk responses, suggesting that AMPA- and NMDA-receptors are both being regulated at the same individual synapses.

Figure 3e. Upon GluD1 KO, overexpression of both Nrxn1betaSS4+ and Nrxn3betaSS4+ appears to be effective in rescuing the reduced NMDA EPSC. This is unlike for AMPAR EPSCs, in which overexpression of neither Nrxn1betaSS4+ nor Nrxn3betaSS4+ is able to reverse the

increase in AMPA EPSC caused by the loss of GluD1. This suggests that there may be GluD1-independent mechanism by which Nrxn3betaSS4+ regulates NMDAR. This point needs to be further clarified, as it is not fully consistent with the present conclusions.

This is an important point that was also questioned by reviewer #1 (second minor point), and that we did not address adequately in the original manuscript. We will first explain our interpretation of the previous data, and then describe new data to clarify this point.

In the original Fig. 3e, the GluD1 KO caused a ~40% decrease in NMDA-EPSC amplitude. Expression of Nrxn1β^{SS4+} in control neurons caused a ~55% increase, whereas Nrxn3β^{SS4+}had no effect. In the GluD1 KO, expression of Nrxn1β^{SS4+} no longer caused any increasein NMDA-EPSCs, so the GluD1 KO abolished the effect of Nrxn1β^{SS4+} on the NMDA- EPSC. However, when comparing the GluD1 KO without or with expression of Nrxn1β^{SS4+}, the GluD1 KO seems to be less effective in suppressing the NMDA-EPSC (although it still suppressed it). Moreover, Nrxn3β^{SS4+} now had the same effect here as Nrxn1β^{SS4+}, even though in control neurons they were dramatically different. We conclude from these resultsthat (1) the GluD1 KO prevented the action of Nrxn1β^{SS4+} on NMDA-EPSCs since there is no longer an Nrxn1β^{SS4+}-dependent enhancement, but that (2) Nrxn1β^{SS4+} and Nrxn3β^{SS4+} both slightly alleviated the suppression of NMDA-EPSCs induced by the GluD1 KO. The latter issue was the question raised by the reviewer.

To address this issue –as well as to get more mechanistic insights as described above–we did not simply repeat the experiment because we felt that we could clarify this issue in experiments would also advance our understanding of the underlying mechanisms. Therefore we performed expression experiments with mutant Nrxn1β^{SS4+} and Nrxn3β^{SS4+} in which the SS4 splice site sequences were swapped (new Fig. 3). Apart from confirming the conclusion (1) above and expanding this conclusion to demonstrate that the alternatively splice SS4 sequence is solely responsible for specificity, the new data now have sufficient power to unequivocally change conclusion (2) to say that neither Nrxn1β^{SS4+} nor Nrxn3β^{SS4+} are able to alleviate the GluD1 KO phenotype, thereby resolving the question of the reviewer.

We hope that with these changes and additions, our paper can be considered for publication. We appreciate the reviewers' and editors' efforts on our behalf.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The reviewer appreciates the additional experiments the authors performed during the revision, which clearly improve the MS.

Specific points:

1) Now the authors demonstrate the critical role of SS4 domain of Nrxn1 and 3 for their cerebellin binding and predict differential affinities. That is an important step. However, the reviewer is still puzzled. How should the reader envisage the downstream mechanisms of the signalling once cerebellin is bound to Nrxn1 or 3 with low/high affinities? Why would GluD1 do something different

if the cerebellin was bound to Nrxn1 or 3?
2) The reviewer appreciates the performed experiment.
3) The new experiments remonstrate the presence of GluD1 in puncta on cultured cells. Whether these are synapses or not and whether such localization is also valid in vivo, remain to be seen.
4) Agreed. There should be a presynaptic mechanism as well.
5) OK.
6) OK
7) The reviewer would argue against this. The data clearly shows that GluD2 can rescue GluD1 KO. There is no problem with this. But assume that in the brain GluD2 is only present in synapses where no cerebellin2 or Nrxn1/3 is present, although, in theory, it could do this job, but it will never do! And the reviewer would argue that biologically that is the relevant. In line 28, I suggest changing 'postsynaptic GluDs' to 'postsynaptic GluD1'. Line 32 is correct.

Minor points/suggestions:
Line 42: I suggest changing 'into intracellular receptor responses' to 'into postsynaptic receptor responses'. Intracellular can be pre- and post-, but more confusing is that someone might relate it to a cytoplasmic receptor.
Line 97: I suggest adding the cell type/brain area where this manipulation was performed.
Even more confusing is the lack of information in the second half of the MS where all GluD mutations and short constructs are tested. The reviewer suggests to write at the beginning of each paragraph that 'in cultured neurons' or 'in subicular cells in acute slices'.
Throughout the MS text, the authors only indicate the mean change (in %) caused by a manipulation. The reviewer suggests adding +-SD as well.
Line 161: 'minimal responses may be mediated by axons forming two synapses on the same postsynaptic neuron. Viewed together, these results suggest that the Cbln2 deletion causes a change in AMPARs and NMDARs at the same synapses similar to the GluD1 deletion.' The first argument 'kills' the second. If indeed the reason of the larger minimal stimulation evoked response is the axons forming multiple synapses, then these experiments cannot provide evidence that the change in AMPA and NMDARs are taking place in the same synapse. However, the reviewer feels that the second interpretation is likely and the fact that single synapses have multiple release sites can easily explain the fact that the average minimal stimulation-evoked response is larger than the quantal size (i.e. mEPSC amplitude).

Referee #2 (Remarks to the Author):

The authors have addressed my prior concerns in the revision. In particular, (1) the demonstration of the dependence of signaling specificity of Nrxn1-Clbn2 or Nrxn3-Clbn2 complexes on the presence of SS4 insert sequences and (2) the identification of the short cytoplasmic motifs in GluD1 sequences and their sufficiency in directing AMPA receptor or NMDA receptor regulation are elegant and provide important advances.

Author Rebuttals to First Revision:

We thank the editors and reviewers for their interest in, and careful assessment of, our work. To address the reviewers' comments, we have revised the manuscript as described in the detailed response to the reviewers' comments below. However, owing to the need to drastically cut the paper size in view of Nature guidelines, we could not always explain everything in the paper in such a length as we would have liked it. In the following, we will cite the reviewers' comments in full in *italic* typeface, and provide our response in blue regular typeface.

Referee #1:

The reviewer appreciates the additional experiments the authors performed during the revision, which clearly improve the MS.

Thank you.

Specific points:

1) Now the authors demonstrate the critical role of SS4 domain of Nrxn1 and 3 for their cerebellin binding and predict differential affinities. That is an important step. However, the reviewer is still puzzled. How should the reader envisage the downstream mechanisms of the signalling once cerebellin is bound to Nrxn1 or 3 with low/high affinities? Why would GluD1 do something different if the cerebellin was bound to Nrxn1 or 3?

Agreed. We are also surprised by the finding of a novel signaling mechanism that totally differs from previously described pathways. All evidence indicates that the differences in cerebellin-binding affinities are responsible for the differential signaling functions of Nrxn1 and Nrxn3, but understanding how those differences in affinity are translated into distinct GluD signals will require major new project. As we hypothesize in the paper, we suggest that the most parsimonious model to account for our findings is that Nrxn1 and Nrxn3 recruit different concentrations of GluD1 to postsynaptic specializations, akin to the differential effects of calcium-concentrations on postsynaptic signaling.

2) The reviewer appreciates the performed experiment.

Thank you.

3) The new experiments remonstrate the presence of GluD1 in puncta on cultured cells. Whether these are synapses or not and whether such localization is also valid in vivo, remain to be seen.

Although it is not guaranteed that GluD1 is also synaptic *in vivo* and not only in cultured neurons, there is no precedent of a protein that exhibits a synaptic localization in cultured neurons but a non-synaptic localization *in vivo*. On the contrary, there is an overwhelming number of examples for the complete correlation of synaptic localizations in cultured neurons and *in vivo*.

4) Agreed. There should be a presynaptic mechanism as well.

Thank you.

5) OK.

Thank you.

6) OK

Thank you.

7) The reviewer would argue against this. The data clearly shows that GluD2 can rescue GluD1 KO. There is no problem with this. But assume that in the brain GluD2 is only present in synapses where no cerebellin2 or Nrxn1/3 is present, although, in theory, it could do this job, but it will never do! And the reviewer would argue that biologically that is the relevant. In line 28, I suggest changing 'postsynaptic GluDs' to 'postsynaptic GluD1'. Line 32 is correct.

We are not quite sure what exactly the reviewer is referring to, but we have the impression that he/she is not convinced that GluD2 has the same function as GluD1. We agree that there is a remote possibility that this is the case, but would like to point out that there are strong clues from the GluD2 literature that its function is similar to the function of GluD1 we uncovered. These previous finding were not explored further, but are supportive of our results. Specifically, extensive earlier studies in the cerebellum showed that application of a GluD2 antibody induces AMPAR endocytosis by an unknown mechanism (Hirai et al., Nat Neurosci 6, 869-876 [2003]), and that the GluD2 deletion in the cerebellum leads to an increase –not a decrease- in AMPARs (Yamasaki et al., J Neurosci 31, 3362-3374 [2011]), similar to what we describe for the GluD1 deletion. Moreover, other studies demonstrated that the GluD2 deletion does not actually impair initial synapse formation, but causes a secondary loss of synapses (Kurihara et al., J Neurosci 17, 9613-9623 [1997]). Given that -as acknowledged by the reviewer- GluD2 also fully rescues the GluD1 deletion phenotype, we feel that the hypothesis that GluD2 functions similar to GluD1 is well supported.

Minor points/suggestions:

Line 42: I suggest changing 'into intracellular receptor responses' to 'into postsynaptic receptor responses'. Intracellular can be pre- and post-, but more confusing is that someone might relate it to a cytoplasmic receptor.

Agreed. Thank you.

Line 97: I suggest adding the cell type/brain area where this manipulation was performed. Even more confusing is the lack of information in the second half of the MS where all GluD mutations and short constructs are tested. The reviewer suggests to write at the beginning of each paragraph that 'in cultured neurons' or 'in subicular cells in acute slices'.

Agreed – we have followed this suggestion as best as we could given the space constraints.

Throughout the MS text, the authors only indicate the mean change (in %) caused by a manipulation. The reviewer suggests adding +-SD as well.

Thank you for the suggestion. In general we would agree, but in our particular case it may not be applicable because following it would make the manuscript too long, and because all the raw data are accessible in the source data files for all readers.

Line 161: 'minimal responses may be mediated by axons forming two synapses on the same postsynaptic neuron. Viewed together, these results suggest that the Cbln2 deletion causes a change in AMPARs and NMDARs at the same synapses similar to the GluD1 deletion.' The first argument 'kills' the second. If indeed the reason of the larger minimal stimulation evoked response is the axons forming multiple synapses, then these experiments cannot provide evidence that the change in AMPA and NMDARs are taking place in the same synapse. However, the reviewer feels that the second interpretation is likely and the fact that single synapses have multiple release sites can easily explain the fact that the average minimal stimulation-evoked response is larger than the quantal size (i.e. mEPSC amplitude).

We do not completely agree with this comment, which we believe is not quite correct for the following reason. Unitary responses elicited by stimulation of single synapses rarely have the same amplitude distribution as mEPSCs because mEPSCs are derived from a heterogeneous set of synapses, whereas the minimal responses are induced by a more homogeneous subset of synapses that could easily have a slightly higher unitary amplitude. For example, a recent paper from leading labs in the field shows this clearly in the hippocampus where minimal stimulation exhibited a similarly larger EPSC amplitude as we describe (Park et al., Nat Commun. 2021 12:413. doi: 10.1038/s41467-020-20523-3). In our recordings, the amplitudes

of mEPSCs and minimal stimulation responses overlap considerably, so clearly a large part – the majority– of the minimal responses must be mediated by single synapses. We formulated our conclusion carefully to include the possibility that a subset of the responses could represent the action of two synapses or multivesicular release, but we feel that the fact that there is a large overlap in amplitudes between mEPSCs and minimal synaptic responses means that overall, single synapses exhibit the same change in AMPAR and NMDAR EPSCs as the total population.

Referee #2:

The authors have addressed my prior concerns in the revision. In particular, (1) the demonstration of the dependence of signaling specificity of Nrxn1-Clbn2 or Nrxn3-Clbn2 complexes on the presence of SS4 insert sequences and (2) the identification of the short cytoplasmic motifs in GluD1 sequences and their sufficiency in directing AMPA receptor or NMDA receptor regulation are elegant and provide important advances.

Thank you very much for the constructive positive response.