The HSPG Syndecan is a core organizer of cholinergic synapses

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Review #1

1. **How much time do you estimate the authors will need to complete the suggested revisions:**

   **Estimated time to Complete Revisions (Required)**

   (Decision Recommendation)

   Between 1 and 3 months

2. **Evidence, reproducibility and clarity:**

   **Evidence, reproducibility and clarity (Required)**

   In this study the authors make use of cutting edge molecular genetic tools, electrophysiology and cell biology to reveal the role of syndecan as a core organizer of (primarily) cholinergic NMJs. They extend their findings along several axis: cholinergic versus GABAergic synapse composition, extracellular versus intracellular scaffold and signaling complexes, and Neuroligin versus Netrin receptor roles. The paper is rich, with many meaningful findings. The richness, which is a strength, makes it really hard to follow at times, and my main advice is a reorganization of the paper and clarification of key points and findings so that it has the broad impact it is meant to have.

   **Major comments:**

   - I found the paper structure confusing. In part, it is confusing because it analyzes simultaneously across three axis: cholinergic versus GABAergic synapse composition, extracellular versus intracellular scaffold and signaling complexes, and Neuroligin versus Netrin receptor roles. In part it is also confusing because it is jargon and does not explain well the system. These broad findings make the paper special and valuable, but special care needs to be put into framing the story, eliminating jargon and providing enough cartoons and explanations of the system that the reader can follow the arguments.

   - The best summary of the paper is given in the first paragraph of the
Discussion. I suggest using that some structure in the presentation of the findings, rather than the current structure, which is focused around SDN-1 but the drifts into a number of genetic analyses that are hard to follow in the context of SDN-1.

• The system needs to be better presented in Figure 1. I assume these are en passant synapses, like many synapses in C. elegans. That neuroanatomy, and the relationship between the GABA and Cholinergic synapses in the context of the examined cells need to be better explained. Are they looking at different cells within the same bundle, innervating the same muscle? Those points are essential to understand the problem, and unclear in the text and figures.

• Is the diffuse localization pattern of L-AchR expected? Given it occupies so much of the neurite, doesn't that affect interpretation of the co-localization studies? If not, why not? The authors argue colocalization in 1D, but neither the line scans nor the images suggest more colocalization as compared to images in which they say the localization was affected, later in the paper.

• In Fig 2A, the authors claim there are no effects on presynaptic sites, but there appears to be a reduction of the examined presynaptic molecule (CLA1) which is not calculated or discussed.

• In Fig 5, were LIN-2 levels examined?

• In page 10, top paragraph, there seems to be a contradiction between what the authors show in the figure (decrease in FMR-3 in sdn-1 mutants) and the Zhou et al study they cite and say is consistent with "FRM-3 level was unaffected by loss of LIN-2". Maybe it is explained in a confusing manner, would benefit from clarification.

• Despite a dramatic reduction of nAChR receptors in sdn-1(0) mutants, authors observed pressure-evoked responses to nicotine, albeit reduced. They suggest two roles for syndecan - that it clusters nAChRs at synapse and stabilizes them at the plasma membrane. Do the authors then claim that nAChR's are present, but below the threshold of detection of their fluorescent marker? Is a similar response seen in acr-16 mutant? Madd-4 mutants?

**Minor comments:**

- In page 7, define "synaptic fluorescence" (name marker used)

- Regarding the electrophysiology experiments, authors should specify whether they observed any facilitation or depression of response after performing a second and third stimulation (in methods)

- AID experiments are used to demonstrate that SDN-1 is mainly from postsynaptic muscle. In S3 it is important to show the AID system working in the DNC, where the bulk of the experiments take place.
-Figure 6 "Hence, despite the loss of LIN-2 at cholinergic synapses in sdn-1(0) mutants, FRM-3 remained partially concentrated in postsynaptic domains of cholinergic synapses" Show cholinergic marker as well? Same with Figure 4C Madd-4B(0)

-Any speculation on what the band at 97kD is in Figure 6I in the FERM-FA pull down with GST-ACR-16?

-The authors make a point to use the milder (as far as axon defects) unc-40(delP3) mutant when demonstrating a change in ACR-16 localization, but do not use the unc-40(delP3) mutant to show changes in SDN-1 localization. This could be interesting to further develop the domain(s) by which UNC-40 and SDN-1 are interacting.

3. Significance:

Significance (Required)

This study advances the understanding of syndecans in their role as postsynaptic organizers. Additionally, this work proposes a novel mechanism for N-AChR localization at the post-synaptic site. This work builds upon previous studies to identify in vivo mechanisms for localizing molecules to the postsynaptic site (Tu et al., 2015). Here, they use elegant structure-function analyses and genetic interactions to make a strong case for the new mechanisms they introduce.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)
Decision Recommendation

Between 3 and 6 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this study, Zhou et al. identify a role for the heparan sulfate proteoglycan (HSPG) Syndecan in organizing acetyl choline receptor (AChR)
clustering at the cholinergic synapses of the C. elegans neuromuscular junction (NMJ). The extracellular matrix protein Ce-Punctin/MADD-4 is a master organizer of cholinergic synapses at the NMJ, but the signaling pathways involved downstream of Punctin at this synapse are less well understood. This article sheds light on these mechanisms and demonstrates that the Punctin/DCC/FRM3/Lin2 signaling pathway, previously identified at GABAergic synapses at the NMJ (Zhou et al., Nature Commun 2020), is also essential for the proper organization of AChRs at cholinergic synapses. The main finding of this article is that the HSPG Syndecan is a novel contributor to this previously described signaling pathway. Syndecan seems broadly expressed and thus is also localized at the NMJ, at both cholinergic and GABAergic synapses. LOF experiments demonstrate a striking reduction of synaptic AChR content (and to a lesser extent synaptic GABA receptor content). The Syndecan C-terminus is able to bind both FRM3 and Lin2 intracellular scaffold proteins and in turn these effectors play a role in the clustering and presumably membrane presentation of the N-AChR subtype. Punctin plays an essential role in the expression/localization of Syndecan at synapses, and further experiments show a genetic interaction between Punctin, UNC-40/DCC and Syndecan. Thus (in a simplified model) it seems that Syndecan could play the same conceptual role at cholinergic synapses that NLG-1 plays in organizing GABA receptors at GABAergic synapses, that is, providing an "anchor" for the clustering of N-AChR.

This study presents robust and convincing findings and identifies the HSPG Syndecan as a novel core organizer of the cholinergic synapse that acts by coupling extra- and intracellular scaffolds to control postsynaptic receptor composition. The study should therefore be of broad interest. A few points need revision, reformulation or further experiments, especially with regard to the specificity of the identified mechanism.

**Major Points**

1. While the study focuses on the role of Syndecan at cholinergic synapses, it also presents many results on its role at GABAergic synapses. While this data is important, streamlining the results, and in particular the figure panels (e.g. by moving GABAergic results to supplemental figures), to reflect the take-home message of the study would facilitate interpretation of the results and improve readability.

2. Figure 4 and 6: the authors should demonstrate that the delta-GAG and delta-KKDEGS mutants of Syndecan are properly trafficked to the cell surface.

3. The genetic interaction of MADD-4 and Syndecan should be tested by a complementary approach, such as the biochemical approach with recombinant proteins previously used by the authors to demonstrate the MADD-4/NLG-1 interaction (Tu et al., Neuron 2015). This would allow testing the contribution of MADD-4L and MADD-4B. In the same assay, the authors can then also test MADD-4 binding to Syndecan lacking HS.

4. Figure 6H would greatly benefit from an in vitro pulldown using a GST-SDN-1 delta-KKDEGS to cement the importance of this domain in the SDN-1 interaction with FRM-3.
5. Questions remain regarding the specificity of the molecular mechanism identified, for example with regard to the more robust effects of Syndecan LOF on N-AChR. Figure 6H, I investigate the molecular link between FRM3 and N-AChR. Is this interaction specific for N-AChR and not L-AChR? Does the SDN1-ECD also interact with N-AChR and could this contribute to the selectivity for N-AChR?

6. All components identified: MADD-4/DCC/FRM3/Lin2, as well as SDN-1, are present at both cholinergic and GABAergic sites. It remains puzzling how the same molecular players can organize specific neurotransmitter receptor composition at both types of synapses. Does SDN-1 display a preference for MADD-4L or MADD-4B? Alternatively, might there be a competitive interaction of the positively charged Punctin Ig domain with NLG-1 or SDN-1 HS? These possibilities, as well as some additional options mentioned on page 14 (Discussion), should be experimentally addressed in order to provide some insight.

7. Figure 8: the authors use an elegant approach to demonstrate that SDN-1 is not only required but also sufficient for the recruiting of N-AChRs specifically. It would be interesting to see if the opposite experiment (SDN-1-ECD/NLG-1-ICD) is also capable of recruiting GABA receptors to cholinergic synapses.

**Minor Points**

1. The title "The Heparan Sulfate Proteoglycan Syndecan Differentially Localizes Acetylcholine Receptor Subtypes at Cholinergic Synapses" suggests that Syndecan uses different mechanisms to localize/cluster N-AChRs and L-AChRs at the NMJ. Yet, most of the paper focuses on the mechanisms specific to N-AChR localization and clustering. By the authors' own admission, Syndecan is not strictly required for L-AChR clustering, rather, it modulates the synaptic content of L-AChRs. The title needs modifications to reflect the different amounts of evidence for Syndecan's role in N-AChR vs L-AChR synaptic localization. Alternatively, the authors can provide a molecular mechanism by which Syndecan regulates L-AChR synaptic content.

2. Figure 1: it seems SDN-1 is broadly expressed at the dorsal cord. It might be due to my incomplete knowledge of C. elegans neurobiology, but it does not seem to me that it is specifically enriched at synapses or NMJs. Such a figure title would require a quantification of the enrichment (e.g. compared to an axonal marker or fill).

3. The broad effect of Syndecan on all types of synapses at the NMJ suggests that it might also have a role in organizing the presynaptic active zones, which in turn would have an effect on the post-synapse. Indeed, Figure 2A shows a visible reduction in the intensity of cholinergic CLA1-BFP in the Syndecan LOF. This would need to be further quantified/characterized.

4. Figure 2: needs a more accurate explanation of the optogenetic stimulation.

5. A clearer cartoon of the various Syndecan constructs used in the study, in the figures where they are used (e.g. the bicistronic one used in Figure...
3), would help interpret results.

6. Figure 3: The methodology here is a bit perplexing, although the main result (that Syndecan at the NMJ is mostly expressed by the muscle cells) is rather convincing. The main way Syndecan expressed e.g. in the epidermis would be localized at the NMJ would be through shedding of the extracellular domain. Indeed, Syndecans, like many transmembrane receptors, are known to be proteolytically cleaved. But in order to determine the localization of the shed extracellular fragments, one needs an N-terminal fluorescent tag. However, the tag used in this experiment is cytoplasmic. I fail to see how the C-terminal domain of SDN-1 produced in the distant epidermis ends up localized at the NMJ (Figures 3B and 3D). More generally, this type of protein degradation experiment could be used to deplete SDN-1 in a more localized manner and observe its effect on AChRs at cholinergic synapses.

7. Figure 5: it seems the RFP-LIN-2B and LIN-2A-GFP are used interchangeably. Please clarify.

8. Figure 6I: why was the ACR16 TM3-TM4 loop selected? Is this a known FRM3/LIN2 binding site?

3. Significance:

Significance (Required)

As mentioned above, this study presents robust and convincing findings and identifies the HSPG Syndecan as a novel core organizer of the cholinergic synapse that acts by coupling extra- and intracellular scaffolds to control postsynaptic receptor composition. The study should therefore be of broad interest to molecular and cellular neuroscientists working on synapse specification, postsynaptic scaffolds, neurotransmitter receptor organization, extracellular matrix proteins and synapse organizers. My background is in molecular/cellular neuroscience, mechanisms of synapse development, synaptic organizers.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months
2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

**Summary:**

This study recognized the syndecan/SDN-1 as a critical cholinergic organizer within NMJ in C. elegans. In particular, they found that SDN-1 is required for the synaptic clustering of homomeric N-AChRs. This investigation also showed that SDN-1 is essential for proper synaptic levels of heteromeric L-AChRs and GABA receptors. Moreover, they noticed that SDN-1 is provided by postsynaptic muscle cells, and cholinergic and GABAergic motoneurons secrete Punctin/MADD-4. By using a series of microscopic and biochemical assays combined with CRISPR/Cas9 knock-in or knock-out strains, the authors proposed that SDN-1 acts by linking the extracellular MADD-4 with intracellular scaffolding proteins LIN-2 and FRM-3. At cholinergic synapses, MADD-4 functions simultaneously to localize SDN-1 at postsynaptic sites and the transmembrane UNC-40. In turn, SDN-1 also stabilizes MADD-4 and UNC-40. Clustered SDN-1 and UNC-40 at NMJs induce the scaffolding proteins LIN-2 and FRM-3 recruitment. Taken together, the data suggested that the LIN-2-FRM-3 complex then mediates the synaptic clustering of the N-AChR, and the electrophysiological analysis confirms the synaptic localization of AChRs.

**Major comments:**

1. Previous studies indicated that motoneurons could secrete agrin, a large HSPG, at the vertebrate NMJs. This HSPG is proposed to be necessary for NMJ differentiation and postsynaptic clustering of AChRs (Burden et al., 2018; Li et al., 2018; Swenarchuk, 2019). In the worm NMJs, did the author assay the function of agrin? Regarding the abnormal distribution of SDN-1, is it possible that it is an indirect effect caused by ECM destruction?

2. Are there any GABA-related functional defects in sdn-1 mutant animals? Is there any evidence that highlighting sdn-1 physiological effects do not have the indirect effects of GABA synapses? As the author stated, in GABA synapses, UNC-40 has also been studied to show its functional effects.

3. In Figure 2B-C, it is important to assay the expression level of AChR in animals or tissues by western blot.

4. In Figure 2D-G, L-AChRs are also clustered, but the stability does not seem to be affected, this phenotype should be explained or tested; in addition, the endocytic degradation of N-AChRs is solely speculated, should be assayed using endocytosis mutants.

5. In Figure 2, sdn-1(0); N-AChR level decreased. Also, in Figure 4, it
showed madd-4(0); SDN-1 level decreased. The direct inference of the above two experiments is that the level of N-AChR in madd-4 mutant should also be significantly reduced, please confirm it.

6. In Figure 4B-C, for the distribution phenotype of SDN-1, it is useful to provide the results of rescue experiments using the two isoforms L and B in madd-4 mutant animals.
7. In Figure 4B-C, why not examine the colocalization between SDN-1 and Ach? Also, the SDN-1 levels in madd-4L(0) and 4B(0) animals are so different from those in (0), please explain it. Maybe the rescue experiment can help explain this discrepancy. Additionally, Page 8 mentioned that the localization and distribution of SDN-1 are regulated by both L and B isoforms. This statement is not accurate; indeed, the distribution of SDN-1 in L(0) is not significantly affected.

8. In Figure S4C, although it is not mandatory, it will be more desirable to use a synapse marker together for colocalization patterns. Another suggestion, Figure 4F-G could be relocated into a supplemental figure and add a western blot to validate that there is no protein expression variation.

9. Figure 5, whether UNC-40 functions in the LIN-2 positioning process, which should be tested.

In Figure 5I, the colocalization of LIN-2B and SDN-1 is perfect. It is worth noting that LIN-2B is known to be located in GABA NMJ too. Thus, this result does not seem to be completely consistent with the result in 5E. Please explain it. Could it be due to the different imaging segments of neuronal cords? Also, delta-EYFA mainly changes the labeling intensity of LIN-2B, but not the pattern, please modify the description accordingly. I speculate that this effect may be related to the influence of LIN-2 on the positioning of SDN-1. Please adjust the description too.

10. Page 11 indicates "UNC-40 remained present at both cholinergic and GABAergic NMJs". However, figure 7D and E only showed the data of GABAergic NMJs.

7C showed that madd(0);sdn-1(0) phenotype is stronger than that of madd(0), although it is not significant, which suggests that both MADD and SDN-1 contribute to the labeling of UNC-40. Please modify the text accordingly.

**Minor comments:**

1. Figure 1, please indicate ventral and dorsal cords; this is also applicable for other figures.

2. Page 9, SDN-1/LIN2CASK, is this typo?

3. Page 8, "SDN-1 contains in its C-terminus an evolutionarily conserved PDZ domain binding site, which was demonstrated to interact with the
scaffolding protein CASK in the mammalian nervous system”. SDN-1 should be modified to syndecan in the mammal.

3. Significance:

Significance (Required)

-This study's findings provide a nice addition to the broader field of understanding of how the extracellular matrix regulates chemical synaptic strength and abundance of receptors.

-Heparan sulfate proteoglycans (HSPG) have been extensively reported to act as synaptic organizers (Yuzaki, 2018). HSPGs are negatively charged and interact with various proteins to regulate synaptogenesis (Song and Kim, 2013). Previous studies indicated that motoneurons secreted agrin/HSPG into vertebrate neuromuscular junctions, promoting postsynaptic clustering of AChRs (Swenarchuk, 2019). Syndecans are a class of transmembrane HSPGs (Saied-Santiago and Bülow, 2018). SDN-1 is the sole syndecan in C. elegans and is found in NMJs. However, the functional mechanism of SDN-1 in the postsynaptic clustering of AChRs remains elusive. The current study deployed CRISPR/cas9 strains and biochemical tools to examine the detailed process. Interestingly, the authors showed that SDN-1/syndecan is localized at NMJs by MADD-4/Punctin. Then, SDN-1 acts to enhance the clustering of N-AChRs via interacting with LIN-2/CASK and FRM-3/FARP.

Although this research was carried out in C. elegans, it will provide meaningful information to learn the functional mechanism of neuromuscular junctions in mammals. Thus, it has a broader neurobiological audience.

-C. elegans; intracellular transport of membrane receptor; cell polarity maintenance. The expertise mentioned above enables a better understanding and a proper scientific evaluation of this research.
I agree with reviewer #1 suggestion on adjusting the article's structure, which will make this study easier for readers to appreciate its findings. Similarly, the reviewer's suggestion that the experimental system requires further explanation is also relevant. This study utilizes a series of colocalization experiments. However, the current interpretations are a bit confusing, and further interpretation should be given in many places.

Reviewer #2 also raises concerns regarding the paper's structure, especially GABAergic results, which is a decent suggestion. The reviewer proposes that it is imperative to increase the biochemical results and the controls of biochemical experiments, which will help strengthen the specificity of the identified mechanism, especially the functional specificity of SDN-1 during regulating cholinergic synapse. Also, the suggested addition of experiments to address the functional difference of SDN-1 in cholinergic and GABAergic synapses is also significant.
REPLY TO THE REVIEWERS - Review Commons

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this study the authors make use of cutting edge molecular genetic tools, electrophysiology and cell biology to reveal the role of syndecan as a core organizer of (primarily) cholinergic NMJs. They extend their findings along several axis: cholinergic versus GABAergic synapse composition, extracellular versus intracellular scaffold and signaling complexes, and Neuroligin versus Netrin receptor roles. The paper is rich, with many meaningful findings. The richness, which is a strength, makes it really hard to follow at times, and my main advice is a reorganization of the paper and clarification of key points and findings so that it has the broad impact it is meant to have.

**Major comments:**

• I found the paper structure confusing. In part, it is confusing because it analyzes simultaneously across three axis: cholinergic versus GABAergic synapse composition, extracellular versus intracellular scaffold and signaling complexes, and Neuroligin versus Netrin receptor roles. In part it is also confusing because it is jargony and does not explain well the system. These broad findings make the paper special and valuable, but special care needs to be put into framing the story, eliminating jargon and providing enough cartoons and explanations of the system that the reader can follow the arguments.

We thank our reviewer for the positive comments and we are sorry that he/she did find the manuscript confusing. We tried to balance precision and comprehensiveness. This is clearly a challenge because the molecular organization that we uncovered is complex. We will take special care to provide a more streamlined revised version and provide material to help the reader going through the complexity of the interactions between individual components.

• The best summary of the paper is given in the first paragraph of the Discussion. I suggest using that some structure in the presentation of the findings, rather than the current structure, which is focused around SDN-1 but the drifts into a number of genetic analyses that are hard to follow in the context of SDN-1.

The core of this story is really the new functions of syndecan at synapses. If the editor agrees with our reviewer's suggestion, we can indeed write the story using a more hierarchical narrative, as in the first paragraph of the discussion.

• The system needs to be better presented in Figure 1. I assume these are en passant synapses, like many synapses in C. elegans. That neuroanatomy, and the relationship between the GABA and Cholinergic synapses in the context of the examined cells need to be better explained. Are they looking at different cells within the same bundle, innervating the same muscle? Those points are essential to understand the problem, and unclear in the text and figures.

We thank our reviewer for this comment. We understand that this system is quite specific to C. elegans researchers and we agree that providing more background would not hurt. If we have enough space, we will provide more information in the text and illustrate the structure of this system in an introductory figure.

• Is the diffuse localization pattern of L-AchR expected? Given it occupies so much of the neurite, doesn't that affect interpretation of the co-localization studies? If not, why not? The authors argue colocalization in 1D, but neither the line scans nor the images suggest more co-localization as compared to images in which they say the localization was affected, later in the paper.

L-AChRs are not diffuse: they are clustered at cholinergic NMJs. Cholinergic NMJs are dense and small, and their size is below the diffraction limit of confocal microscopy. Because GABA NMJs are bigger and sparser, they are easier to individualize. Over the years, we developed quantitative analysis methods that efficiently measured clustering and delocalization features. It is very important to use such quantitative analysis because our visual impression can sometimes be biased by the spatial structure of the signals.
• In Fig 2A, the authors claim there are no effects on presynaptic sites, but there appears to be a reduction of the examined presynaptic molecule (CLA1) which is not calculated or discussed. The intensity of this marker is quite variable, and we agree that this specific image is confusing. We are now providing a revised version of the figure, which does not show such dramatic difference between wild-type and sdn-1(0) mutant:

![Figure 2A](image)

Our point is that the loss of syndecan-1 does not modify the number of presynaptic boutons, which might have impacted the postsynaptic localization of receptors, yet we have not performed an extensive analysis of presynaptic boutons structure. This will be emphasized in the text.

• In Fig 5, were LIN-2 levels examined? LIN-2 level was quantified in Fig5G.

• In page 10, top paragraph, there seems to be a contradiction between what the authors show in the figure (decrease in FMR-3 in sdn-1 mutants) and the Zhou et al study they cite and say is consistent with "FMR-3 level was unaffected by loss of LIN-2". Maybe it is explained in a confusing manner, would benefit from clarification.

We agree that such phrasing might be confusing. In our previous study, we analyzed the epistatic interactions between lin-2 and frm-3 and we demonstrated that LIN-2 was dependent on the presence of FRM-3 for its synaptic localization, while FRM-3 was not affected by the loss of LIN-2. FRM-3 appeared to depend on its interaction with DCC/UNC-40. Similarly, in sdn-1(0) mutants, LIN-2 is gone but FRM-3 remains partially localized at synapses and depends on DCC/UNC-40. We will clarify this point in the text.

• Despite a dramatic reduction of nAChR receptors in sdn-1(0) mutants, authors observed pressure-evoked responses to nicotine, albeit reduced. They suggest two roles for syndecan that it clusters nAChRs at synapse and stabilizes them at the plasma membrane. Do the authors then claim that nAChRs are present, but below the threshold of detection of their fluorescent marker? Is a similar response seen in cr-16 mutants? Madd-4 mutants?

Our reviewer is perfectly right. As we previously observed in L-AChR clustering mutants (Galli et al; 2004; Gendrel et al, 2009) we are no longer able to detect dispersed receptors by confocal microscopy, but we can still record currents. In acr-16(0) mutants, there is no fluorescence detected and no ACR-16-dependent current remains. In madd-4(0) mutants, we previously reported a strong decrease of N-AChR using a transgene overexpressing ACR-16-GFP in muscle (Pinan-Lucarré et al, 2014, Extended figure 2, i-j). We revisited these data with our wmScarlett-ACR-16 knock-in and observed similar results.

**Minor comments:**

- In page 7, define "synaptic fluorescence" (name marker used)
  We changed the statement to "SDN-1-AID-mNG knock in fluorescence".

- Regarding the electrophysiology experiments, authors should specify whether they observed any facilitation or depression of response after performing a second and third stimulation (in methods)
  The goal of our protocol was not to test facilitation or depression. Between each stimulation we waited a few seconds. During the first stimulation, we measured the amplitude of evoked currents through L-AChR and N-AChR. Then, we applied DHBE and measured again the amplitude of the current, only through L-AChR this time. We waited for the third stimulation to be sure that N-AChRs were indeed inhibited.
- AID experiments are used to demonstrate that SDN-1 is mainly from post-synaptic muscle. In S3 it is important to show the AID system working in the DNC, where the bulk of the experiments take place. These data are provided in Fig. 3.

- Figure 6 "Hence, despite the loss of LIN-2 at cholinergic synapses in sdn-1(0) mutants, FRM-3 remained partially concentrated in postsynaptic domains of cholinergic synapses" Show cholinergic marker as well? Same with Figure 4C Madd-4B(0).

We agree that it might be more straightforward to show cholinergic markers, but as shown in previous publications and in Fig. 8 for example, the cholinergic and GABAergic domains are mutually exclusive at this resolution. We could perform the requested experiments, but this is a lot of additional work, which will not provide additional information. If this is an absolute request, we can do it, however.

- Any speculation on what the band at 97kD is in Figure 6I in the FERM-FA pull down with GST-ACR-16?

We think that it is an unspecific band that can be repeatedly detected by the HA antibody. We have no further explanation.

- The authors make a point to use the milder (as far as axon defects) unc-40(delP3) mutant when demonstrating a change in ACR-16 localization, but do not use the unc-40(delP3) mutant to show changes in SDN-1 localization. This could be interesting to further develop the domain(s) by which UNC-40 and SDN-1 are interacting.

This is an excellent suggestion. We have now completed the analysis of SDN-1 localization in unc-40(delP3) mutant background. The results show that deleting the P3 domain causes a similar decrease of SDN-1 as in unc-40(0) and in frm-3(0). These data further support our model in which UNC-40 recruits FRM-3 at NMJs through its P3 domain, which in turn stabilizes the subsynaptic scaffold bridging SDN-1 and ACR-16. These new data will be included in the manuscript.
synapses, and further experiments show a genetic interaction between Punctin, UNC-40/DCC and Syndecan. Thus (in a simplified model) it seems that Syndecan could play the same conceptual role at cholinergic synapses that NLG-1 plays in organizing GABA receptors at GABAergic synapses, that is, providing an "anchor" for the clustering of N-AChR.

This study presents robust and convincing findings and identifies the HSPG Syndecan as a novel core organizer of the cholinergic synapse that acts by coupling extra- and intracellular scaffolds to control postsynaptic receptor composition. The study should therefore be of broad interest. A few points need revision, reformulation or further experiments, especially with regard to the specificity of the identified mechanism.

**Major Points**

1. While the study focuses on the role of Syndecan at cholinergic synapses, it also presents many results on its role at GABAergic synapses. While this data is important, streamlining the results, and in particular the figure panels (e.g. by moving GABAergic results to supplemental figures), to reflect the take-home message of the study would facilitate interpretation of the results and improve readability.

   The *C. elegans* NMJ system provides an interesting means to assess the specific contribution of synaptic component with respect to synapse identity. Describing phenotypes at GABA synapses provides information on the more specific role of SDN-1 at cholinergic synapses. Since there is a need for reorganizing the manuscript, we will consider this suggestion and propose a different organization depending on the final format required by the journal where the work will be published.

2. Figure 4 and 6: the authors should demonstrate that the delta-GAG and delta-KKDEGS mutants of Syndecan are properly trafficked to the cell surface.

   We have no indication that these mutants are trapped intracellularly. However, this question is highly relevant. We could fuse pHluorin to the extracellular domain of SDN-1, to further verify that it is properly trafficked. It would be very difficult to redo everything by CRISPR, but we can express these mutant versions in muscle using standard transgenesis.

3. The genetic interaction of MADD-4 and Syndecan should be tested by a complementary approach, such as the biochemical approach with recombinant proteins previously used by the authors to demonstrate the MADD-4/NLG-1 interaction (Tu et al., Neuron 2015). This would allow testing the contribution of MADD-4L and MADD-4B. In the same assay, the authors can then also test MADD-4 binding to Syndecan lacking HS.

   The proposed experiments are of course extremely sound and, when possible, we always try to complement genetics with biochemistry. However, SDN-1 is way more difficult to manipulate than NLG-1 because of GAG chains. We made preliminary attempts, but we do not think that we will be able to perform such experiments.

4. Figure 6H would greatly benefit from an in vitro pulldown using a GST-SDN-1 delta-KKDEGS to cement the importance of this domain in the SDN-1 interaction with FRM-3.

   We have now performed this experiment and the results confirm the importance of the KKDEGS domain for the interaction between SDN-1 and FRM-3.

   ![Image](image_url)

   These data will be included in figure 6.
5. Questions remain regarding the specificity of the molecular mechanism identified, for example with regard to the more robust effects of Syndecan LOF on N-AChR. Figure 6H, I investigate the molecular link between FRM3 and N-AChR. Is this interaction specific for N-AChR and not L-AChR? Does the SDN1-ECD also interact with N-AChR and could this contribute to the selectivity for N-AChR?

Our data indicate that syndecan ICD is necessary and sufficient for N-AChR clustering. In figure 6H, I, we wanted to test if FRM-3 would engage direct interactions with syndecan and N-AChR, or if it only contributed to stabilize LIN-2 at synapses. Our results support a model in which FRM-3, together with LIN-2, forms a complex that physically bridges SDN-1 and N-AChR. Whether SDN-1-ECD interacts with N-AChR is not excluded, but at least we show in fig. 8 that SDN-1-ICD specifically recruits N- but not L-AChR, independently from the ECD. L-AChR clustering relies on an extracellular scaffold containing LEV-9, LEV-10 and OIG-4. How FRM-3 might modulate L-AChR synaptic content is complicated by the interdependency of the different components, namely MAD4-4, SDN-1, LIN-2, FRM-3, UNC-40, as stressed out in the discussion. The firm conclusions are that SDN-1 is an obligatory component for N-AChR clustering in vivo, and LEV-9 and LEV-10 for L-AChR clustering. Unfortunately, the relative contribution of the other components in the modulation of L-AChR synaptic contents is difficult to test in this system.

6. All components identified: MAD4-4/DCC/FRM3/Lin2, as well as SDN-1, are present at both cholinergic and GABAergic sites. It remains puzzling how the same molecular players can organize specific neurotransmitter receptor composition at both types of synapses. Does SDN-1 display a preference for MAD4-4L or MAD4-4B? Alternatively, might there be a competitive interaction of the positively charged Punctin Ig domain with NLG-1 or SDN-1 HS? These possibilities, as well as some additional options mentioned on page 14 (Discussion), should be experimentally addressed in order to provide some insight.

We agree with our reviewer that this the burning question of this work and we would love to answer it directly. SDN-1 does not display a preference for MAD4-4L vs MAD4-4B as shown in Fig. 4C-D. We already crossed SDN-1-mNG in a nlg-1(0) mutant and we do not see major differences. We can provide a more quantitative analysis. Testing if there is distinct oligomerization at the nanoscale or a differential phosphorylation of SDN-1 at distinct synapses is unfortunately beyond technical feasibility at the moment in the context of this study.

7. Figure 8: the authors use an elegant approach to demonstrate that SDN-1 is not only required but also sufficient for the recruiting of N-AChRs specifically. It would be interesting to see if the opposite experiment (SDN-1-ECD/NLG-1-ICD) is also capable of recruiting GABA receptors to cholinergic synapses.

Yes! This is a great experiment that we tried in a slightly different way. Because GABARs are randomly distributed along the cord in nlg-1(0) mutants, it would be very difficult to interpret the distribution of the receptors after expression of a SDN-1-ECD/NLG-1-ICD chimera, which will localize at both types of synapses. Therefore, we expressed in nlg-1(0) mutants a LEV-10-ECD/NLG-1-ICD, which was supposed to bring the NLG-1-ICD at cholinergic synapses. Unfortunately, this chimera is unstable, and gets diffuse when overexpressed. This remains, however, an extremely interesting suggestion that we would like to follow up in the future if we can develop better tools.

**Minor Points**

1. The title "The Heparan Sulfate Proteoglycan Syndecan Differentially Localizes Acetylcholine Receptor Subtypes at Cholinergic Synapses" suggests that Syndecan uses different mechanisms to localize/cluster N-AChRs and L-AChRs at the NMJ. Yet, most of the paper focuses on the mechanisms specific to N-AChR localization and clustering. By the authors' own admission, Syndecan is not strictly required for L-AChR clustering, rather, it modulates the synaptic content of L-AChRs. The title needs modifications to reflect the different amounts of evidence for Syndecan's role in N-AChR vs L-AChR synaptic localization. Alternatively, the authors can provide a molecular mechanism by which Syndecan regulates L-AChR synaptic content.

This is very embarrassing but the genuine title of the manuscript, as loaded on the Review Commons web site is: "The HSPG Syndecan is a core organizer of cholinergic synapses in C. elegans". For unexplained reasons, the PDF document that was uploaded contained an old title. We hope that the actual title addresses our reviewer's concerns.
2. Figure 1: it seems SDN-1 is broadly expressed at the dorsal cord. It might be due to my incomplete knowledge of C. elegans neurobiology, but it does not seem to me that it is specifically enriched at synapses or NMJs. Such a figure title would require a quantification of the enrichment (e.g. compared to an axonal marker or fill). SDN-1 is expressed in multiple tissues, especially in the nearby epidermis, which might confuse the visual impression (see for example fig 3B, third row, where the pattern is more punctate after degradation of the epidermis component). In addition, SDN-1 is present at both cholinergic and GABAergic NMJs, which altogether are dense along the nerve cords. However, analysis of fluorescence peaks clearly indicates synaptic enrichment, as shown in fig. C-D and all along the paper.

3. The broad effect of Syndecan on all types of synapses at the NMJ suggests that it might also have a role in organizing the presynaptic active zones, which in turn would have an effect on the post-synapse. Indeed, Figure 2A shows a visible reduction in the intensity of cholinergic CLA1-BFP in the Syndecan LOF. This would need to be further quantified/characterized.

4. Figure 2: needs a more accurate explanation of the optogenetic stimulation. We gave more details of the protocol in the M&M section. However, if the reviewer thinks it is mandatory to add them in the legends, we can do it.

5. A clearer cartoon of the various Syndecan constructs used in the study, in the figures where they are used (e.g. the bicistronic one used in Figure 3), would help interpret results. Depending on the final format of the manuscript as conditioned by the Journal policy, we will add cartoons to better illustrate the constructs we used.

6. Figure 3: The methodology here is a bit perplexing, although the main result (that Syndecan at the NMJ is mostly expressed by the muscle cells) is rather convincing. The main way Syndecan expressed e.g. in the epidermis would be through shedding of the extracellular domain. Indeed, Syndecans, like many transmembrane receptors, are known to be proteolytically cleaved. But in order to determine the localization of the shed extracellular fragments, one needs an N-terminal fluorescent tag. However, the tag used in this experiment is cytoplasmic. I fail to see how the C-terminal domain of SDN-1 produced in the distant epidermis ends up localized at the NMJ (Figures 3B and 3D). More generally, this type of protein degradation experiment could be used to deplete SDN-1 in a more localized manner and observe its effect on AChRs at cholinergic synapses. Our reviewer is perfectly correct. However, the reason why we tested if some of the signal detected at NMJs was contributed by the epidermis is because of the C. elegans anatomy. As shown on these transversal EM sections (https://www.wormatlas.org/SW/SW.php/), the dorsal cord (DC) and the ventral cord (VC) are in intimate contact with the epidermis (Hyp 7). Confocal photonic microscopy does not have the resolution to discriminate SDN-1 at NMJs from SDN-1 on the epidermis membrane. Tissue-specific degradation provides a means to solve this question. This will be clarified. However, we do see some potential cleavage of SDN-1 by western blot analysis (Fig. S4) and we do not exclude that it is functionally relevant, although this was not directly tested.

7. Figure 5: it seems the RFP-LIN-2B and LIN-2A-GFP are used interchangeably. Please clarify. We do apologize because errors were inadvertently introduced in the figure labeling. In these experiments, the RFP-tagged LIN-2 protein was expressed from our krSi35 allele, which indeed corresponds to RFP-LIN-2A as described in the material and methods. Also, in Fig 5K, we should read GFP-LIN-2A instead of LIN-2A-GFP considering the tag position. This will be corrected.
10. cholinergic NMJ. We previously analyzed mutants deeply affecting cholinergic neurotransmission such as otherwise, we have no argument to think that the GABA NMJ.

We do not see any behavioral phenotype that can be attributed to functional defects of GABA synapses.

2. Are there any GABA localization of addition SDN could not identify any electron tomography after high resolution analysis.

With respect to the second part of the question, the ultrastructure of the NMJs was analyzed in madd-4 mutants by electron tomography after high-pressure freezing of adult animals (Pinan et al., Nature, 2014, extended data Fig.3). We could not identify any synaptic morphological defect, even at this very high resolution, despite the disappearance of SDN-1 in these mutants. However, it will certainly be interesting in the future to test if MADD-4 might control the localization of additional ECM components.

2. Are there any GABA-related functional defects in sdn-1 mutant animals? Is there any evidence that highlighting sdn-1 physiological effects do not have the indirect effects of GABA synapses? As the author stated, in GABA synapses, UNC-40 has also been studied to show its functional effects.

We do not see any behavioral phenotype that can be attributed to functional defects of GABA synapses. We have not performed electrophysiological recording because this is a lot of work and we already know that defects are limited at GABA NMJs based on the moderate decrease of GABAR content. Otherwise, we have no argument to think that the phenotypes at GABA NMJs are a consequence of defects at cholinergic NMJ. We previously analyzed mutants deeply affecting cholinergic neurotransmission such as lev-9 or lev-10 and saw no electrophysiological nor morphological defects at GABA NMJs. A recent work from the group of Mike...
Francis (Barbagallo et al, Development, 2017) indicates that impairing cholinergic synaptogenesis by disruption of cholinergic innervation either by manipulating cholinergic motoneuron differentiation or synaptic transmission tends to decrease the density of GABA NMJs and increase GABA receptor synaptic content (Fig. 4), which is not what to we observe in sdn-1 mutants.

In Figure 2B-C, it is important to assay the expression level of AChR in animals or tissues by western blot. We do not have antibodies to detect N-AChRs by western blot. In addition, these receptors represent minute amounts of the total worm proteins and cannot be detected in whole worm extracts. If requested, we can IP the CRISPR-tagged N- and L-AChRs in WT and mutants. Yet, we think that electrophysiological measurements are more quantitative to measure surface receptor content in this system. We agree with our reviewer that estimating the overall protein content will be an interesting information, which will not change, however, our interpretation on the role of SDN-1 in the regulation of synaptic receptor content.

In Figure 2D-G, L-AChRs are also clustered, but the stability does not seem to be affected, this phenotype should be explained or tested; in addition, the endocytic degradation of N-AChRs is solely speculated, should be assayed using endocytosis mutants.

The phenotype of L-AChRs is consistent with our previous observations in clustering mutants such as lev-9 and lev-10 where synaptic currents are drastically reduced while the global response to pressure-applied levamisole is unchanged, indicating that receptors redistribute at the surface of the muscle cell. We can comment this point furthermore in the discussion. We agree that we speculate that N-AChRs might be endocytosed and degraded. We will cross a mutant allele of dpy-23, which encodes the AP2 µ1 subunit, into sdn-1(0) and test if N-AChR levels are increased.

In Figure 2, sdn-1(0): N-AChR level decreased. Also, in Figure 4, it showed madd-4(0); SDN-1 level decreased. The direct inference of the above two experiments is that the level of N-AChR in madd-4 mutant should also be significantly reduced, please confirm it.

In madd-4(0) mutants, we previously reported a strong decrease of N-AChRs using a transgene overexpressing ACR-16-GFP in muscle (Pinan-Lucarré et al, 2014, Extended figure 2, i-j). We revisited these data with our wmScarlett-ACR-16 knock-in and observed similar results. These data will be mentioned in the text.

In Figure 4B-C, for the distribution phenotype of SDN-1, it is useful to provide the results of rescue experiments using the two isoforms L and B in madd-4 mutant animals.

In madd-4L(0) MADD-4B is still expressed, while in madd-4B(0) MADD-4L remains, which is equivalent to the rescue experiments requested by our reviewer. We will further detail each genetic background to make it clearer.

In Figure 4B-C, why not examine the colocalization between SDN-1 and Ach? Also, the SDN-1 levels in madd-4L(0) and 4B(0) animals are so different from those in (0), please explain it. Maybe the rescue experiment can help explain this discrepancy. Additionally, Page 8 mentioned that the localization and distribution of SDN-1 are regulated by both L and B isoforms. This statement is not accurate; indeed, the distribution of SDN-1 in L(0) is not significantly affected. As shown in Fig. 8 for example, the cholinergic and GABAergic domains are mutually exclusive at this resolution and showing one of the two markers provides similar information in this context. We chose to show GABAergic domains in Figure 4C because they are easier to individualize and more easily demonstrate the relocalization of SDN-1 out of GABAergic domains by the MADD-4L isoform, which is exclusively present at cholinergic synapses (Pinan-Lucarré et al, 2014; p. 7 of the manuscript). Our data indicate that the C-terminal moiety of MADD-4, which is identical in MADD-4-L and -B, is necessary to concentrate SDN-1 at NMJs. In the absence of MADD-4L, the expression of MADD-4B is sufficient to localize SDN-1 at both NMJ types. In the absence of MADD-4B, MADD-4 is absent from GABAergic NMJs and MADD-4L relocalizes SDN-1 at cholinergic NMJs. Hence, we think that it is accurate to say that MADD-4L also controls SDN-1 localization. To be more precise, we will specify in the text "likely through its C-terminal moiety".

In Figure S4C, although it is not mandatory, it will be more desirable to use a synapse marker together for colocalization patterns. Another suggestion, Figure 4F-G could be relocated into a supplemental figure and add a western blot to validate that there is no protein expression variation. Although the amount of the SDN-1 variants is decreased after the mutation of the different HS attachment sites, the cords are readily identified in the animals. The aim of these experiments is really to check how much SDN-1 remains at the cord. Providing a presynaptic marker will not really change the conclusions. However, we think that it is important to show in the main figures that SDN-1 modulates the amount of MADD-4 at NMJs because this is the first protein shown to regulate the amount of MADD-4 at the synapse. Because the effect is modest, we do not think that western blot analysis will be conclusive, especially because MADD-4 is very difficult to solubilize, and we doubt that we will reliably see a 30 % change in the total protein level.
9. Figure 5, whether UNC-40 functions in the LIN-2 positioning process, which should be tested. In Figure 5I, the colocalization of LIN-2B and SDN-1 is perfect. It is worth noting that LIN-2B is known to be located in GABA NMJ too. Thus, this result does not seem to be completely consistent with the result in 5E. Please explain it. Could it be due to the different imaging segments of neuronal cords? Also, delta-EYFA mainly changes the labeling intensity of LIN-2B, but not the pattern, please modify the description accordingly. I speculate that this effect may be related to the influence of LIN-2 on the positioning of SDN-1. Please adjust the description too.

We showed in a previous study that LIN-2 is reduced by 55% in unc-40(0). (Fig 3C, Zhou et al., 2020). As written p.9, we previously published that LIN-2 is present at GABAergic and cholinergic NMJs, similar to SDN-1 as shown in 5I. Consistently in figure 5E, LIN-2 is located at GABA synapses labeled by neuroligin NLG-1 and at cholinergic NMJs outside of GABA synapses in a wild-type background (control). By contrast, LIN-2 is only detected at GABA synapses in sdn-1(0) mutants, which is a new result. As stated in the text based on Figure 5I-J and Figure S5B-D, we do show that introducing the delta EYFA mutation in SDN-1 changes the pattern of LIN-2, which becomes concentrated at GABA NMJs, most probably by interacting with NLG-1. Accordingly, LIN-2-GFP was almost undetectable at the nerve cord of nlg-1(0); sdn-1(0) double mutants (Fig. 5K).

10. Page 11 indicates "UNC-40 remained present at both cholinergic and GABAergic NMJs". However, figure 7D and E only showed the data of GABAergic NMJs. 7C showed that madd(0);sdn-1(0) phenotype is stronger than that of madd(0), although it is not significant, which suggests that both MADD and SDN-1 contribute to the labeling of UNC-40. Please modify the text accordingly.

UNC-40 has been demonstrated to localize on both synapses (Tu et al., 2015; Zhou et al., 2020). As stated above, the cholinergic and GABAergic domains are mutually exclusive at this resolution and showing one of the two markers provides similar information in this context. With respect to Figure 7C, we are not sure about the comment of our reviewer because the results do indicate that there is no difference between madd-4(0) and madd-4(0); sdn-1(0) mutants.

**Minor comments:**

1. Figure 1, please indicate ventral and dorsal cords; this is also applicable for other figures. Ventral and dorsal cords were labeled in fig 1B. In the next panels and all the following figures images were taken from the dorsal cord as mentioned in the figure legend.

2. Page 9, SDN-1/LIN2CASK, is this typo? Thank you for pointing this out. We modified the text to “SDN-1 and LIN-2/CASK interaction”

3. Page 8, "SDN-1 contains in its C-terminus an evolutionarily conserved PDZ domain binding site, which was demonstrated to interact with the scaffolding protein CASK in the mammalian nervous system". SDN-1 should be modified to syndecan in the mammal.

We will modify the text accordingly.

Reviewer #3 (Significance (Required)):

-This study's findings provide a nice addition to the broader field of understanding of how the extracellular matrix regulates chemical synaptic strength and abundance of receptors.

-Heperan sulfate proteoglycans (HSPG) have been extensively reported to act as synaptic organizers (Yuzaki, 2018). HSPGs are negatively charged and interact with various proteins to regulate synaptogenesis (Song and Kim 2013). Previous studies indicated that motoneurons secreted agrin/HSPG into vertebrate neuromuscular junctions, promoting postsynaptic clustering of AChRs (Swenarchuk, 2019). Syndecans are a class of transmembrane HSPGs (Saied-Santiago and Bülow, 2018). SDN-1 is the sole syndecan in C. elegans and is found in NMJs. However, the functional mechanism of SDN-1 in the postsynaptic clustering of AChRs remains elusive. The current study deployed CRISPR/cas9 strains and biochemical tools to examine the detailed process. Interestingly, the authors showed that SDN-1/syndecan is localized at NMJs by MADD-4/Punctin. Then, SDN-1 acts to enhance the clustering of N-AChRs via interacting with LIN-2/CASK and FRM-3/FARP.
Although this research was carried out in C. elegans, it will provide meaningful information to learn the functional mechanism of neuromuscular junctions in mammals. Thus, it has a broader neurobiological audience.

-C. elegans; intracellular transport of membrane receptor; cell polarity maintenance. The expertise mentioned above enables a better understanding and a proper scientific evaluation of this research.

**Referees cross-commenting**

I agree with reviewer #1 suggestion on adjusting the article's structure, which will make this study easier for readers to appreciate its findings. Similarly, the reviewer's suggestion that the experimental system requires further explanation is also relevant. This study utilizes a series of colocalization experiments. However, the current interpretations are a bit confusing, and further interpretation should be given in many places.

We will follow the reviewer's comment to clarify the text, keeping in mind that this system involves several molecular players and that an oversimplified model needs to be avoided.

Reviewer #2 also raises concerns regarding the paper's structure, especially GABAergic results, which is a decent suggestion. The reviewer proposes that it is imperative to increase the biochemical results and the controls of biochemical experiments, which will help strengthen the specificity of the identified mechanism, especially the functional specificity of SDN-1 during regulating cholinergic synapse. Also, the suggested addition of experiments to address the functional difference of SDN-1 in cholinergic and GABAergic synapses is also significant.

Depending on the final format of the manuscript, we might restrict the main focus of the paper on the functions of SDN-1 at cholinergic junctions and we will complement genetic experiments with biochemical data when feasible.
January 13, 2021

Re: JCB manuscript #202011144T

Prof. Jean-Louis Bessereau
Claude Bernard University Lyon 1
UMR 5534
8 avenue Rockefeller
Lyon 69008
France

Dear Prof. Bessereau,

Thank you for submitting your manuscript entitled "The HSPG Syndecan is a core organizer of cholinergic synapses in C. elegans" from Review Commons, and for your patience while we assessed your study and response to the reviewer comments. Overall, we agree that your manuscript provides interesting insight into the role of the ECM at the synapse which is of interest to the readership of JCB. Therefore, we invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

While for the most part we agree with your proposed revisions, for resubmission to JCB you must also address the following:

Reviewer 1:
The plan to address the questions of reviewer 1 are adequate (mostly textual changes and reorganization).

Reviewer 2:

1) In his/her third question the reviewer asks to demonstrate a biochemical interaction between MADD4 and Syndecan. I understand the difficulty of promising the include biochemical interactions if one of the proteins is difficult to manipulate. I would, however, appreciate the author's best effort.

2) In the fifth question the reviewer asks about the specificity of the observed molecular mechanism. The authors state that it is difficult to go much further in the current system. I can follow the author's argument but urge them to address this and alternative possibilities clearly in the discussion.

4) I do believe that the 6th comment of reviewer 2 is valid, and for the JCB, additional experimental detail on how the same molecular players that are studied in this manuscript ultimately organize different transmitter receptor types at both synapses is an interesting and relevant point to address. This is an element that can be at least partially addressed with the technology available.

5) Given that the experiment proposed in the 7th question of reviewer 2 is a logical and very interesting experiment, and that the authors have carried out a slightly modified version of this, I would ask to mention this in the text and state that the experiment in the end did not work because the chimeric protein was unstable.
The proposed plans to address the additional questions by reviewer 2 are adequate.

Reviewer 3:
1) to address the first question of reviewer 3 it would be appropriate to analyze the ECM using a limited number of markers.

2) The question to determine AChR expression levels the reviewer asks for in question 3 is important to address. IP of the tagged protein is an option; at minimum Q-RT-PCR could be included.

The proposed plans to address the additional questions by reviewer 3 are adequate.

In addition, please carefully reorganize your manuscript as discussed by the reviewers.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Transfer is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Transfers may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Transfers may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments
point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you’ve had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Patrik Verstreken, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this study the authors make use of cutting edge molecular genetic tools, electrophysiology and cell biology to reveal the role of syndecan as a core organizer of (primarily) cholinergic NMJs. They extend their findings along several axis: cholinergic versus GABAergic synapse composition, extracellular versus intracellular scaffold and signaling complexes, and Neuroligin versus Netrin receptor roles. The paper is rich, with many meaningful findings. The richness, which is a strength, makes it really hard to follow at times, and my main advice is a reorganization of the paper and clarification of key points and findings so that it has the broad impact it is meant to have.

**Major comments:**

•I found the paper structure confusing. In part, it is confusing because in analyzes simultaneously across three axis: cholinergic versus GABAergic synapse composition, extracellular versus intracellular scaffold and signaling complexes, and Neuroligin versus Netrin receptor roles. In part it is also confusing because it is jargony and does not explain well the system. These broad findings make the paper special and valuable, but special care needs to be put into framing the story, eliminating jargon and providing enough cartoons and explanations of the system that the reader can follow the arguments.

We thank our reviewer for the positive comments and we are sorry that he/she did find the manuscript confusing. We tried to balance precision and comprehensiveness. This is clearly a challenge because the molecular organization that we uncovered is complex. We have taken special care to provide a more streamlined revised version and provide material to help the reader going through the complexity of the interactions between individual components (see also the two next points).

•The best summary of the paper is given in the first paragraph of the Discussion. I suggest using that some structure into framing the story, eliminating jargon and providing enough cartoons and explanations of the system that the reader can follow the arguments.

We followed this suggestion and extensively reframed the Results section to follow a more hierarchical narrative, as in the first paragraph of the discussion. As a consequence, the order and the content of the figures have been changed:

- New Fig. 1 corresponds to previous Fig. 2, in which we have added a cartoon explaining the experimental system (see next comment)
- New Fig. S1 corresponds to previous Fig. S2
- New Fig. S2 corresponds to previous Fig. S1
- New Fig. S1 corresponds to previous Fig. 1D-H
- New Fig. S4 corresponds to previous Fig. S3
- New Fig. 3 corresponds to previous Fig. S4
- New Fig. 5 corresponds to previous Fig. 7
- New Fig. 6 corresponds to previous Fig. 5
- New Fig. 7 corresponds to previous Fig. 6

•The system needs to be better presented in Figure 1. I assume these are en passant synapses, like many synapses in C. elegans. That neuroanatomy, and the relationship between the GABA and Cholinergic synapses in the context of the examined cells need to be better explained. Are they looking at different cells within the same bundle, innervating the same muscle? Those points are essential to understand the problem, and unclear in the text and figures.

We understand that this system is quite specific to C. elegans researchers and we agree that providing more background would help. We have added cartoons in the new figures 1 and 2 to present the experimental system.

•Is the diffuse localization pattern of L-AchR expected? Given it occupies so much of the neurite, doesn’t that affect interpretation of the co-localization studies? If not, why not? The authors argue colocalization in 1D, but neither the line scans nor the images suggest more co-localization as compared to images in which they say the localization was affected, later in the paper.

L-AChRs are not diffuse: they are clustered at cholinergic NMJs. Cholinergic NMJs are dense and small, and their size is below the diffraction limit of confocal microscopy. Because GABA NMJs are bigger and sparser, they are easier to individualize. Over the years, we developed quantitative analysis methods that efficiently measured clustering and delocalization features. It is very important to use such quantitative analysis because our visual impression can sometimes be biased by the spatial structure of the signals.
• In Fig 2A, the authors claim there are no effects on presynaptic sites, but there appears to be a reduction of the examined presynaptic molecule (CLA1) which is not calculated or discussed. The intensity of this marker is quite variable, and we agree that the image in the first version was confusing. We are now providing a revised version of the figure, which does not show such dramatic difference between wild-type and sdn-1(0) mutant. Our point is that the loss of syndecan-1 does not modify the number of presynaptic boutons, which might have impacted the postsynaptic localization of receptors, yet we have not performed an extensive analysis of presynaptic boutons structure.

• In Fig 5, were LIN-2 levels examined? LIN-2 level was quantified in Fig 6G (previous Fig. 5G).

• In page 10, top paragraph, there seems to be a contradiction between what the authors show in the figure (decrease in FMR-3 in sdn-1 mutants) and the Zhou et al study they cite and say is consistent with "FRM-3 level was unaffected by loss of LIN-2". Maybe it is explained in a confusing manner, would benefit from clarification. We agree that such phrasing might be confusing. In our previous study, we analyzed the epistatic interactions between lin-2 and frm-3 and we demonstrated that LIN-2 was dependent on the presence of FRM-3 for its synaptic localization, while FRM-3 was not affected by the loss of LIN-2. FRM-3 appeared to depend on its interaction with DCC/UNC-40. Similarly, in sdn-1(0) mutants, LIN-2 is gone but FRM-3 remains partially localized at synapses and depends on DCC/UNC-40. This paragraph was extensively edited in the current version (p. 9).

• Despite a dramatic reduction of nAChR receptors in sdn-1(0) mutants, authors observed pressure-evoked responses to nicotine, albeit reduced. They suggest two roles for syndecan - that it clusters nAChRs at synapse and stabilizes them at the plasma membrane. Do the authors then claim that nAChRs are present, but below the threshold of detection of their fluorescent marker? Is a similar response seen in acr-16 mutant? Madd-4 mutants? Our reviewer is perfectly right. As we previously observed in L-AChR clustering mutants (Galli et al; 2004; Gendrel et al. 2009) we are no longer able to detect dispersed receptors by confocal microscopy, but we can still record currents. In contrast with sdn-1(0) mutants, there is no response to nicotine in acr-16(0) mutants. In madd-4(0) mutants, we previously reported a strong decrease of N-AChRs using a transgene overexpressing ACR-16-GFP in muscle (Pinan-Lucarré et al, 2014, Extended figure 2, i,j). We revisited these data with our wmscarlett-ACR-16 knock-in and observed similar results.

**Minor comments:**

- In page 7, define "synaptic fluorescence" (name marker used)
  We changed the statement to "SDN-1-AID-mNG knock in fluorescence".

- Regarding the electrophysiology experiments, authors should specify whether they observed any facilitation or depression of response after performing a second and third stimulation (in methods)
  The goal of our protocol was not to test facilitation or depression. Between each stimulation we waited a few seconds. During the first stimulation, we measured the amplitude of evoked currents through L-AChR and N-AChR. Then, we applied DHBE and measured again the amplitude of the current, only through L-AChR this time. We waited for the third stimulation to be sure that of N-AChRs were indeed inhibited.

- AID experiments are used to demonstrate that SDN-1 is mainly from post-synaptic muscle. In S3 it is important to show the AID system working in the DNC, where the bulk of the experiments take place.
  These data are provided in new Fig. 2F, third raw: after activation of the AID system in neurons, we can observe that the SDN-1 fluorescence is less bright and less dense at the nerve cord, in agreement with the control experiments in Figure S4 showing that Prab-3::tir-1-bfp is efficiently degrading an AID-GFP in all neurons.

- Figure 6 "Hence, despite the loss of LIN-2 at cholinergic synapses in sdn-1(0) mutants, FRM-3 remained partially concentrated in postsynaptic domains of cholinergic synapses" Show cholinergic marker as well? Same with Figure 4C Madd-4B(0)
  We agree that it might be more straightforward to show cholinergic markers, but as shown in previous publications and in Fig. 8E for example, the cholinergic and GABAergic domains are mutually exclusive at this resolution. We could perform the requested experiments, but this is a lot of additional work, which will not provide additional information. If this is an absolute request, we can do it, however.
Any speculation on what the band at 97kD is in Figure 6I in the FERM-FA pull down with GST-ACR-16?
We think that it is an unspecific band that can be repeatedly detected by the HA antibody. We have no further explanation.

The authors make a point to use the milder (as far as axon defects) unc-40(delP3) mutant when demonstrating a change in ACR-16 localization, but do not use the unc-40(delP3) mutant to show changes in SDN-1 localization. This could be interesting to further develop the domain(s) by which UNC-40 and SDN-1 are interacting.

This is an excellent suggestion. We have now analyzed SDN-1 localization in unc-40(delP3) mutant background. The results show that deleting the P3 domain causes a similar decrease of SDN-1 as in unc-40(0) and in frm-3(0). These data further support our model in which UNC-40 recruits FRM-3 at NMJs through its P3 domain, which in turn stabilizes the subsynaptic scaffold bridging SDN-1 and ACR-16. These new data are now included in new Fig. 5B and summarized in the graphical model in Fig. 9.

Reviewer #1 (Significance (Required)):

This study advances the understanding of syndecans in their role as post-synaptic organizers. Additionally, this work proposes a novel mechanism for N-AChR localization at the post-synaptic site. This work builds upon previous studies to identify in vivo mechanisms for localizing molecules to the postsynaptic site (Tu et al., 2015). Here, they use elegant structure-function analyses and genetic interactions to make a strong case for the new mechanisms they introduce.
Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this study, Zhou et al. identify a role for the heparan sulfate proteoglycan (HSPG) Syndecan in organizing acetyl choline receptor (AChR) clustering at the cholinergic synapses of the C. elegans neuromuscular junction (NMJ). The extracellular matrix protein Ce-Punctin/MADD-4 is a master organizer of cholinergic synapses at the NMJ, but the signaling pathways involved downstream of Punctin at this synapse are less well understood. This article sheds light on these mechanisms and demonstrates that the Punctin/DCC/FRM3/Lin2 signaling pathway, previously identified at GABAergic synapses at the NMJ (Zhou et al., Nature Commun 2020), is also essential for the proper organization of AChRs at cholinergic synapses. The main finding of this article is that the HSPG Syndecan is a novel contributor to this previously described signaling pathway. Syndecan seems broadly expressed and thus is also localized at the NMJ, at both cholinergic and GABAergic synapses. LOF experiments demonstrate a striking reduction of synaptic AChR content (and to a lesser extent synaptic GABA receptor content). The Syndecan C-terminus is able to bind both FRM3 and Lin2 intracellular scaffold proteins and in turn these effectors play a role in the clustering and presumably membrane presentation of the N-AChR subtype. Punctin plays an essential role in the expression/localization of Syndecan at synapses, and further experiments show a genetic interaction between Punctin, UNC-40/DCC and Syndecan. Thus (in a simplified model) it seems that Syndecan could play the same conceptual role at cholinergic synapses that NLG-1 plays in organizing GABA receptors at GABAergic synapses, that is, providing an "anchor" for the clustering of N-AChR.

This study presents robust and convincing findings and identifies the HSPG Syndecan as a novel core organizer of the cholinergic synapse that acts by coupling extra- and intracellular scaffolds to control postsynaptic receptor composition. The study should therefore be of broad interest. A few points need revision, reformulation or further experiments, especially with regard to the specificity of the identified mechanism.

**Major Points**

1. While the study focuses on the role of Syndecan at cholinergic synapses, it also presents many results on its role at GABAergic synapses. While this data is important, streamlining the results, and in particular the figure panels (e.g. by moving GABAergic results to supplemental figures), to reflect the take-home message of the study would facilitate interpretation of the results and improve readability.

The C. elegans NMJ system provides an interesting means to assess the specific contribution of synaptic components with respect to synapse identity. Describing phenotypes at GABA synapses provides information on the more specific role of SDN-1 at cholinergic synapses. However, we agree that it can also be distracting: we followed our reviewer's recommendation and moved the GABA data to the new Fig. S1, S2 and S3.

2. Figure 4 and 6: the authors should demonstrate that the delta-GAG and delta-KKDEGS mutants of Syndecan are properly trafficked to the cell surface.

We have no indication that these mutants are trapped intracellularly. However, to verify that these Syndecan mutants are properly trafficked to the cell surface, we fused pHluorin to the extracellular domain of SDN-1 and expressed these mutant versions in muscle using standard transgenesis. The results are shown below. Both mutant SDN-1 versions were properly expressed and enriched at neuromuscular junctions (indicated by arrows). Scale bar:10 μm. These results are not included in the manuscript because of space limitation.

![Pmyo-3::SP::pHluorin-SDN-1delGAG](image1.png)

![Pmyo-3::SP::pHluorin-SDN-1delKKDEGS](image2.png)

3. The genetic interaction of MADD-4 and Syndecan should be tested by a complementary approach, such as the biochemical approach with recombinant proteins previously used by the authors to demonstrate the MADD-4/NLG-1 interaction (Tu et al., Neuron 2015). This would allow testing the contribution of MADD-4L and MADD-4B. In the same assay, the authors can then also test MADD-4 binding to Syndecan lacking HS.
The proposed experiments are of course extremely sound and, when possible, we always try to complement genetics with biochemistry. However, as compared to NLG-1, SDN-1 is way more difficult to express and manipulate, maybe because of the presence of the GAG chains. After several attempts, we succeeded in co-expressing MADD-4B-eGFP and HA-SDN-1 in HEK293 EBNA cells and we were able to colP these 2 proteins, using anti-GFP antibodies. These results are now presented in Fig 4H.

4. Figure 6H would greatly benefit from an in vitro pulldown using a GST-SDN-1 delta-KKDEGS to cement the importance of this domain in the SDN-1 interaction with FRM-3.

We have now performed this experiment and the results confirm the importance of the KKDEGS domain for the interaction between SDN-1 and FRM-3. The results are shown in Fig 7H.

5. Questions remain regarding the specificity of the molecular mechanism identified, for example with regard to the more robust effects of Syndecan LOF on N-AChR. Figure 6H, I investigate the molecular link between FRM3 and N-AChR. Is this interaction specific for N-AChR and not L-AChR? Does the SDN1-ECD also interact with N-AChR and could this contribute to the selectivity for N-AChR?

Our data indicate that syndecan ICD is necessary and sufficient for N-AChR clustering. In figure 6H, I (i.e. new Fig. 7 H, I), we wanted to test if FRM-3 would engage direct interactions with syndecan and N-AChR, or if it only contributed to stabilize LIN-2 at synapses. Our results support a model in which FRM-3, together with LIN-2, forms a complex that physically bridges SDN-1 and N-AChR. Whether SDN-1-ECD interacts with N-AChR is not excluded, but at least we show in fig. 8 that SDN-1-ICD specifically recruits N- but not L-AChR, independently from the ECD.

L-AChR clustering relies on an extracellular scaffold containing LEV-9, LEV-10 and OIG-4. How FRM-3 might modulate L-AChR synaptic content is complicated by the interdependency of the different components, namely MADD-4, SDN-1, LIN-2, FRM-3, UNC-40, as stressed out in the discussion. The firm conclusions are that SDN-1 is an obligatory component for N-AChR clustering in vivo, and that LEV-9 and LEV-10 are at the core of L-AChR clustering. Unfortunately, the relative contribution of the other components in the modulation of L-AChR synaptic contents is difficult to test in this system.

6. All components identified: MADD-4/DCC/FRM3/Lin2, as well as SDN-1, are present at both cholinergic and GABAergic sites. It remains puzzling how the same molecular players can organize specific neurotransmitter receptor composition at both types of synapses. Does SDN-1 display a preference for MADD-4L or MADD-4B? Alternatively, might there be a competitive interaction of the positively charged Punctin Ig domain with NLG-1 or SDN-1 HS? These possibilities, as well as some additional options mentioned on page 14 (Discussion), should be experimentally addressed in order to provide some insight.

We agree with our reviewer that this the burning question of this work and we would love to answer it directly. SDN-1 does not display a preference for MADD-4L vs MADD-4B as shown in Fig.4C-D. To test if NLG-1 could outcompete SDN-1 for LIN-2/CASK binding and stabilize a complex that preferentially binds GABA<sub>R</sub>As, we crossed wrmScarlet-AChR-16 in a nlg-1(0) mutant and saw no major differences. However, this is a negative result, which is difficult to interpret. Testing if there is distinct oligomerization at the nanoscale or a differential phosphorylation of SDN-1 at distinct synapses is unfortunately beyond technical feasibility at the moment in the context of this study.

7. Figure 8: the authors use an elegant approach to demonstrate that SDN-1 is not only required but also sufficient for the recruiting of N-AChRs specifically. It would be interesting to see if the opposite experiment (SDN-1-ECD/NLG-1-ICD) is also capable of recruiting GABA receptors to cholinergic synapses.

Yes! This is a great experiment that we tried in a slightly different way. Because GABARs are randomly distributed along the cord in nlg-1(0) mutants, it would be very difficult to interpret the distribution of the receptors after expression of a SDN-1-ECD/NLG-1-ICD chimera, which will localize at both types of synapses. Therefore, we expressed in nlg-1(0) mutants a LEV-10-ECD/NLG-1-ICD, which was supposed to bring the NLG-1-ICD at cholinergic synapses. Unfortunately, this chimera is unstable, and gets diffuse when overexpressed. This remains, however, an extremely interesting suggestion that we would like to follow up in the future if we can develop better tools. Following reviewer’s and editor’s comment, we have now mentioned in the last paragraph of page 12 that we performed this experiment.

**Minor Points**

1. The title “The Heparan Sulfate Proteoglycan Syndecan Differentially Localizes Acetylcholine Receptor Subtypes at...
Cholinergic Synapses suggests that Syndecan uses different mechanisms to localize/cluster N-AChRs and L-AChRs at the NMJ. Yet, most of the paper focuses on the mechanisms specific to N-AChR localization and clustering. By the authors' own admission, Syndecan is not strictly required for L-AChR clustering, rather, it modulates the synaptic content of L-AChRs. The title needs modifications to reflect the different amounts of evidence for Syndecan's role in N-AChR vs L-AChR synaptic localization. Alternatively, the authors can provide a molecular mechanism by which Syndecan regulates L-AChR synaptic content.

This is very embarrassing but the genuine title of the manuscript, as loaded on the Review Commons web site is: “The HSPG Syndecan is a core organizer of cholinergic synapses in C. elegans” For unexplained reasons, the PDF document that was uploaded contained an old title. We hope that the actual title addresses our reviewer's concerns.

2. Figure 1: It seems SDN-1 is broadly expressed at the dorsal cord. It might be due to my incomplete knowledge of C. elegans neurobiology, but it does not seem to me that it is specifically enriched at synapses or NMJs. Such a figure title would require a quantification of the enrichment (e.g. compared to an axonal marker or fill).

SDN-1 is expressed in multiple tissues, especially in the nearby epidermis, which might confuse the visual impression (see for example new fig 3F, fourth row, where the pattern is more punctate after degradation of the epidermis component). In addition, SDN-1 is present at both cholinergic and GABAergic NMJs, which altogether are dense along the nerve cords. However, analysis of fluorescence peaks clearly indicates synaptic enrichment, as shown in new fig. 2C and all along the paper.

3. The broad effect of Syndecan on all types of synapses at the NMJ suggests that it might also have a role in organizing the presynaptic active zones, which in turn would have an effect on the post-synapse. Indeed, Figure 2A shows a visible reduction in the intensity of cholinergic CLA1-BFP in the Syndecan LOF. This would need to be further quantified/characterized.

The intensity of this marker is quite variable, and we agree that the image in the first version was confusing. We are now providing a revised version of the figure, which does not show such dramatic difference between wild-type and sdn-1(0) mutant. Our point is that the loss of syndecan-1 does not modify the number of presynaptic boutons, which might have impacted the postsynaptic localization of receptors, yet we have not performed an extensive analysis of presynaptic boutons structure.

4. Figure 2: Needs a more accurate explanation of the optogenetic stimulation.

We gave more details of the protocol in the M&M section. However, if the reviewer thinks it is mandatory to add them in the legends, we can do it.

5. A clearer cartoon of the various Syndecan constructs used in the study, in the figures where they are used (e.g. the bicistronic one used in Figure 3), would help interpret results.

We reorganized the figures, and we now believe that the cartoon that we provide is now more helpful to understand the experiments that follow.

6. Figure 3: The methodology here is a bit perplexing, although the main result (that Syndecan at the NMJ is mostly expressed by the muscle cells) is rather convincing. The main way Syndecan expressed e.g. in the epidermis would be localized at the NMJ would be through shedding of the extracellular domain. Indeed, Syndecans, like many transmembrane receptors, are known to be proteolytically cleaved. But in order to determine the localization of the shed extracellular fragments, one needs an N-terminal fluorescent tag. However, the tag used in this experiment is cytoplasmic. I fail to see how the C-terminal domain of SDN-1 produced in the distant epidermis ends up localized at the NMJ (Figures 3B and 3D). More generally, this type of protein degradation experiment could be used to deplete SDN-1 in a more localized manner and observe its effect on AChRs at cholinergic synapses.

Our reviewer is perfectly correct. However, the reason why we tested if some of the signal detected at NMJs was contributed by the epidermis is because of the C. elegans anatomy. As shown on these transversal EM sections (https://www.wormatlas.org/SW/SW.php/), the dorsal cord (DC) and the ventral cord (VC) are in intimate contact with the epidermis (Hyp 7). Confocal photonic microscopy does not have the resolution to discriminate SDN-1 at NMJs from SDN-1 on the epidermis membrane. Tissue-specific degradation provides a means to solve
this question. To clarify this point, we added a cartoon in new Fig. 2D showing the *C. elegans* anatomy of this region. We agree that SDN-1 can be cleaved, but we cannot target its extracellular region with the AID system.

7. Figure 5: it seems the RFP-LIN-2B and LIN-2A-GFP are used interchangeably. Please clarify.
We do apologize because errors were inadvertently introduced in the figure labeling. In these experiments, the RFP-tagged LIN-2 protein was expressed from our krSi35 allele, which indeed corresponds to RFP-LIN-2A as described in the material and methods. Also, in Fig 6N, we should read GFP-LIN-2A instead of LIN-2A-GFP considering the tag position. This was corrected.

8. Figure 6I: why was the ACR16 TM3-TM4 loop selected? Is this a known FRM3/LIN2 binding site?
In all Cys-loop receptors, there are very few amino-acids between TM-1 and TM2 and the TM3-TM4 loop represents the only large cytoplasmic region of these receptors. This loop did not contain obvious FRM-3/LIN-2 binding sites, but if any physical interaction would occur with ACR-16, it was predicted to involve an interaction with the TM3-TM4 loop.

Reviewer #2 (Significance (Required)):

As mentioned above, this study presents robust and convincing findings and identifies the HSPG Syndecan as a novel core organizer of the cholinergic synapse that acts by coupling extra- and intracellular scaffolds to control postsynaptic receptor composition. The study should therefore be of broad interest to molecular and cellular neuroscientists working on synapse specification, postsynaptic scaffolds, neurotransmitter receptor organization, extracellular matrix proteins and synapse organizers. My background is in molecular/cellular neuroscience, mechanisms of synapse development, synaptic organizers.
Reviewer #3 (Evidence, reproducibility and clarity (Required)):

**Summary:**

This study recognized the syndecan/SDN-1 as a critical cholinergic organizer within NMJ in C. elegans. In particular, they found that SDN-1 is required for the synaptic clustering of homomeric N-AChRs. This investigation also showed that SDN-1 is essential for proper synaptic levels of heteromeric L-AChRs and GABA receptors. Moreover, they noticed that SDN-1 is provided by postsynaptic muscle cells, and cholinergic and GABAergic motoneurons secrete Punctin/MADD-4. By using a series of microscopic and biochemical assays combined with CRISPR/Cas9 knock-in or knock-out strains, the authors proposed that SDN-1 acts by linking the extracellular MADD-4 with intracellular scaffolding proteins LIN-2 and FRM-3. At cholinergic synapses, MADD-4 functions simultaneously to localize SDN-1 at postsynaptic sites and the transmembrane UNC-40. In turn, SDN-1 also stabilizes MADD-4 and UNC-40. Clustered SDN-1 and UNC-40 at NMJs induce the scaffolding proteins LIN-2 and FRM-3 recruitment. Taken together, the data suggested that the LIN-2-FRM-3 complex then mediates the synaptic clustering of the N-AChR, and the electrophysiological analysis confirms the synaptic localization of AChRs.

**Major comments:**

1. Previous studies indicated that motoneurons could secret agrin, a large HSPG, at the vertebrate NMJs. This HSPG is proposed to be necessary for NMJ differentiation and postsynaptic clustering of AChRs (Burden et al., 2018; Li et al., 2018; Swenarchuk, 2019). In the worm NMJs, did the author assay the function of agrin? Regarding the abnormal distribution of SDN-1, is it possible that it is an indirect effect caused by ECM destruction?

We tested agrin a long time ago and saw no impact on NMJs. Analysis of agrin function in C. elegans was later published in PLoS One (Hrus et al., 2007) agreeing with our unpublished observations: “C. elegans Agrin Is Expressed in Pharynx, IL1 Neurons and Distal Tip Cells and Does Not Genetically Interact with Genes Involved in Synaptogenesis or Muscle Function”.

With respect to the second part of the question, the ultrastructure of the NMJs was analyzed in madd-4 mutants by electron tomography after high-pressure freezing of adult animals (Pinan et al., Nature, 2014, extended data Fig.3). We could not identify any synaptic morphological defect, even at this very high resolution, despite the disappearance of SDN-1, which we have now documented in madd-4(0) mutants.

However, to meet our reviewer's request, we obtained 4 knock-in strains expressing mNeon-Green-tagged components of the ECM: the agrin AGR-1, the Nidogen NID-1, the collagens CLE-1 and EMB-9 (Keeley DP, Hastie E, Jayadev R, et al. Comprehensive Endogenous Tagging of Basement Membrane Components Reveals Dynamic Movement within the Matrix Scaffolding. Developmental Cell, 2020;54(1):60-74.e67. doi:10.1016/j.devcel.2020.05.022). These markers were crossed into sdn-1(0) mutants and we observed no obvious defects (see pictures; scale bar is 10 um, yellow arrows indicate NMJ, yellow arrowheads point to muscle arms).
2. Are there any GABA-related functional defects in sdn-1 mutant animals? Is there any evidence that highlighting sdn-1 physiological effects do not have the indirect effects of GABA synapses? As the author stated, in GABA synapses, UNC-40 has also been studied to show its functional effects.

We do not see any behavioral phenotype that can be attributed to functional defects of GABA synapses. We have not performed electrophysiological recording because this is a lot of work and we already know that defects are limited at GABA NMJs based on the moderate decrease of GABAR content.

Otherwise, we have no argument to think that the phenotypes at GABA NMJs are a consequence of defects at cholinergic NMJ. We previously analyzed mutants deeply affecting cholinergic neurotransmission such as lev-9 or lev-10 and saw no electrophysiological or morphological defects at GABA NMJs. A recent work from the group of Mike Francis (Barbagallo et al. Development, 2017) indicates that impairing cholinergic synaptogenesis by disruption of cholinergic innervation either by manipulating cholinergic motoneuron differentiation or synaptic transmission tends to decrease the density of GABA NMJs and increase GABA receptor synaptic content (Fig. 4), which is not what to we observe in sdn-1 mutants.

3. In Figure 2B-C, it is important to assay the expression level of AChR in animals or tissues by western blot.

We agree with our reviewer that estimating the overall protein content will be an interesting information but we do not have antibodies to detect N-AChRs by western blot. So we performed an IP experiment of the CRISPR-tagged N-AChRs in WT and mutants, followed by a western blot. The result shows no major different of the expression level of ACR-16 in sdn-1(0) mutants as compare to wild type (see beside) These results are not included in the manuscript because of space limitation.

4. In Figure 2D-G, L-AChRs are also clustered, but the stability does not seem to be affected, this phenotype should be explained or tested; in addition, the endocytic degradation of N-AChRs is solely speculated, should be assayed using endocytosis mutants.

The phenotype of L-AChRs is consistent with our previous observations in clustering mutants such as lev-9 and lev-10 where synaptic currents are drastically reduced while the global response to pressure-applied levamisole is unchanged, indicating that receptors redistribute at the surface of the muscle cell.

We have crossed a mutant allele of dpy-23, which encodes the AP2 µ1 subunit, into sdn-1(0) and test if N-AChR levels are increased. As shown in the following image, the dpy-23 mutation didn’t rescue the level of L-AChRs. Unfortunately, we had to trim the manuscript extensively to comply with the J. Cell. Biol. format and we no longer discuss this possibility.

5. In Figure 2, sdn-1(0); N-AChR level decreased. Also, in Figure 4, it showed madd-4(0); SDN-1 level decreased.

The direct inference of the above two experiments is that the level of N-AChR in madd-4 mutant should also be significantly reduced, please confirm it.

We previously reported a strong decrease of N-AChRs in madd-4(0) mutants using a transgene overexpressing ACR-16-GFP in muscle (Pinañ-Lucarré et al., 2014, Extended figure 2, i-j). We revisited these data using our wmScarlett-ACR-16 knock-in and observed similar results. The synaptic level of ACR-16 in madd-4(0) mutant shows more than 50% reduction (see beside), with additional mis-localization defects.

6. In Figure 4B-C, for the distribution phenotype of SDN-1, it is useful to provide the results of rescue experiments using the two isoforms L and B in madd-4 mutant animals.

In madd-4L(0) MADD-4B is still expressed, while in madd-4B(0) MADD-4L(0) remains, which is equivalent to the rescue experiments requested by our reviewer. This is now stated in the second paragraph of page 7.

7. In Figure 4B-C, why not examine the colocalization between SDN-1 and Ach? Also, the SDN-1 levels in madd-4L(0) and 4B(0) animals are so different from those in (0), please explain it. Maybe the rescue experiment can help explain this discrepancy. Additionally, Page 8 mentioned that the localization and distribution of SDN-1 are regulated by both L and B isoforms. This statement is not accurate; indeed, the distribution of SDN-1 in L(0) is not significantly affected. As shown in Fig. 8E for example, the cholinergic and GABAergic domains are mutually exclusive at this resolution and showing one of the two markers provides similar information in this context. We chose to show GABAergic domains in Figure 4C because they are easier to individualize and more easily demonstrate the relocalization of SDN-1 out of
GABAergic domains by the MADD-4L isofrom, which is exclusively present at cholinergic synapses (Pinan-Lucarré et al., 2014; p. 7 of the manuscript). Our data indicate that the C-terminal moiety of MADD-4, which is identical in MADD-4L and -B, is necessary to concentrate SDN-1 at NMJs. In the absence of MADD-4L, the expression of MADD-4B is sufficient to localize SDN-1 at both NMJ types. In the absence of MADD-4B, MADD-4 is absent from GABAergic NMJs and MADD-4L relocalizes SDN-1 at cholinergic NMJs. Hence, we think that it is accurate to say that MADD-4L controls SDN-1 localization.

To be more accurate, we now state in the text that SDN-1 "localization can be instructed by any of the two MADD-4 isoforms, likely through its C-terminal moiety." (p. 7).

8. In Figure S4C, although it is not mandatory, it will be more desirable to use a synapse marker together for colocalization patterns. Another suggestion, Figure 4F-G could be relocated into a supplemental figure and add a western blot to validate that there is no protein expression variation. Although the amount of the SDN-1 variants is decreased after mutation of the different HS attachment sites, the cords are readily identified in the animals. The aim of these experiments is really to check how much SDN-1 remains at the cord. Providing a presynaptic marker will not really change the conclusions. However, we think that it is important to show in the main figures that SDN-1 modulates the amount of MADD-4 at NMJs because this is the first protein shown to regulate the amount of MADD-4 at the synapse. Because the effect is modest, we do not think that western blot analysis will be conclusive, especially because MADD-4 is very difficult to solubilize, and we doubt that we will reliably see a 30% change in the total protein level.

9. Figure 5, whether UNC-40 functions in the LIN-2 positioning process, which should be tested. In Figure 5I, the colocalization of LIN-2B and SDN-1 is perfect. It is worth noting that LIN-2B is known to be located in GABA NMJs too. Thus, this result does not seem to be completely consistent with the result in 5E. Please explain it. Could it be due to the different imaging segments of neuronal cords? Also, delta-EYFA mainly changes the labeling intensity of LIN-2B, but not the pattern, please modify the description accordingly. I speculate that this effect may be related to the influence of LIN-2 on the positioning of SDN-1. Please adjust the description too.

We showed in a previous study that LIN-2 is reduced by 55% in unc-40(0) (Fig 3C, Zhou et al., 2020). As written p. 8, we previously published that LIN-2 is present at GABAergic and cholinergic NMJs, similar to SDN-1 as shown in 6L. Consistently in figure 6F, LIN-2 is located at GABA synapses labeled by neureilin NLG-1 and at cholinergic NMJs outside of GABA synapses in a wild-type background (control). By contrast, LIN-2 is only detected at GABA synapses in sdn-1(0) mutants, which is a new result. As stated in the text based on Figure 6L-M and Figure S5B-D, we do show that introducing the delta EYFA mutation in SDN-1 changes the pattern of LIN-2, which becomes concentrated at GABA NMJs, most probably by interacting with NLG-1. Accordingly, LIN-2-GFP was almost undetectable at the nerve cord of nlg-1(0); sdn-1(0) double mutants (new Fig. 6N).

10. Page 11 indicates "UNC-40 remained present at both cholinergic and GABAergic NMJs". However, figure 7D and E only showed the data of GABAergic NMJs. 7C showed that madd(0); sdn-1(0) phenotype is stronger than that of madd(0), although it is not significant, which suggests that both MADD and SDN-1 contribute to the labeling of UNC-40. Please modify the text accordingly.

UNC-40 has been demonstrated to localize on both synapses (Tu et al., 2015; Zhou et al., 2020). As stated above, the cholinergic and GABAergic domains are mutually exclusive at this resolution and showing one of the two markers provides similar information in this context. With respect to Figure 7C, we are not sure about the comment of our reviewer because the results do indicate that there is no difference between madd-4(0) and madd-4(0); sdn-1(0) mutants.

**Minor comments:**

1. Figure 1, please indicate ventral and dorsal cords; this is also applicable for other figures. Ventral and dorsal cords were labeled in fig 1B. In the next panels and all the following figures images were taken from the dorsal cord as mentioned in the figure legend.

2. Page 9, SDN-1/LIN2CASK, is this typo? Thank you for pointing this out. We modified the text to “SDN-1 and LIN-2/CASK interaction”

3. Page 8, "SDN-1 contains in its C-terminus an evolutionarily conserved PDZ domain binding site, which was demonstrated to interact with the scaffolding protein CASK in the mammalian nervous system". SDN-1 should be modified to syndecan in the mammal. We have modified the text accordingly.
Reviewer #3 (Significance (Required)):

- This study’s findings provide a nice addition to the broader field of understanding of how the extracellular matrix regulates chemical synaptic strength and abundance of receptors.

- Heparan sulfate proteoglycans (HSPG) have been extensively reported to act as synaptic organizers (Yuzaki, 2018). HSPGs are negatively charged and interact with various proteins to regulate synaptogenesis (Song and Kim 2013). Previous studies indicated that motoneurons secreted agrin/HSPG into vertebrate neuromuscular junctions, promoting postsynaptic clustering of AChRs (Swenarchuk, 2019). Syndecans are a class of transmembrane HSPGs (Saied-Santiago and Bülow, 2018). SDN-1 is the sole syndecan in C. elegans and is found in NMJs. However, the functional mechanism of SDN-1 in the postsynaptic clustering of AChRs remains elusive. The current study deployed CRISPR/cas9 strains and biochemical tools to examine the detailed process. Interestingly, the authors showed that SDN-1/syndecan is localized at NMJs by MADD-4/Punctin. Then, SDN-1 acts to enhance the clustering of N-AChRs via interacting with LIN-2/CASK and FRM-3/FARP.

- Although this research was carried out in C. elegans, it will provide meaningful information to learn the functional mechanism of neuromuscular junctions in mammals. Thus, it has a broader neurobiological audience.

- C. elegans; intracellular transport of membrane receptor; cell polarity maintenance. The expertise mentioned above enables a better understanding and a proper scientific evaluation of this research.

**Referees cross-commenting**

I agree with reviewer #1 suggestion on adjusting the article’s structure, which will make this study easier for readers to appreciate its findings. Similarly, the reviewer’s suggestion that the experimental system requires further explanation is also relevant. This study utilizes a series of colocalization experiments. However, the current interpretations are a bit confusing, and further interpretation should be given in many places. We have followed the reviewer’s comment and extensively re-organized the manuscript to clarify the text, keeping in mind that this system involves several molecular players and that an oversimplified model needs to be avoided. We are also now providing a cartoon to present the experimental system in new Fig. 1.

Reviewer #2 also raises concerns regarding the paper’s structure, especially GABAergic results, which is a decent suggestion. The reviewer proposes that it is imperative to increase the biochemical results and the controls of biochemical experiments, which will help strengthen the specificity of the identified mechanism, especially the functional specificity of SDN-1 during regulating cholinergic synapse. Also, the suggested addition of experiments to address the functional difference of SDN-1 in cholinergic and GABAergic synapses is also significant. We have moved the GABAergic results to the supplemental materials. We have complemented genetic experiments with biochemical data when feasible (new Fig. 4H, new Fig. 6D, new Fig. 7H).
May 18, 2021

RE: JCB Manuscript #202011144R

Prof. Jean-Louis Bessereau
Claude Bernard University Lyon 1
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8 avenue Rockefeller
Lyon 69008
France

Dear Prof. Bessereau:

Thank you for submitting your revised manuscript entitled "The HSPG Syndecan is a core organizer of cholinergic synapses in C. elegans". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision as mentioned by reviewer 1 please include the additional data in your supplementary information.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."
5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

* Unless absolutely necessary C. elegans should be removed from your title

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
   a. Make and model of microscope
   b. Type, magnification, and numerical aperture of the objective lenses
   c. Temperature
   d. Imaging medium
   e. Fluorochromes
   f. Camera make and model
   g. Acquisition software
   h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstructions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider
providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB’s Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

**It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.**

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Patrik Verstreken, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology
Reviewer #1 (Comments to the Authors (Required)):

Zhou et al. have responded to many of my critiques. Particularly, the article's structure has been appropriately re-organized. Overall, I think it fits the scope of JCB. However, I remain confused that the authors did not incorporate many suggested and newly acquired data into the revised manuscript, which will help address concerns from broad fields readers. Indeed, JCB supplemental data section can contain these data. Also, there are still some typos in the figures, and authors should go through them to fix them.

Reviewer #2 (Comments to the Authors (Required)):

The authors make use of cutting edge molecular genetic tools, electrophysiology and cell biology to reveal the role of syndecan as a core organizer of (primarily) cholinergic NMJs. They extend their findings along several axis: cholinergic versus GABAergic synapse composition, extracellular versus intracellular scaffold and signaling complexes, and Neuroligin versus Netrin receptor roles. The paper is rich, with many meaningful findings. My comments upon their first submission to Review Commons had to do with organization of the arguments. They addressed all my comments, improving clarity, and it is a beautiful piece of scholarship. My only (minor) suggestion is to re-organize some of the figures (like Fig 6 so that the panels follow a logical flow (J through K transition). That figure could also benefit from a cartoon diagram of the proteins "in the cell", with lesions used in study highlighted (space permitting).

Reviewer #3 (Comments to the Authors (Required)):

I appreciate the efforts of the authors to address my points. Their experimental additions, clarifications, and reorganization of the manuscript have greatly enhanced their study. This is very interesting and elegant work that is suitable for publication in JCB in my opinion.