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Review COMMONS

Neuronal MT1-MMP mediates ECM clearance and Lrp4 cleavage for agrin deposition and signaling in presynaptic development

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Review timeline

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Reviewer 1

Evidence, reproducibility and clarity

This is an interesting paper by Oetaryo et al that presents some compelling data to support their model. However, while I find some of their data of good quality and interesting, several conclusions are not well supported by experimental findings.

The authors suggest that as Xenopus spinal neurons mature in culture (some of which are motor neurons, but not all), they secrete Agrin, which is involved in maturation of ACh receptors. In their model, they suggest that MMP-mediated cleavage of the Agrin-receptor, Lrp4 (? And ECM) is necessary for presynaptic differentiation. The authors first show that as spinal neurons mature in culture, they slow, and begin to express Agrin. The Agrin staining in Fig 2 is robust, but much less so in later figures. This may be due to fact that not all neurons in spinal cord cultures are MNs, so may not all express Agrin. Agrin expression fits roughly with the MMP-mediated degradation, so it is suggested these two processes are linked. I find this argument weak.

It is also suggested that since Agrin slows extension of young axons, but not mature neurons that are already static, that this means that Agrin is involved in neuronal maturation. To prove this, the authors should test the effect of Agrin KD. If Agrin KD neurons remain motile at later times in culture, but still respond to Agrin, this would be good evidence that Agrin matures neurons in a paracrine fashion. The role of Agrin in the formation of presynaptic specializations was tested using Agrin-beads and the targeting of mitochondria and SV2. I am not convinced these are accurate measures of presynaptic development.

A link between MMP activity and synaptic maturation is made because MMP inhibitors disrupt SV2 and mitos at bead contact sites. I find this weak as well. If the authors could show increased MMP activity at bead contact sites (with gelatin degradation) or MMP release at bead contact sites, this would be stronger evidence. MT1-MMP trafficking was used to suggest that MT1-MMP targets to presynaptic sites of Agrin-bead contact. I don't find vesicle stalling at bead contact sites very compelling case for presynaptic development. Also, if these neurons are expressing endogenous Agrin all over (as Fig 2 indicates), how does additional Agrin on a bead matter? ACh receptor clustering at sites of muscle contact was more convincing, especially the MT-MMP MO experiment. However, I would have liked to see the evidence that MT1-MMP Morpholinos work (cited paper, Chan et al. 2019, is a meeting abstract). A blot or ICC showing MT1-MMP KD would be nice. Also, the specific MT1-MMP antibody that was used should be indicated.

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Significance

MMPs are important secreted and trans membrane enzymes that are poorly understood. They likely have important functions in neural development. I have expertise in neural development.

Reviewer 2

Evidence, reproducibility and clarity

Summary:

The development of the NMJ is largely regulated by agrin, an HSPG secreted from motor neurons, through its receptor and effector, Lrp4 and muscle-specific kinase MuSK, respectively, in the muscle fiber. In addition, a plethora of other factors investigated mostly in cell culture have been suggested to modulate/facilitate agrin-driven NMJ formation, including matrix metalloproteinases associated with neurons. They are thought to act by proteolytically releasing synaptogenic molecules and/or by degrading ECM components involved in cell-cell interactions, possibly releasing growth factors from the ECM. The mechanisms involved are poorly understood.

The present experiments, performed on cultures of dissociated motor neurons or cocultures of neurons and muscle cells derived from Xenopus embryos, suggest a mechanism how the matrix metalloproteinase, MT1-MMP, controls/facilitates differentiation of presynaptic nerve terminals. It has been known for some time that localized presentation of agrin to cultured cholinergic neurons can induce presynaptic differentiation. The present paper suggests a mechanism, how this might occur. Specifically, MT1-MMP secreted from cultured neurons is shown to degrade the culture substrate, thus facilitating the deposition of agrin at the developing NMJ and promoting its formation, a process inhibited by pharmacological MMP blocker or by MT1-MMP knock-down. Local agrin stimulation through agrin-coated beads contacting neurites induced local accumulation of mitochondria and of synaptic vesicles, i.e. markers of presynaptic differentiation; this process was inhibited by knock-down of neuronal Lrp4. Likewise, the same treatments inhibited agrin deposition and AChR clustering in nerve- muscle cocultures and in cocultures of MMP knock-down neurons and w.t. muscle cells. The authors conclude a novel, previously not appreciated role of agrin to promote presynaptic differentiation through MMP's proteolytic activity via ECM degradation and cleavage of neuronal Lrp4.

Major comments:

1) In general, the experiments appear to support the conclusions and the working model shown in Fig. 9. However, it should be noted that in principle, cultured cells can show potential mechanisms only, but not their physiological relevance in an in vivo environment. For example, agrin is the sole organizer of NMJ formation in vivo, whereas bFGF has at best a modulatory role in this process, yet they have similar effects on presynaptic differentiation in the culture system used. The paper is thus a preliminary, but nevertheless potentially useful communication of how MT1-MMP might be involved in presynaptic differentiation. The authors would probably agree that validation of their model in vivo, e.g. through genetic approaches such as inducible knock-out of MT1-MMP selectively in motor neurons, would make their story considerably stronger.

Minor comments:

There are a number of issues and (apparent) inconsistencies that should be addressed at the present stage

1) How long can neurons in culture be kept viable? This is essential to estimate whether the rapid axon growth and growth cones in young neurons vs. the slow growth and grown cone collapse do not reflect a culture artifact such as limited/impaired viability in dissociated cell culture. In vivo, such phases do not exist, but motor axons keep growing until they make synapses rather than following an intrinsic neural property.

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2) Given that physiologically native agrin at NMJs is thought to attach to synaptic basal lamina by binding to laminin, and that MT1-MMP degrades laminin, how can MT1-MMP activity promote agrin deposition in the cultures?

3) in Fig. 2A,B, it is stated that soluble agrin causes growth cone collapse in young, but not old neurons. Given that in old neurons as many as 70% of growth cones are collapsed, i.e. that collapse is almost complete in the absence of agrin, how can the authors expect a dramatic increase in collapses in its presence? Thus, the absence of further growth cone collapse by agrin in longer cultured neurons may not reflect a new state of neuronal differentiation as suggested.

4) In the introduction (p.5) and Results (p.6) and Figure 6E, it is claimed that MT1-MMP-mCherry vesicles migrate bidirectionally along neurites until they are captured by agrin bead contacted sites. On p.10/11 it is stated that "nevertheless, we also observed bidirectional transport of some MT1-MMP-mCherry vesicles passing through the bead-neurite contacts without stopping". The authors should indicate what the ratio of "captured" to "bead passing" MT1-MMP-mCherry vesicles was, and how many bead contacts were analyzed. For captured vesicles. Is there a way to estimate whether occasional stopping at bead contacts was significant rather than just accidental? Furthermore, in some of the panels (Fig. 6E), fluorescence puncta are barely visible and should be enhanced. Finally, it would help understanding the figure to indicate bead location by an asterisk and puncta with an arrow. In Figure 6F, it is not clear what arrows point to.

5) In the last para of p. 14 (Discussion), it is argued that
"Local presentation of some heparan sulfate proteoglycan (HSPG)-bound growth factors (e.g. bFGF and heparin-binding growth-associated molecule) via polystyrene beads can effectively induce both cytoplasmic and membranous presynaptic specializations at the bead neurite contact sites in a spatiotemporally controllable manner (Dai and Peng, 1995; Rauvala and Peng, 1997). In this study, we found that polystyrene beads coated with the C-terminal fragment of recombinant agrin proteins locally induce presynaptic differentiation to an extent similar to bFGF-coated beads. Agrin, the major HSPG at NMJs, may control the extracellular distribution of diffusible growth factors at developing NMJs (Matsuo and Kimura- Yoshida, 2014). Like other HSPGs, agrin contains heparan sulfate glycosaminoglycan chains that play an important role in a diverse array of biological functions (Ruoslahti, 1989).
Therefore, agrin-coated beads could serve to concentrate and localize endogenous bFGF and other growth factors for the induction of presynaptic differentiation."
To my knowledge the minimal NMJ-inducing C-terminal fragment of agrin effective in vivo does not contain glycosaminoglycan side chains. Thus, if the C-terminal agrin fragment used here to coat beads, was indeed minimal, it does not contain glycosaminoglycan side chains; as a consequence the para quoted above would not make sense. The authors should specify precisely in the Methods section, which agrin fragment they used to coat beads.

6) In the same context, as a first step to dissect the mechanism of bFGF action on presynaptic differentiation in the cultures, the authors should examine whether soluble recombinant ecto-Lrp4 treatment can rescue the inhibitory effects of the MMP inhibitor or MT1-MMP knockdown as it does on agrin-induced presynaptic differentiation.

Significance

See above in summary

My expertise: cell biology on NMJ formation

Reviewer 3

Evidence, reproducibility and clarity

In this work the authors correlate neuronal outgrowth and synaptogenesis with agrin deposition along the axon. During an early phase of neurite outgrowth there is little agrin deposited along the axon, which increases with time in culture. Agrin deposition correlates further with MMP mediated ECM degradation along the neurites. The authors found that proteolytic activity MT1-MMP and Lrp4

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was necessary for agrin induced presynaptic differentiation. Together, this study suggests agrin to reduce axonal outgrowth but to promote synaptogenesis- a mechanism that involves MT1-MMP and Lrp4. I find this topic highly relevant and the findings are in principle very interesting. However, in the current state of manuscript the mechanism leading to synaptogenesis is not clear.
Questions:
1. Is it known which form of agrin is secreted by the spinal neurons used in this work? Is it the same isoform as presented on the beads?
2. Does agrin deposition on axons depend on MT1-MMP activity, agrin expression or expression of agrin receptor(s)? This could be addressed e.g. by adding soluble agrin to young neurons and quantifying its association to neurites or by blocking MMP activity in older cultures and testing agrin association.
3. What are the targets of MT1-MMP in this system? Is agrin proteolytically cleaved by MT1-MMP as it is e.g. by neurotrypsin? Or is Lrp4 proteolytically cleaved by MT1-MMP? And related to that- what is the source of Lrp4 ectodomain that seem to necessary for synaptogenesis?
4. Inhibition of MT-MMP1 is preventing synaptogenesis, which can rescued by application of MT-MMP1. This suggests Lrp4 to be target of the protease (see point 3). Is therefore neuronal, transmembrane Lrp4 inhibiting synapse formation?
5. Figure 1 demonstrates the accumulation of agrin along the axon, which seems to quite homogenous in 1-2 days old cultures. This in mind I wonder how presynaptic vesicles can be clustered at specific sites or whether MT1-MMP vesicles are directed to adhesion sites (maybe here artificially induced via agrin and bFGF beads) and this may be the main (and no less interesting) mechanism.

Comments:

In some figures labeling of the axes are not intuitive or misleading. E.g Figure 1b: Normalized extension can not have a label in um but probably in %.

Significance

This work highlights the role of again and proteolysis in the transmission from axon extension to synapse formation.

Author response to reviewers' comments

Author Response (Refereed Preprint RC-2019-00130)

We sincerely thank the reviewers for their critical evaluation and constructive comments on our work. We are encouraged by their overall positive view of our work from all reviewers, including "*an interesting paper*" and "*data of good quality and interesting*" from Reviewer #1, "*potentially useful communication of how MT1-MMP might be involved in presynaptic differentiation*" from Reviewer #2, and "*this topic highly relevant and the findings are in principle very interesting*" from Reviewer #3. The reviewers, however, raised some important points on the study. We have considered their comments carefully and have planned several sets of new experiments to address key issues raised by the reviewers, which are summarized below in the first section. We anticipate that all new experiments listed in the revision plan will be completed within 2-3 months.

In addition, detailed point-to-point responses to individual reviewer's comments are also provided. We believe that we have now addressed most, if not all, of the reviewers' questions and have incorporated all suggestions from the reviewers either in this initial response or in the revision plan. We hope that the reviewers find our revision plan and responses to be satisfactory. With the constructive comments from the reviewers, we are happy to see that our work will be significantly strengthened by the addition of new data in our substantial revision plan below.

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Major experiments in the revision plan:

1. **Agrin knockdown and inhibition.** Agrin expression and activity in cultured spinal neurons will be manipulated by antisense morpholino and functional blocking antibody, respectively. We will then determine if neural agrin synthesis and deposition induce presynaptic differentiation via autocrine/paracrine regulation. (See Reviewer #1, Point #3 and Reviewer #2, Point #4).

2. **Knockdown of neuronal MT1-MMP in *Xenopus* embryos *in vivo*.** Our recent study showed that MT1-MMP knockout mice exhibit an obvious presynaptic defect in embryonic diaphragm muscles (Chan et al., 2020). To further test the possibility of neuronal MT1-MMP in regulating axonal outgrowth and presynaptic differentiation, we will perform morpholino-mediated knockdown of neuronal MT1-MMP (as determined by the well- established lineage tracking and fate mapping in *Xenopus* embryos). Then, immunostaining of whole-mount embryos will be performed to examine the effects of neuronal MT1-MMP knockdown on the structural features of presynaptic and postsynaptic markers at developing NMJs *in vivo*. (See Reviewer #2, Point #1).

3. **MT1-MMP vesicular trafficking and surface insertion.** Quantitative analyses will be performed using MT1-MMP-mCherry to study the axonal trafficking of MT1-MMP vesicles along neurites in response to agrin bead stimulation. In addition, MT1-MMP- pHluorin will be used to visualize the exact location of MT1-MMP surface insertion upon agrin bead stimulation. (See Reviewer #1, Points #5 & #6 and Reviewer #2, Point #5).

4. **Ubiquitously expressed versus synapse-specific ECM proteins.** To further support our hypothesis that MT1-MMP degrades ubiquitously expressed ECM proteins for allowing deposition of synaptogenic factors at the initial stage of synaptogenesis, we will perform ECM degradation assay using other ubiquitously expressed ECM protein (e.g. fluorescent collagen) in response to MMP inhibition. Next, we will perform immunostaining experiments to examine the spatial and temporal patterns of laminin β2, one of the key synapse-specific ECM proteins at the synaptic basal lamina, before and after agrin bead stimulation. Lastly, we will test whether laminin β2 proteins are resistant to the proteolytic degradation by recombinant MT1-MMP treatment. (See Reviewer #2, Point #3).

5. **MT1-MMP activity on agrin expression and deposition.** To address whether agrin synthesis and deposition in axons depend on MT1-MMP activity, we will examine the effects of MMP inhibition on agrin deposition (by immunostaining), and that on agrin and Lrp4 mRNA levels (by Northern blot analysis or semi-quantitative reverse transcription PCR) in young versus old cultured neurons. (See Reviewer #3, Points #3 & #4)

6. **Sources of ecto-Lrp4 and possible effects of non-cleaved Lrp4 on NMJ formation**. To further determine the source of soluble Lrp4 ectodomain that regulates presynaptic differentiation, we will examine the effects of morpholino-mediated knockdown of neuronal and/or muscle Lrp4 expression on the clustering of presynaptic markers and postsynaptic AChRs in nerve-muscle co-cultures. After that, we will test if ecto-Lrp4 treatment can reverse the inhibitory effects, if any, of Lrp4 knockdown from neuron and/or muscle origin. (See Reviewer #3, Point #4).

To address whether neuronal, transmembrane Lrp4 inhibits synaptic formation, spinal neurons will be over-expressed with full-length Lrp4, and then co-cultured with wild-type muscle cells. We will then determine the effects of Lrp4 overexpression on nerve-induced AChR clustering in the presence or absence of the MMP inhibitor. (See Reviewer #3, Point #5).

Point-to-point responses to reviewers' comments:

Reviewer #1:

1. *The authors first show that as spinal neurons mature in culture, they slow, and begin to express Agrin. The Agrin staining in Fig 2 (Fig 1) is robust, but much less so in later figures. This may be due to fact that not all neurons in spinal cord cultures are MNs, so may not all express Agrin.*

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We thank the reviewer for raising this concern. In **Figure 1C**, agrin immunostaining showed a time-dependent increase in the signals of secreted agrin proteins along the neurites of spinal neurons over 2 days in culture. As the reviewer pointed out, the pattern of agrin deposition is robust, primarily in 2-day old cultures. Since our study sets out to understand the transition from neuronal outgrowth to synaptogenesis, all our subsequent experiments were performed using either 4-8 hours or 1-day old neuronal cultures instead. On the other hand, we do agree with the reviewer that not all neurons cultured from dissociated neural tube tissues of *Xenopus* embryos are motor neurons. Previous electrophysiological studies showed that more than 60% of the neuronal population in cultured *Xenopus* spinal neurons release acetylcholine upon their direct muscle contact (Chow and Poo, 1985). Importantly, imaging studies further demonstrated that postsynaptic acetylcholine receptor clustering could be detected at up to 90% of nerve-muscle contacts (Peng et al., 2003). These studies therefore indicate that the major neuronal type in *Xenopus* spinal neuronal cultures is indeed motor neurons. We have now clarified these points in lines 324-330.

2. Agrin expression fits roughly with the MMP-mediated degradation, so it is suggested these two processes are linked. I find this argument weak.

To better illustrate the spatial and temporal correlation between ECM degradation and agrin deposition in neurons over 2 days in culture, we have now modified Figure 1C, in which the merge images were constructed by overlaying binary images of agrin signals (after thresholding at a fixed value across different experimental groups) and grayscale images of fluorescent gelatin. In addition, we have marked the segment of neurites with both agrin deposition and gelatin degradation by arrows, whereas arrowheads indicate the absence or minimal level of agrin deposition and gelatin degradation in other neurites of the same cultures.

In the revision plan, we will also incorporate the suggestion from Reviewer #3 (point 6) to include immunostaining of synapsin 1 for showing the localization of synaptic vesicles, in relationship to agrin deposition and gelatin degradation, along the neurites in cultured neurons of different ages. With the new triple-channel imaging data, quantitative analyses will be added in Figure 1C to further strengthen our argument. For example, line profiles of fluorescent gelatin, agrin, and synaptic vesicle signals along the neurites will be plotted to further prove the coupling events between MMP-mediated ECM degradation and presynaptic differentiation.

3. It is also suggested that since Agrin slows extension of young axons, but not mature neurons that are already static, that this means that Agrin is involved in neuronal maturation. To prove this, the authors should test the effect of Agrin KD. If Agrin KD neurons remain motile at later times in culture, but still respond to Agrin, this would be good evidence that Agrin matures neurons in a paracrine fashion.

We thank the reviewer for this excellent suggestion. In the revision plan, we will perform antisense morpholino-mediated knockdown of endogenous agrin expression in cultured *Xenopus* spinal neurons. The knockdown efficiency will be validated by western blot analysis. As suggested by the reviewer, we will test if agrin knockdown neurons remain motile and exhibit normal growth cone structures at later times in culture, but they are still responsive to exogenous agrin-induced growth cone collapse. As a parallel approach, we will also use an agrin functional blocking antibody (Millipore, MAB5204), which has recently been demonstrated to inhibit agrin-induced MuSK phosphorylation in cultured C2C12 muscle fibers and tumor cells (Chakraborty et al., 2015). We anticipate that cultured neurons treated with this agrin functional blocking antibody remain motile and exhibit normal growth cone structures at later times in culture, but they are expected to be unresponsive to exogenous agrin treatment. These two parallel approaches would provide good evidence to support our proposed autocrine/paracrine action of neural agrin in presynaptic differentiation.

4. The role of Agrin in the formation of presynaptic specializations was tested using Agrin-beads and the targeting of mitochondria and SV2. I am not convinced these are accurate measures of presynaptic development.

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We thank the reviewer for raising this concern. We would like to emphasize that synaptic vesicle and mitochondrial clusters are two well-established markers of presynaptic differentiation as previously demonstrated (Dai and Peng, 1995; Dai and Peng, 1996; Lee and Peng, 2006; Lee and Peng, 2008). In this revision, we have provided additional data to further support that presynaptic differentiation can be locally induced by agrin-coated beads, including (1) stimulation-induced loading, and then followed by unloading, of lipophilic styryl dye FM1-43 to show the functional releasable pools of synaptic vesicle clusters at the bead-neurite contacts (**Supplemental Figure 1A**); (2) fluorescent phalloidin staining to show the enrichment of filamentous actin (F-actin) scaffold for stabilizing presynaptic components at the bead-neurite contacts (**Supplemental Figure 1B**, top panels); (3) immunostaining of phosphotyrosine to show the enrichment of tyrosine phosphorylated presynaptic proteins at the bead-neurite contacts (**Supplemental Figure 1B**, bottom panels); and (4) immunostaining of bassoon and piccolo (awaiting for order shipment, will be performed in our revision plan) to show the presence of active zone markers localized at the bead-neurite contacts. With all these new data, we hope that the reviewer is now convinced that focal agrin stimulation induces presynaptic differentiation in cultured spinal neurons.

5. A link between MMP activity and synaptic maturation is made because MMP inhibitors disrupt SV2 and mitos at bead contact sites. I find this weak as well. If the authors could show increased MMP activity at bead contact sites (with gelatin degradation) or MMP release at bead contact sites, this would be stronger evidence.

We agree that the link between MMP activity and presynaptic differentiation should be further strengthened, and we thank the reviewer for his/her suggested experiments.
Regarding the first suggestion to examine if there is a spatial gelatin degradation at the bead-neurite contacts, unfortunately a technical issue makes this experiment unfeasible. As agrin beads are added onto the top surface of spinal neurons, the increased MMP activity at bead-contacted sites do not affect the fluorescent gelatin-coated substratum, which is in contact with the basal surface of cultured spinal neurons. Alternatively, we will perform the second suggestion from this reviewer to examine the spatial insertion of MT1-MMP to the agrin bead-neurite contact sites using pH-sensitive GFP-tagged MT1-MMP (MT1-MMP- pHluorin). In our recent study, we have successfully used this probe to convincingly demonstrate the surface insertion of MT1-MMP at aneural acetylcholine receptor clusters in cultured *Xenopus* muscle cells (Chan et al., 2020). Here, our preliminary data below also show the spatial localization of surface MT1-MMP signals at agrin bead-neurite contacts. In the revision plan, we will further perform fluorescence recovery after photobleaching (FRAP) experiment to determine the spatial and temporal events of MT1-MMP surface insertion at the bead-contacted versus non-contacted sites along the neurites.

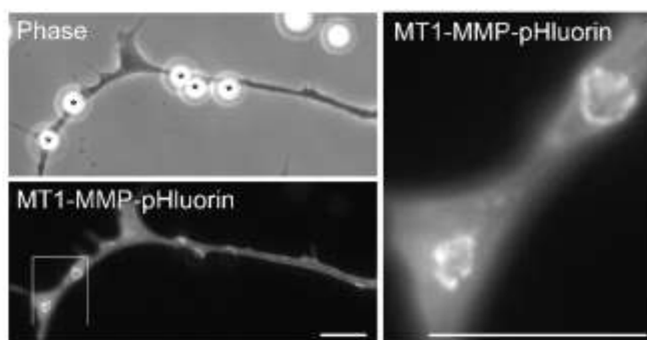

Figure 1. Spatial localization of surface MT1-MMP induced by agrin-coated beads.
Representative images showing the spatial enrichment of surface MT1-MMP, as visualized by a pH-sensitive GFP variant pHluorin, at the agrin bead-neurite contact sites (asterisks). For clarity, the boxed region in MT1-MMP-pHluorin image was magnified and shown in the right panel for illustrating the localization of surface MT1- MMP at two of the bead-neurite contacts.
Scale bars represent 10 μm.

6. MT1-MMP trafficking was used to suggest that MT1-MMP targets to presynaptic sites of Agrin-bead contact. I don't find vesicle stalling at bead contact sites very compelling case for

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presynaptic development.

We agree with the reviewer that by simply showing MT1-MMP-mCherry vesicles stalling at agrin bead-contacted sites falls short to support the essential role of MMP- mediated ECM degradation in presynaptic differentiation. As MT1-MMP is an integral membrane protein, its proteolytic activity in modulating ECM proteins requires the precise control of not only vesicular trafficking, but also surface insertion, of MT1-MMP proteins. As stated in our response to point #5 above, we will use MT1-MMP-pHluroin to study the spatial and temporal events of MT1-MMP surface insertion upon agrin bead stimulation.

7. Also, if these neurons are expressing endogenous Agrin all over (as Fig 2 (Fig 1) indicates), how does additional Agrin on a bead matter?

We would like to clarify that endogenous agrin expression and deposition were pronouncedly detected along the entire length of neurites only in neurons cultured for 2 days or longer. In contrast, agrin deposition in younger cultures is minimal and unevenly detected along the neurites. Therefore, only neurons cultured for 1 day or less were used in all our subsequent experiments for investigating the induction of presynaptic differentiation by agrin beads. Importantly, the bead assay serves as a spatiotemporally controllable mean for us to test a potential candidate of presynaptic differentiation inducer (i.e. agrin in this study). We have now clarified these points in lines 143-144 and 186-189.

8. ACh receptor clustering at sites of muscle contact was more convincing, especially the MT-MMP MO experiment. However, I would have liked to see the evidence that MT1- MMP Morpholinos work (cited paper, Chan et al. 2019, is a meeting abstract). A blot or ICC showing MT1-MMP KD would be nice. Also, the specific MT1-MMP antibody that was used should be indicated.

We thank the reviewer for commenting our work to be convincing. The cited morpholino work in a meeting abstract has been recently accepted for publication (Chan et al., 2020), in which western blot analysis was performed to demonstrate that MT1-MMP antisense morpholino significantly reduces the endogenous MT1-MMP protein level in *Xenopus* embryos. We have now updated this citation. In addition, we have included the detailed information of MT1-MMP antibody used in the Materials and Methods section.

Reviewer #2:

Major comments:

1. In general, the experiments appear to support the conclusions and the working model shown in Fig. 9. However, it should be noted that in principle, cultured cells can show potential mechanisms only, but not their physiological relevance in an in vivo environment. For example, agrin is the sole organizer of NMJ formation in vivo, whereas bFGF has at best a modulatory role in this process, yet they have similar effects on presynaptic differentiation in the culture system used. The paper is thus a preliminary, but nevertheless potentially useful communication of how MT1-MMP might be involved in presynaptic differentiation. The authors would probably agree that validation of their model in vivo, e.g. through genetic approaches such as inducible knock-out of MT1-MMP selectively in motor neurons, would make their story considerably stronger.

We thank the reviewer for considering our work as potentially useful communication of how MT1-MMP might be involved in presynaptic differentiation. We agree with the reviewer regarding the importance of validating our model by an *in vivo* system. In fact, our recent study has already provided *in vivo* data using MT1-MMP$^{-/-}$ knockout embryos to demonstrate the role of MT1-MMP in regulating the recruitment of aneural acetylcholine receptor clusters for the assembly of postsynaptic differentiation at developing NMJs (Chan et al., 2020). Interestingly, we found that MT1-MMP$^{-/-}$ knockout mice also exhibit an obvious presynaptic defect in embryonic diaphragm muscles (Figure 2 of this response letter), suggesting a possible involvement of neuronal MT1-MMP in axonal growth and differentiation that warrants further

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investigation. Consistent with that observation, our present study further demonstrated that either pharmacological inhibition of MMP activity or knockdown expression of MT1-MMP greatly promotes growth cone collapse, leading to a significant reduction in neurite outgrowth of cultured *Xenopus* spinal neurons. This new result is now presented in **Supplemental Figure 5** and lines 378-382.

We are aware of the importance of developing motor neuron-specific and inducible MT1-MMP knockout models. Considering the long generation time of tissue-specific knockout mice or frogs, we feel that this experiment is outside the scope of our revision plan. Alternative, we will address this concern about the *in vivo* validation by perform morpholino- mediated knockdown of neuronal MT1-MMP (as determined by the well-established lineage tracking and fate mapping in *Xenopus* embryos). Then, immunostaining of whole- mount embryos will be performed to examine the effects of neuronal MT1-MMP knockdown on the structural features of presynaptic and postsynaptic markers at developing NMJs *in vivo*. Results of this experiment should provide a further validation of MT1-MMP (primarily from the neuronal origin) in presynaptic development *in vivo*, which can be done in a reasonable revision timeframe.

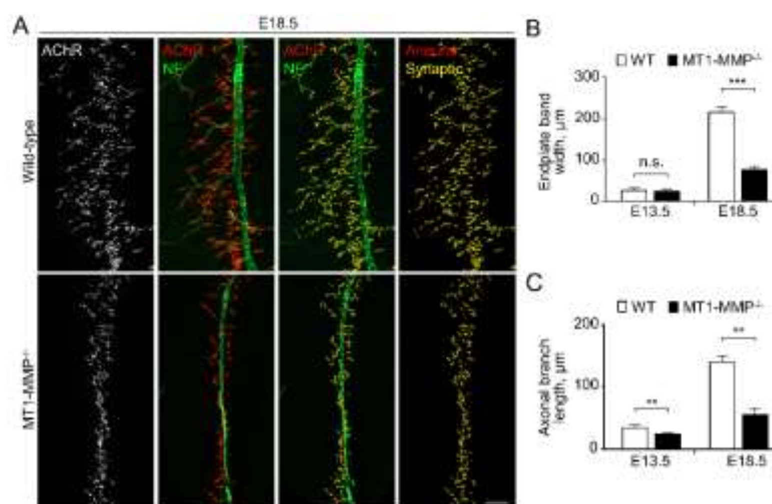

Figure 2. Presynaptic defects observed in diaphragm muscles from MT1-MMP$^{-/-}$ knockout mouse embryos.
(A) Representative confocal images showing aneural versus synaptic acetylcholine receptor (AChR) clusters in whole- mount diaphragms from wild- type control and MT1-MMP$^{-/-}$ knockout mice at E18.5. Whole-mount tissues were stained for AChR and neurofilament (NF). The superimposed 3D reconstruction images were generated by z-stack images.
Synaptic AChR clusters (yellow, right panels) were identified when signals of AChR and NF overlapped with each other, whereas all other AChR signals (red, right panels) represent aneural AChR clusters. Scale bar represents 100 µm. (B-C) Quantification on the width of end-plate bands (B), and the length of axonal branches (C) in diaphragm muscles between wild-type (WT) and MT1-MMP$^{-/-}$ mouse embryos at E13.5 and E18.5. n = 4 (E13.5, WT) and 5 (E13.5, MT1-MMP$^{-/-}$), 5 (E18.5, WT), and 3 (E18.5, MT1-MMP$^{-/-}$) embryos from 3 independent experiments. Data are represented as mean ± SEM. Two-way ANOVA with Sidak's multiple comparison test, **, *** represent $p \le 0.01$, and 0.001 respectively. n.s.: non-significant.

Minor comments:
2. *How long can neurons in culture be kept viable? This is essential to estimate whether the rapid axon growth and growth cones in young neurons vs. the slow growth and grown cone collapse do not reflect a culture artifact such as limited/impaired viability in dissociated cell culture. In vivo, such phases do not exist, but motor axons keep growing until they make synapses rather than following an intrinsic neural property.*

A previous study has shown that *Xenopus* spinal neurons can survive up to 5 days in culture,

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provided that no tropic molecule is added (Peng et al., 2003). In contrast, it is true that motor axons keep growing until they make synapses under *in vivo* environment. As explained by the classical neurotrophic hypothesis (Davies, 1988; Henderson, 1996), the survival, outgrowth, and/or pathfinding of neurons are influenced by various neurotrophic factors secreted from target cells. On the other hand, our *in vitro* studies employ a methodological reduction approach to convincingly indicate a novel intrinsic neural property underlying the transition from axonal outgrowth to synaptogenesis, which cannot be easily studied in the complex *in vivo* environment. We have now further clarified these points in lines 348-359.

3. Given that physiologically native agrin at NMJs is thought to attach to synaptic basal lamina by binding to laminin, and that MT1-MMP degrades laminin, how can MT1-MMP activity promote agrin deposition in the cultures?

We would like to clarify that laminins are major components of the basal lamina, and they are a large family of glycoproteins responsible for structural scaffolding in different tissues. Among different laminin family members, "synaptic" laminin (also known as laminin β2) is localized at the synaptic basal lamina that interacts with agrin at NMJs. Our working hypothesis suggests that MT1-MMP degrades different ubiquitously expressed ECM proteins that clear the surrounding extracellular environment for the subsequent deposition of synapse-specific proteins (e.g. synaptogenic factors agrin and neuregulin, synapse-specific ECM proteins laminin β2 and collagen IV). We think that this process could be regulated by: (1) temporal segregation between the degradation of ubiquitously expressed ECM proteins and the deposition of synapse-specific ECM proteins; and/or (2) the resistance of synaptic ECM proteins against MMP-mediated degradation.

To further support our model, we will perform the following new experiments in the revision plan: (1) Apart from using fluorescent gelatin, we will perform ECM degradation assay using another ubiquitously expressed ECM protein, fluorescent collagen, for substratum coating. This experiment could provide further support that different ubiquitously expressed ECM proteins are MMP proteolytic targets in our model; (2) We will also perform laminin β2 immunostaining to examine the spatiotemporal changes in synapse-specific ECM proteins before and after agrin bead stimulation or nerve-muscle contact; and (3) We will test whether laminin β2 proteins are resistant to the proteolytic degradation by recombinant MT1- MMP treatment.

4. In Fig. 2A,B, it is stated that soluble agrin causes growth cone collapse in young, but not old neurons. Given that in old neurons as many as 70% of growth cones are collapsed, i.e. that collapse is almost complete in the absence of agrin, how can the authors expect a dramatic increase in collapses in its presence? Thus, the absence of further growth cone collapse by agrin in longer cultured neurons may not reflect a new state of neuronal differentiation as suggested.

We thank the reviewer for this excellent question. As suggested by Reviewer #1 (Point #3), we will perform antisense morpholino-mediated knockdown of agrin in cultured spinal neurons, and then determine if agrin knockdown neurons remain motile and exhibit normal growth cone structures at later times in culture, but still causes growth cone collapse in response to exogenous treatment of agrin. This new experiment will provide a stronger support on the autocrine/paracrine action of agrin deposition, as an age-dependent molecular switch from neuronal outgrowth to synaptogenesis.

5. In the introduction (p.5) and Results (p.6) and Figure 6E, it is claimed that MT1-MMP-mCherry vesicles migrate bidirectionally along neurites until they are captured by agrin bead contacted sites. On p.10/11 it is stated that "nevertheless, we also observed bidirectional transport of some MT1-MMP-mCherry vesicles passing through the bead-neurite contacts without stopping". The authors should indicate what the ratio of "captured" to "bead passing" MT1-MMP-mCherry vesicles was, and how many bead contacts were analyzed. For captured vesicles. Is there a way to estimate whether occasional stopping at bead contacts was significant rather than just accidental?

We thank the reviewer for this suggestion. We will perform additional quantitative analyses to determine the percentage of MT1-MMP-mCherry vesicles that pass through the bead contacts

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without contact versus local capture and immobilization at the bead contacts. The effects by using agrin beads will also be compared with a negative control (BSA beads) to rule out the possibility of MT1-MMP vesicle stopping is just accidental.

It is also important to note that the proteolytic activity of MT1-MMP requires the surface targeting of vesicular MT1-MMP proteins. As stated in our response to Reviewer #1 (Points #5 and #6) above, we believe that our proposed experiments in the revision plan using MT1-MMP-pHluroin will provide a stronger evidence on the spatiotemporal events of MT1- MMP surface insertion to the sites induced by agrin beads.

6. Furthermore, in some of the panels (Fig. 6E), fluorescence puncta are barely visible and should be enhanced.

We have now further enhanced the contrast of images in Figure 6E to better show the small structures and weak signals of MT1-MMP-mCherry vesicles along the neurites.

7. Finally, it would help understanding the figure to indicate bead location by an asterisk and puncta with an arrow. In Figure 6F, it is not clear what arrows point to.

We thank the reviewer for this suggestion. We have now marked the bead location by an asterisk and puncta with an arrow in all figures as suggested. In the legend of Figure 6F, we have further clarified that white arrows point to examples of the local capturing of multiple MT1-MMP-mCherry vesicles at the bead-neurite contacts. For clarity, we have also added white dotted lines to indicate the bead location, and a yellow dotted line to indicate the time of photobleaching in the kymograph in Figure 6F.

8. In the last para of p. 14 (Discussion), it is argued that "Local presentation of some heparan sulfate proteoglycan (HSPG)-bound growth factors (e.g. bFGF and heparin-binding growth-associated molecule) via polystyrene beads can effectively induce both cytoplasmic and membranous presynaptic specializations at the bead neurite contact sites in a spatiotemporally controllable manner (Dai and Peng, 1995; Rauvala and Peng, 1997). In this study, we found that polystyrene beads coated with the C-terminal fragment of recombinant agrin proteins locally induce presynaptic differentiation to an extent similar to bFGF-coated beads. Agrin, the major HSPG at NMJs, may control the extracellular distribution of diffusible growth factors at developing NMJs (Matsuo and Kimura-Yoshida, 2014). Like other HSPGs, agrin contains heparan sulfate glycosaminoglycan chains that play an important role in a diverse array of biological functions (Ruoslahti, 1989). Therefore, agrin-coated beads could serve to concentrate and localize endogenous bFGF and other growth factors for the induction of presynaptic differentiation."

To my knowledge the minimal NMJ-inducing C-terminal fragment of agrin effective in vivo does not contain glycosaminoglycan side chains. Thus, if the C-terminal agrin fragment used here to coat beads, was indeed minimal, it does not contain glycosaminoglycan side chains; as a consequence the para quoted above would not make sense. The authors should specify precisely in the Methods section, which agrin fragment they used to coat beads.

We thank the reviewer for bringing up this critical point. We have double checked the product information of recombinant agrin protein (purchased from R&D, Cat# 550-AG). The C-terminal agrin fragment contains amino acid sequence from Ala1153 to Pro1959, with an N-terminal Met and 6-His tag. With this information, we agree with the reviewer that this agrin fragment does not contain heparan sulfate glycosaminoglycan chains that can interact with other growth factors. That explains why bath application of bFGF at different concentration did not cause any effects on presynaptic differentiation induced by beads coated with this agrin fragment. While the experimental results are not contradictory to our working model, our experimental design fails to differentiate the primary functional role of agrin in inducing presynaptic differentiation from the possible secondary role through regulating extracellular distribution of diffusible growth factors. In addition, we agree with this reviewer stating that, "*agrin is the sole organizer of NMJ formation in vivo, whereas bFGF has at best a modulatory role in this process*" (Point #1 above). Considering the minor modulatory role of bFGF for NMJ development *in vivo*, we have decided to focus primarily on the role of agrin in presynaptic differentiation.

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Therefore, we have now removed the quoted text and the experimental data related to bFGF (in original Supplemental Figure 5) in the revision.

9. In the same context, as a first step to dissect the mechanism of bFGF action on presynaptic differentiation in the cultures, the authors should examine whether soluble recombinant ecto-Lrp4 treatment can rescue the inhibitory effects of the MMP inhibitor or MT1-MMP knockdown as it does on agrin-induced presynaptic differentiation.

As stated in our response to Point #8 above, we agree with the reviewer that bFGF plays a minor modulatory role in NMJ development *in vivo*. Therefore, we will not pursue the suggested experiment to examine whether recombinant ecto-Lrp4 treatment can rescue the inhibitory effects of MMP inhibitor or MT1-MMP knockdown in bFGF-induced presynaptic differentiation.

Reviewer #3:

Major comments:

1. I find this topic highly relevant and the findings are in principle very interesting. However, in the current state of manuscript the mechanism leading to synaptogenesis is not clear.

We thank the reviewer for praising our topic and findings to be highly relevant and very interesting. We apologize that some findings presented in our initial submission are not clear to support our proposed mechanisms. With the new experiments and analyses proposed in the revision plan, we hope that the reviewer considers our work to be satisfactory for publication after revision.

2. Is it known which form of agrin is secreted by the spinal neurons used in this work? Is it the same isoform as presented on the beads?

Previous studies showed that all four isoforms of agrin that differ in an insert of 0, 8, 11, or 19 amino acids at the B site (also known as Z site in mammalian agrin) near the C terminal are expressed in cultured *Xenopus* spinal neurons (Peng et al., 2003). As for the bead coating, the recombinant rat agrin proteins from R&D (Cat# 550-AG) was used, which contain amino acid sequence from Ala1153 to Pro1959. This recombinant protein containing a nine amino acid insert at the Z site, which is highly conserved in different species, is also known as one of the neural agrin isoforms produced selectively by motor neurons for inducing acetylcholine receptor clustering in muscle cells (Ferns et al., 1992; Gesemann et al., 1995; Ruegg et al., 1992). We have now included this information in lines 402-406.

3. Does agrin deposition on axons depend on MT1-MMP activity, agrin expression or expression of agrin receptor(s)? This could be addressed e.g. by adding soluble agrin to young neurons and quantifying its association to neurites or by blocking MMP activity in older cultures and testing agrin association.

We thank the reviewer for this question and suggestion. Since bath application of soluble agrin results in growth cone collapse and outgrowth inhibition of young neurons (Figure 2), this would be difficult to interpret the results if we examine the effects of soluble agrin treatment and MMP inhibition simultaneously. Alternatively, we will examine the effects of MMP inhibition on agrin deposition (by immunostaining), and on agrin and Lrp4 mRNA levels (by Northern blot analysis or semi-quantitative reverse transcription PCR) in young versus old cultured neurons.

4. What are the targets of MT1-MMP in this system? Is agrin proteolytically cleaved by MT1-MMP as it is e.g. by neurotrypsin? Or is Lrp4 proteolytically cleaved by MT1-MMP? And related to that- what is the source of Lrp4 ectodomain that seem to necessary for synaptogenesis?

In our working model (Figure 9), ubiquitously expressed ECM proteins and neuronal Lrp4 are both the targets of MT1-MMP for the induction of presynaptic differentiation at developing NMJs. It

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is noteworthy that MMP-3 activity can proteolytically degrade and remove agrin from synaptic basal lamina at adult NMJs (VanSaun and Werle, 2000).

However, our recent study demonstrated that the pattern and intensity of endogenous agrin tracks were largely unaffected by MMP inhibitors (Chan et al., 2020) (also shown in Figure 3 of this response letter), ruling out that agrin is a target of muscle MMP-mediated degradation at developing NMJs. We have further clarified this point in the revised manuscript, lines 386-390.

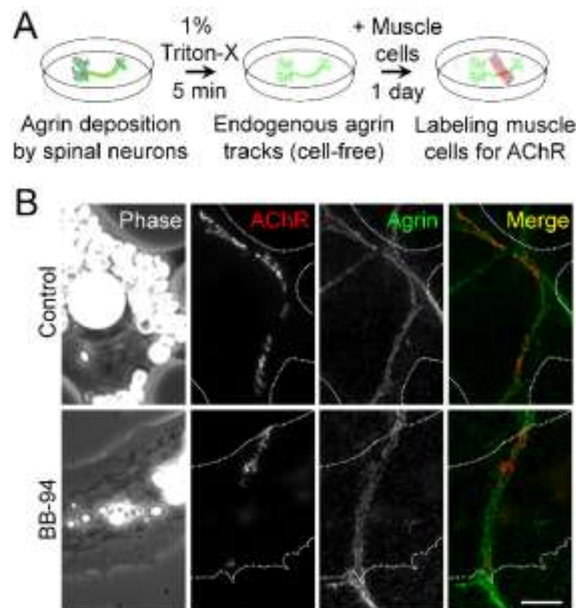

Figure 3. MMP inhibition suppresses nerve-induced AChR clustering, not through agrin degradation. (A) A schematic diagram illustrating the preparation of endogenous agrin tracks on ECM-coated substratum for inducing synaptic AChR clustering in cultured muscle cells. (**B**) Representative images showing the inhibitory effects of MMP inhibitor BB-94 on AChR clustering induced by endogenous agrin tracks. The location of agrin tracks was visualized by agrin immunostaining and then confirmed if neurites were not found in the phase contrast images. Dotted lines outline the periphery of muscle cells. Scale bar represents 10 µm.

To further investigate the possible action of neuronal MMPs in agrin cleavage and degradation, we will determine if MT1-MMP activity affects agrin deposition along the neurites. Specifically, we will incubate 1-day-old cultured neurons with the MMP inhibitor, BB-94, for 24 hours. The pattern and intensity of agrin deposition along the neurites will then be compared between BB-94-treated and control groups.

Intriguingly, Lrp4 is expressed in both motor neurons and skeletal muscles (Wu et al., 2012). In the revision plan, we will further determine the source of soluble Lrp4 ectodomain that regulates presynaptic differentiation. Specifically, we will examine the effects of morpholino-mediated knockdown of neuronal and/or muscle Lrp4 expression on the clustering of presynaptic markers and postsynaptic AChRs in nerve-muscle co-cultures.
After that, we will test if ecto-Lrp4 treatment can reverse the inhibitory effects, if any, of Lrp4 knockdown from neuron and/or muscle origin. In the revised manuscript, we have clarified this point in lines 429-430.

5. Inhibition of MT-MMP1 is preventing synaptogenesis, which can rescued by application of MT-MMP1. This suggests Lrp4 to be target of the protease (see point 4). Is therefore neuronal, transmembrane Lrp4 inhibiting synapse formation?

We thank the reviewer for this question. To address whether neuronal, transmembrane Lrp4 inhibits synaptic formation, spinal neurons will be over-expressed with Lrp4, and then co-cultured with wild-type muscle cells. We will then determine the effects of Lrp4 overexpression on nerve-induced AChR clustering in the presence or absence of the MMP inhibitor.

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6. Figure 1 demonstrates the accumulation of agrin along the axon, which seems to quite homogenous in 1-2 days old cultures. This in mind I wonder how presynaptic vesicles can be clustered at specific sites or whether MT1-MMP vesicles are directed to adhesion sites (maybe here artificially induced via agrin and bFGF beads) and this may be the main (and no less interesting) mechanism.

In the revision plan, we will include immunostaining of synapsin 1 to show the localization of synaptic vesicle (SV) clusters, in relationship to agrin deposition and gelatin degradation, along the neurites in cultured neurons of different ages in Figure 1. In addition, we will also perform more quantitative analyses, e.g. line profiles of fluorescent gelatin, agrin, and SV signals along the neurites will be plotted to further prove the coupling events between MMP-mediated ECM degradation and presynaptic differentiation in cultured neurons of different ages.

To further test whether SVs are simply clustered at adhesion sites induced by bead stimulation, here we coated the beads with an ubiquitously expressed ECM protein, laminin- 111, and found that they are not able to induce SV clustering at the bead-neurite contacts. Interestingly, integrin β1 immunostaining indicated that neither agrin- nor laminin-coated beads can induce the formation of focal adhesion at the bead-neurite contacts. In contrast to a previous study using other cell types revealing that MT1-MMP vesicles are directed to focal adhesion sites (Stehbens et al., 2014), our results therefore suggest that presynaptic differentiation, together with directed MT1-MMP vesicular trafficking and surface insertion, are specifically regulated by the intracellular signaling pathways induced by agrin, but not by the secondary effects of integrin-mediated matrix adhesion sites induced by the physical bead contact *per se*.

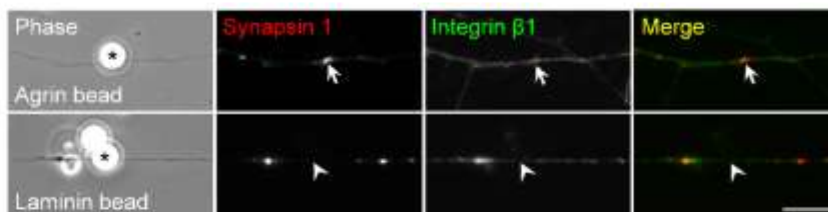

Figure 4. Agrin bead specifically induces SV clustering, but not integrin-mediated matrix adhesion. Agrin-coated bead induces synapsin 1, but not integrin β1, clustering at the bead-neurite contact (asterisk). In contrast, laminin-coated beads are not able to induce neither synapsin 1 nor integrin β1 localization at the bead-neurite contact (asterisk). Arrows point to the sites of SV clusters induced by agrin beads. Arrowheads point to the absence of SV or integrin β1 clusters at laminin bead-neurite contacts. Scale bar represents 10 μm.

7. In some figures labeling of the axes are not intuitive or misleading. E.g Figure 1b: Normalized extension can not have a label in um but probably in %.

We thank the reviewer for pointing out the mistakes. We have now changed the labelling of the axes in **Figures 1B and 2C**, as suggested.

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First decision letter

MS ID#: JOCES/2020/246710

MS TITLE: Neuronal MT1-MMP regulates focal ECM degradation and agrin deposition in presynaptic development

AUTHORS: Marilyn Janice Oentaryo, Anna Chung-Kwan Tse and Chi Wai Lee
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript based on the information you have provided. Overall, we think that the paper is suitable for JCS if you revise according to your proposed plan. However, one issue that was apparent after reading the manuscript was "what determines what": agrin deposition driving MMP secretion and activity, or otherwise MMP secretion driving agrin localisation? In revising your MS, I feel that you really need to tackle this point in text with robust writing in additional to experimentallyIf you think that you can deal satisfactorily with this criticism on revision, then I would be pleased to see a revised manuscript and make a quick decision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

First **revision**

Author response to reviewers' comments

Author Response (JOCES/2020/246710, RC-2019-00130)

We sincerely thank the reviewers for their critical evaluation and constructive comments on our work. We are encouraged by their overall positive view of our work from all reviewers, including "*an interesting paper*" and "*data of good quality and interesting*" from Reviewer #1, "*potentially useful communication of how MT1-MMP might be involved in presynaptic differentiation*" from Reviewer #2**, and** "*this topic highly relevant and the findings are in principle very interesting*" from Reviewer #3. The reviewers, however, raised some important points on the study. We have performed many new experiments to address key issues raised by the reviewers, which are summarized below in the first section. In addition, detailed point-to-point responses to the editor's and individual reviewer's comments are also provided.

We believe that we have now addressed most, if not all, of the reviewers' questions and have incorporated all suggestions from the reviewers in this revised manuscript. We hope that the reviewers find our new data and responses to be satisfactory. With the constructive comments from the reviewers, we are happy to see that our work will be significantly strengthened by the addition of new data in this substantial revision.

A list of new data in the revision:

1. ***Gelatin degradation is spatially and temporally correlated with agrin deposition and synapsin localization along the neurites (Fig. 1C-G).*** To further demonstrate that agrin is a developmentally regulated synaptogenic factor for inducing presynaptic differentiation, we have replaced the original data in Fig. 1C by including synapsin I immunostaining as well. In addition, we have constructed line profiles of fluorescent gelatin, agrin, and synapsin I signals (Fig. 1D) to convincingly illustrate the close spatial correlation between gelatin degradation and localization of agrin and synapsin I in different segments of neurite in 1-day old cultured neurons. (See Reviewer #1, Point #2 and Reviewer #3, Point #6).

2. ***Endogenous agrin knockdown or inhibition promotes neurite outgrowth and inhibits growth cone collapse in old neurons (Fig. 2G-I).*** To test whether nerve-derived endogenous agrin induces presynaptic differentiation in cultured spinal neurons via autocrine/paracrine regulation, agrin expression and activity in cultured spinal neurons were manipulated by antisense morpholino and functional blocking antibody, respectively. Our new data indicated that inhibition of agrin expression or activity promotes neuronal outgrowth and inhibits growth cone collapse in 1-day old cultured neurons, further supporting our hypothesis that secretion of endogenous agrin is a time- dependent molecular switch from neuronal outgrowth to synaptogenesis. (See Reviewer #1, Point #3 and Reviewer #2, Point #4).

3. ***Agrin beads focally induce the clustering of various presynaptic markers at the bead- neurite contact sites (Fig. 3C-D).*** To further prove that agrin stimulation locally induces presynaptic differentiation, we have performed immunostaining of several different presynaptic markers (including F-actin, phosphotyrosine-containing proteins, synapsin I, bassoon, and piccolo), in addition to mitochondrial and synaptic vesicle clustering presented in our initial submission. We found that all these markers are highly localized at the bead-

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neurite contact sites. Moreover, FM1-43 loading and unloading experiments on live cultured neurons further indicated that agrin beads induce the clustering of synaptic vesicles that are functionally releasable in response to high-potassium stimulation. (See Reviewer #1, Point #4)

4. ***MMP inhibition suppresses gelatin degradation, agrin deposition, and synapsin localization (Fig. 4A-D).*** To demonstrate if MMP-mediated ECM degradation is required for agrin deposition and synapsin I localization along the neurites, we used BB-94, a broad-spectrum MMP inhibitor. While BB-94 completely abolished the degradation of fluorescent gelatin, agrin deposition and synapsin I localization were also significantly inhibited along the neurites. This result indicated the possible causal relationship between MMP-mediated ECM degradation and agrin deposition (and subsequently accumulation of presynaptic markers). (See Editor, Point #1; Reviewer #3, Point #3)

5. ***Secreted agrin is not a target of MMP proteolytic activity (Fig. 4E-F).*** To address whether agrin is proteolytically degraded by MMP activity, we first performed agrin immunostaining, followed by live-cell imaging to examine the effects of BB-94 treatment on the fluorescence intensity of secreted agrin signals. Our new data indicated that agrin immunostaining signals are comparable in the presence or absence of BB-94, after 1 day. This further suggested that agrin is not a proteolytic target of MMPs in spinal neurons. (See Reviewer #2, Point #3 and Reviewer #3, Point #4)

6. ***MT1-MMP-mediated ECM degradation is required for agrin deposition along the neurites (Fig. 5C-D).*** To further test whether agrin synthesis and deposition in axons depend on MT1-MMP activity, we have performed immunostaining experiments that show a significant reduction of agrin deposition by MT1-MMP knockdown. (See Reviewer #3, Points #3 & #4)

7. ***MT1-MMP is spatially inserted at agrin bead-neurite contacts (Fig. 6G-H).*** To demonstrate that MT1-MMP vesicles are locally delivered to agrin bead-neurite contact sites, we used MT1-MMP tagged with a pH-sensitive GFP variant, pHluorin. Our new data showed the spatial insertion of MT1-MMP at the agrin bead-neurite contact sites. (See Reviewer #1, Points #5 & #6 and Reviewer #2, Point #5).

8. ***Neuronal MT1-MMP is essential for presynaptic development in vivo (Fig. 8G-H).*** Our recent study showed that MT1-MMP knockout mice exhibit an obvious presynaptic defect in embryonic diaphragm muscles (Chan et al., 2020). To further test the possibility of neuronal MT1-MMP in regulating axonal outgrowth and presynaptic differentiation *in vivo*, we have performed morpholino-mediated knockdown of MT1- MMP in the spinal cord preferentially (as determined by the well-established lineage tracing and fate mapping in *Xenopus* embryos), followed by immunostaining of whole- mount embryos. Our new data showed that neuronal MT1-MMP knockdown affects the structural features of presynaptic and postsynaptic markers at developing *Xenopus* NMJs *in vivo*. (See Reviewer #2, Point #1).

9. ***Sources of ecto-Lrp4 regulate NMJ formation (Fig. S8).*** To further determine the source of soluble Lrp4 ectodomain that regulates presynaptic differentiation, we have examined the effects of morpholino-mediated knockdown of neuronal and/or muscle Lrp4 expression on the clustering of presynaptic and postsynaptic markers in nerve- muscle co-cultures. Our new data showed that ecto-Lrp4 treatment can reverse the inhibitory effects of Lrp4 knockdown from neuron and/or muscle origin. (See Reviewer #3, Point #4).

Point-to-point responses to the editor's comments:

1. Overall, we think that the paper is suitable for JCS if you revise according to your proposed plan. However, one issue that was apparent after reading the manuscript was "what determines what": agrin deposition driving MMP secretion and activity, or otherwise MMP secretion driving agrin localisation? In revising your MS, I feel that you really need to tackle this point in text with robust writing in additional to experimentally.

We thank Dr Way for raising this important concern. In the revised manuscript, we have provided new data showing that inhibition of ECM degradation by either MMP inhibitor BB-94 (Fig. 4A-D) or MT1-MMP knockdown (Fig. 5C-D) significantly suppresses agrin deposition along

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the neurites, further supporting our hypothesis that MMP-mediated ECM degradation determines agrin deposition. Interestingly, local agrin stimulation via bead application is also found to direct the vesicular trafficking and surface insertion of MT1- MMP (Fig. 6), indicating a feedback regulation of localized MT1-MMP surface expression by agrin stimulation at the presynaptic regions. Localized MT1-MMP activity at the nascent synaptic sites is believed to mediate proteolytic activation of Lrp4 for presynaptic development. To better show our working model, we have now further clarified in text, modified our schematic diagram (Fig. 8I), and revised the title of our manuscript as "*Neuronal MT1-MMP mediates ECM clearance and Lrp4 cleavage for agrin deposition and signaling in presynaptic development*" to better reflect the main findings of our present study.

Point-to-point responses to reviewers' comments:

Reviewer #1:
9. The authors first show that as spinal neurons mature in culture, they slow, and begin to express Agrin. The Agrin staining in Fig 2 (Fig 1) is robust, but much less so in later figures. This may be due to fact that not all neurons in spinal cord cultures are MNs, so may not all express Agrin.

We thank the reviewer for raising this concern. In Fig. 1C, agrin immunostaining showed a time-dependent increase in the signals of secreted agrin proteins along the neurites of spinal neurons over 2 days in culture. As the reviewer pointed out, the pattern of agrin deposition is robust, primarily in 2-day old cultures. Since our study sets out to understand the transition from neuronal outgrowth to synaptogenesis, all our subsequent experiments were performed using either 4-8 hours or 1-day old neuronal cultures instead. Therefore, the agrin staining is less robust in later figures.

On the other hand, we do agree with the reviewer that not all neurons cultured from dissociated neural tube tissues of *Xenopus* embryos are motor neurons. Previous electrophysiological studies showed that more than 60% of the neuronal population in cultured *Xenopus* spinal neurons release acetylcholine upon their direct muscle contact (Chow and Poo, 1985). Importantly, imaging studies further demonstrated that postsynaptic acetylcholine receptor clustering could be detected at up to 90% of nerve-muscle contacts (Peng et al., 2003). These studies therefore indicate that the major neuronal type in *Xenopus* spinal neuronal cultures is indeed motor neurons. We have now clarified these points in lines 408-414.

10. Agrin expression fits roughly with the MMP-mediated degradation, so it is suggested these two processes are linked. I find this argument weak.

To better illustrate the spatial correlation between ECM degradation and agrin deposition, we have now included line profiles of fluorescent gelatin and agrin signals in Fig. 1D to further demonstrate that ECM degradation is spatially correlated with agrin deposition along the neurites in 1-day old cultured spinal neurons. For clarity, we have also marked the segment of neurites with both gelatin degradation and agrin deposition by arrows, whereas arrowheads indicate the absence or minimal level of gelatin degradation and agrin deposition in other segment of the same neurite in Fig. 1C. With the new analysis and annotation, we hope that the reviewer finds our argument more convincing.

11. It is also suggested that since Agrin slows extension of young axons, but not mature neurons that are already static, that this means that Agrin is involved in neuronal maturation. To prove this, the authors should test the effect of Agrin KD. If Agrin KD neurons remain motile at later times in culture, but still respond to Agrin, this would be good evidence that Agrin matures neurons in a paracrine fashion.

We thank the reviewer for this excellent suggestion. In this revision, we have performed antisense morpholino-mediated knockdown of endogenous agrin expression in cultured *Xenopus* spinal neurons, as suggested by the reviewer. We found that agrin knockdown neurons remain motile and exhibit normal growth cone structures at later times in culture, but they are still responsive to exogenous agrin-induced growth cone collapse and outgrowth inhibition (Fig. 2G-I). As a parallel approach, a similar trend was observed when an agrin functional blocking

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antibody (Millipore, MAB5204), which has recently been demonstrated to inhibit agrin-induced MuSK phosphorylation in cultured C2C12 muscle fibers and tumor cells (Chakraborty et al., 2015), was used. While the functional blocking antibody-treated neurons remain motile and exhibit normal growth cone structures at later times in culture, they are unresponsive to exogenous agrin treatment, as expected. These two parallel approaches provide good evidence to support our hypothesis on the time-dependent deposition of neural agrin that regulates presynaptic differentiation in an autocrine/paracrine fashion.

12. The role of Agrin in the formation of presynaptic specializations was tested using Agrin-beads and the targeting of mitochondria and SV2. I am not convinced these are accurate measures of presynaptic development.

We thank the reviewer for raising this concern. We would like to emphasize that synaptic vesicle and mitochondrial clusters are two well-established markers of presynaptic differentiation as previously demonstrated (Dai and Peng, 1995; Dai and Peng, 1996; Lee and Peng, 2006; Lee and Peng, 2008). In this revision, we have also provided additional data to further support that presynaptic differentiation can be locally induced by agrin-coated beads, including (1) stimulation-induced loading, and then followed by unloading, of lipophilic styryl dye FM1-43 that show the functional releasable pools of synaptic vesicle clusters at the bead-neurite contacts (Fig. 3C); (2) fluorescent phalloidin staining that shows the enrichment of filamentous actin (F-actin) scaffold for stabilizing presynaptic components at the bead-neurite contacts (top row, Fig. 3D); (3) phosphotyrosine immunostaining that shows the enrichment of tyrosine phosphorylated presynaptic proteins at the bead-neurite contacts (second row, Fig. 3D); and (4) bassoon and piccolo immunostaining that show the presence of presynaptic active zone markers localized at the bead-neurite contacts (3rd and bottom rows, Fig. 3D). With all these new data, we hope that the reviewer is now convinced that focal agrin stimulation induces presynaptic differentiation in cultured spinal neurons.

13. A link between MMP activity and synaptic maturation is made because MMP inhibitors disrupt SV2 and mitos at bead contact sites. I find this weak as well. If the authors could show increased MMP activity at bead contact sites (with gelatin degradation) or MMP release at bead contact sites, this would be stronger evidence.

We agree that the link between MMP activity and presynaptic differentiation should be further strengthened, and we thank the reviewer for his/her suggested experiments.
Regarding the first suggestion to examine if fluorescent gelatin is spatially degraded at the bead-neurite contacts, unfortunately a technical issue makes this experiment unfeasible. As agrin beads are added onto the top surface of spinal neurons, the increased MMP activity at bead-contacted sites is incapable to affect the fluorescent gelatin-coated substratum, which is in contact with the basal surface of cultured spinal neurons.

Alternatively, we have performed the second suggestion from this reviewer to examine the spatial insertion of MT1-MMP to the agrin bead-neurite contact sites using pH- sensitive GFP-tagged MT1-MMP (MT1-MMP-pHluorin). In our recent study, we have successfully used this probe to convincingly demonstrate the surface insertion of MT1-MMP at aneural acetylcholine receptor clusters in cultured *Xenopus* muscle cells (Chan et al., 2020). Here, we have provided new data to demonstrate the spatial localization of surface MT1-MMP signals at agrin bead-neurite contacts (Fig. 6G). In addition, fluorescence recovery after photobleaching (FRAP) experiment was performed to demonstrate that MT1- MMP vesicular trafficking and surface insertion are directed by localized agrin stimulation, and the spatially inserted MT1-MMP proteins are then incorporated into the existing MT1- MMP clusters induced by agrin beads (Fig. 6H).

14. MT1-MMP trafficking was used to suggest that MT1-MMP targets to presynaptic sites of Agrin-bead contact. I don't find vesicle stalling at bead contact sites very compelling case for presynaptic development.

We agree with the reviewer that by simply showing MT1-MMP-mCherry vesicles stalling at agrin bead-contacted sites falls short to support the essential role of MMP-mediated ECM degradation in presynaptic differentiation. As MT1-MMP is an integral membrane protein, its

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proteolytic activity in modulating ECM proteins requires the precise control of not only vesicular trafficking, but also surface insertion, of MT1-MMP proteins. As stated in our response to point #5 above, our new data using MT1-MMP-pHluroin also showed the spatial and temporal events of MT1-MMP surface insertion upon agrin bead stimulation.

15. Also, if these neurons are expressing endogenous Agrin all over (as Fig 2 (Fig 1) indicates), how does additional Agrin on a bead matter?

As stated in point #1 above, we would like to clarify that endogenous agrin deposition was pronouncedly detected along the entire length of neurites only in neurons cultured for 2 days or longer. In contrast, agrin deposition in younger cultures is unevenly detected along the neurites (Fig. 1C, first two rows). Therefore, we have used neurons cultured for 1 day or less in all our subsequent experiments for investigating the induction of presynaptic differentiation by agrin beads. Importantly, the bead assay allows us to investigate how localized agrin stimulation induces presynaptic differentiation in a spatiotemporally controllable manner. We have now clarified these points in lines 157-159 and 215-218.

16. ACh receptor clustering at sites of muscle contact was more convincing, especially the MT-MMP MO experiment. However, I would have liked to see the evidence that MT1- MMP Morpholinos work (cited paper, Chan et al. 2019, is a meeting abstract). A blot or ICC showing MT1-MMP KD would be nice. Also, the specific MT1-MMP antibody that was used should be indicated.

We thank the reviewer for commenting our work to be convincing. The cited morpholino work in a meeting abstract has recently been published (Chan et al., 2020), in which western blot analysis was performed to demonstrate that the same sequence of MT1- MMP antisense morpholino significantly reduces the endogenous MT1-MMP protein level in *Xenopus* embryos. We have now updated this citation in the revision. In addition, we have included the detailed information of MT1-MMP antibody used in the Materials and Methods section.

<u>*Reviewer #2:*</u>

<u>Major comments:</u>

10. In general, the experiments appear to support the conclusions and the working model shown in Fig. 9. However, it should be noted that in principle, cultured cells can show potential mechanisms only, but not their physiological relevance in an in vivo environment. For example, agrin is the sole organizer of NMJ formation in vivo, whereas bFGF has at best a modulatory role in this process, yet they have similar effects on presynaptic differentiation in the culture system used. The paper is thus a preliminary, but nevertheless potentially useful communication of how MT1-MMP might be involved in presynaptic differentiation. The authors would probably agree that validation of their model in vivo, e.g. through genetic approaches such as inducible knock-out of MT1-MMP selectively in motor neurons, would make their story considerably stronger.

We thank the reviewer for considering our work as potentially useful communication of how MT1-MMP might be involved in presynaptic differentiation. We agree with the reviewer regarding the importance of validating our model by an *in vivo* system. In fact, our recent study has already provided *in vivo* data using MT1-MMP$^{-/-}$ knockout embryos to demonstrate the role of MT1-MMP in regulating the recruitment of aneural acetylcholine receptor clusters for the assembly of postsynaptic differentiation at developing NMJs (Chan et al., 2020). In that study, we found that MT1-MMP$^{-/-}$ knockout mice also exhibit an obvious presynaptic defect in embryonic diaphragm muscles, suggesting a possible involvement of neuronal MT1-MMP in axonal growth and differentiation that warrants further investigation. Consistent with that observation, our present study further demonstrated that either pharmacological inhibition of MMP activity or knockdown expression of MT1-MMP greatly promotes growth cone collapse, leading to a significant reduction in neurite outgrowth of cultured *Xenopus* spinal neurons. This new result is now presented in Fig. S7 and lines 398-401.

We are aware of the importance of developing motor neuron-specific and inducible MT1-MMP

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knockout models. Considering the long generation time of tissue-specific knockout mice or frogs, we feel that this experiment is outside the scope of our revision plan. Alternative, we have tried to address this concern about the *in vivo* validation by perform morpholino-mediated knockdown of neuronal MT1-MMP (as determined by the well- established lineage tracing and fate mapping in *Xenopus* embryos), followed by immunostaining of whole-mount embryos to examine the effects of neuronal MT1-MMP knockdown on the structural features of presynaptic and postsynaptic markers at developing NMJs *in vivo*. Our new data indicated that synaptic specializations, including postsynaptic AChR clustering and presynaptic SV clustering, are largely reduced in whole-mount *Xenopus* embryos with MT1-MMP knockdown preferentially at the spinal cord region (Fig. 8G-H).

In contrast to the generation of tissue-specific knockdown animal models, results of our experiment provide a further validation of MT1-MMP (primarily from the neuronal origin) in presynaptic development *in vivo*, which can be done in a reasonable revision timeframe.

Minor comments:

11. How long can neurons in culture be kept viable? This is essential to estimate whether the rapid axon growth and growth cones in young neurons vs. the slow growth and grown cone collapse do not reflect a culture artifact such as limited/impaired viability in dissociated cell culture. In vivo, such phases do not exist, but motor axons keep growing until they make synapses rather than following an intrinsic neural property.

A previous study showed that *Xenopus* spinal neurons can survive up to 5 days in culture, provided that no tropic molecule is added (Peng et al., 2003). In contrast, it is true that motor axons keep growing until they make synapses under *in vivo* environment. As explained by the classical neurotrophic hypothesis (Davies, 1988; Henderson, 1996), the survival, outgrowth, and/or pathfinding of neurons are influenced by various neurotrophic factors secreted from target cells. On the other hand, our *in vitro* studies using a methodological reduction approach convincingly reveal a novel intrinsic neuronal property underlying the transition from axonal outgrowth to synaptogenesis, which cannot be easily studied in the complex *in vivo* environment. We have now further clarified these points in lines 432-444.

12. Given that physiologically native agrin at NMJs is thought to attach to synaptic basal lamina by binding to laminin, and that MT1-MMP degrades laminin, how can MT1-MMP activity promote agrin deposition in the cultures?

We would like to clarify that laminins are major components of the basal lamina, and they are a large family of glycoproteins responsible for structural scaffolding in different tissues. Among different laminin family members, “synaptic” laminin (also known as laminin β2) is localized at the synaptic basal lamina that interacts with agrin at NMJs. Our working hypothesis suggests that MT1-MMP degrades different ubiquitously expressed ECM proteins that clear the surrounding extracellular environment for the subsequent deposition of synapse-specific proteins (e.g. synaptogenic factors agrin and neuregulin, synapse-specific ECM proteins laminin β2 and collagen IV). We think that this process could be regulated by:
(1) temporal segregation between the degradation of ubiquitously expressed ECM proteins and the deposition of synapse-specific ECM proteins; and/or (2) the resistance of synaptic ECM proteins against MMP-mediated degradation. We have now included these in the discussion section, lines 478-483.

To further support our model, we have also included new data in this revision: (1) Apart from using fluorescent gelatin, we also performed ECM degradation assay using another ubiquitously expressed ECM protein, fluorescent collagen, for substratum coating. Our new data showed a close spatial correlation between fluorescent collagen degradation and agrin deposition along the neurites (Fig. S1C-D), further suggesting that different ubiquitously expressed ECM proteins are MMP proteolytic targets in our model; (2) We have performed agrin immunostaining, followed by live-cell imaging to examine the effects of BB-94 treatment on the fluorescence intensity of secreted agrin signals (Fig. 4E-F). Our new data indicated that agrin immunostaining signals are comparable in the presence or absence of BB-94, after 1 day. This further suggested that synaptic ECM proteins (e.g. agrin) are not proteolytic targets of MMPs in

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spinal neurons.

It is important to note that we have also performed immunostaining experiments to study the localization of laminin β2. For four different commercially available antibodies against laminin β2 that we have tested, unfortunately none of them works in *Xenopus*.
Therefore, whether laminin β2, like agrin, is also resistant to MMP-mediated degradation remains to be investigated.

13. In Fig. 2A,B, it is stated that soluble agrin causes growth cone collapse in young, but not old neurons. Given that in old neurons as many as 70% of growth cones are collapsed, i.e. that collapse is almost complete in the absence of agrin, how can the authors expect a dramatic increase in collapses in its presence? Thus, the absence of further growth cone collapse by agrin in longer cultured neurons may not reflect a new state of neuronal differentiation as suggested.

We thank the reviewer for this excellent question. As suggested by Reviewer #1 (Point #3), we have now performed antisense morpholino-mediated knockdown of endogenous agrin in cultured spinal neurons. We found that agrin knockdown neurons remain motile and exhibit normal growth cone structures at later times in culture, but they are still responsive to exogenous agrin-induced growth cone collapse and outgrowth inhibition (Fig. 2G-I). As a parallel approach, a similar trend was observed when an agrin functional blocking antibody (Millipore, MAB5204), which has recently been demonstrated to inhibit agrin-induced MuSK phosphorylation in cultured C2C12 muscle fibers and tumor cells (Chakraborty et al., 2015), was used. While the functional blocking antibody-treated neurons remain motile and exhibit normal growth cone structures at later times in culture, they are unresponsive to exogenous agrin treatment, as expected. These two parallel approaches provide good evidence to support our hypothesis on the time-dependent secretion and deposition of neural agrin that regulates presynaptic differentiation in an autocrine/paracrine fashion.

14. In the introduction (p.5) and Results (p.6) and Figure 6E, it is claimed that MT1-MMP-mCherry vesicles migrate bidirectionally along neurites until they are captured by agrin bead contacted sites. On p.10/11 it is stated that "nevertheless, we also observed bidirectional transport of some MT1-MMP-mCherry vesicles passing through the bead-neurite contacts without stopping". The authors should indicate what the ratio of "captured" to "bead passing" MT1-MMP-mCherry vesicles was, and how many bead contacts were analyzed. For captured vesicles. Is there a way to estimate whether occasional stopping at bead contacts was significant rather than just accidental?

We thank the reviewer for this suggestion. We have now provided additional quantitative data on the percentage of MT1-MMP-mCherry vesicles that pass through the bead contacts without contact versus local capture and immobilization at the bead contacts in lines 331-334.

It is also important to note that the proteolytic activity of MT1-MMP requires the surface targeting of vesicular MT1-MMP proteins. As stated in our response to Reviewer #1 (Points #5 and #6) above, we believe that our new data using MT1-MMP-pHluroin provides stronger evidence on the spatiotemporal events of MT1-MMP surface insertion to the sites induced by agrin beads (Fig. 6G-H).

15. Furthermore, in some of the panels (Fig. 6E), fluorescence puncta are barely visible and should be enhanced.

We have now enhanced the contrast of images in Fig. 6E to better show the small structures and weak signals of MT1-MMP-mCherry vesicles along the neurites.

16. Finally, it would help understanding the figure to indicate bead location by an asterisk and puncta with an arrow. In Figure 6F, it is not clear what arrows point to.
We thank the reviewer for this suggestion. We have now marked the bead location by an asterisk and puncta with an arrow in all figures as suggested. In the figure legend, we have further clarified that white arrows point to examples of the local capturing of multiple MT1-MMP-mCherry vesicles at the bead-neurite contacts. For clarity, we have also added white

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dotted lines to indicate the bead location, and a yellow dotted line to indicate the time of photobleaching in the kymograph in Fig. 6F.

17. In the last para of p. 14 (Discussion), it is argued that "Local presentation of some heparan sulfate proteoglycan (HSPG)-bound growth factors (e.g. bFGF and heparin-binding growth-associated molecule) via polystyrene beads can effectively induce both cytoplasmic and membranous presynaptic specializations at the bead neurite contact sites in a spatiotemporally controllable manner (Dai and Peng, 1995; Rauvala and Peng, 1997). In this study, we found that polystyrene beads coated with the C-terminal fragment of recombinant agrin proteins locally induce presynaptic differentiation to an extent similar to bFGF-coated beads. Agrin, the major HSPG at NMJs, may control the extracellular distribution of diffusible growth factors at developing NMJs (Matsuo and Kimura-Yoshida, 2014). Like other HSPGs, agrin contains heparan sulfate glycosaminoglycan chains that play an important role in a diverse array of biological functions (Ruoslahti, 1989). Therefore, agrin-coated beads could serve to concentrate and localize endogenous bFGF and other growth factors for the induction of presynaptic differentiation."

To my knowledge the minimal NMJ-inducing C-terminal fragment of agrin effective in vivo does not contain glycosaminoglycan side chains. Thus, if the C-terminal agrin fragment used here to coat beads, was indeed minimal, it does not contain glycosaminoglycan side chains; as a consequence the para quoted above would not make sense. The authors should specify precisely in the Methods section, which agrin fragment they used to coat beads.

We thank the reviewer for bringing up this critical point. We have double checked the product information of recombinant agrin protein (purchased from R&D, Cat# 550-AG). The C-terminal agrin fragment contains amino acid sequence from Ala1153 to Pro1959, with an N-terminal Met and 6-His tag. With this information, we agree with the reviewer that this agrin fragment does not contain heparan sulfate glycosaminoglycan chains that can interact with other growth factors. That explains why bath application of bFGF at different concentration did not cause any effects on presynaptic differentiation induced by beads coated with this agrin fragment. While the experimental results are not contradictory to our working model, the experimental design of our previous experiment fails to differentiate between the primary functional role of agrin in inducing presynaptic differentiation and the possible secondary role through regulating extracellular distribution of diffusible growth factors. In addition, we agree with this reviewer stating that, "*agrin is the sole organizer of NMJ formation in vivo, whereas bFGF has at best a modulatory role in this process*" (Point
#1 above). Considering the minor modulatory role of bFGF for NMJ development *in vivo*, we have decided to focus primarily on the role of agrin in presynaptic differentiation.
Therefore, we have now removed the quoted text and the experimental data related to agrin-bFGF interaction (presented in the original Figure S5) in the revision.

18. In the same context, as a first step to dissect the mechanism of bFGF action on presynaptic differentiation in the cultures, the authors should examine whether soluble recombinant ecto-Lrp4 treatment can rescue the inhibitory effects of the MMP inhibitor or MT1-MMP knockdown as it does on agrin-induced presynaptic differentiation.

As stated in our response to Point #8 above, we agree with the reviewer that bFGF plays a minor modulatory role in NMJ development *in vivo*, and the role of agrin in presynaptic differentiation is the primary focus in this revision. Therefore, we have not pursued the suggested experiment for investigating whether recombinant ecto-Lrp4 treatment can rescue the inhibitory effects of MMP inhibitor or MT1-MMP knockdown in bFGF- induced presynaptic differentiation.

Reviewer #3:

Major comments:

8. I find this topic highly relevant and the findings are in principle very interesting. However, in the current state of manuscript the mechanism leading to synaptogenesis is not clear.

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We thank the reviewer for praising our topic and findings to be highly relevant and very interesting. We apologize that some findings presented in our initial submission are not clear to support our proposed mechanisms. With all the new experiments and analyses listed in the first section, we hope that the reviewer considers our substantially revised manuscript to be satisfactory for publication.

9. Is it known which form of agrin is secreted by the spinal neurons used in this work? Is it the same isoform as presented on the beads?

Previous studies showed that all four isoforms of agrin that differ in an insert of 0, 8, 11, or 19 amino acids at the B site (also known as Z site in mammalian agrin) near the C terminal are expressed in cultured *Xenopus* spinal neurons (Peng et al., 2003). As for the bead coating, the recombinant rat agrin proteins from R&D (Cat# 550-AG) was used, which contain amino acid sequence from Ala1153 to Pro1959. This recombinant protein containing a nine amino acid insert at the Z site, which is highly conserved in different species, is also known as one of the neural agrin isoforms produced selectively by motor neurons for inducing acetylcholine receptor clustering in muscle cells (Ferns et al., 1992; Gesemann et al., 1995; Ruegg et al., 1992). We have now included this information in lines 493-497.

10. Does agrin deposition on axons depend on MT1-MMP activity, agrin expression or expression of agrin receptor(s)? This could be addressed e.g. by adding soluble agrin to young neurons and quantifying its association to neurites or by blocking MMP activity in older cultures and testing agrin association.

We thank the reviewer for this question and suggestion. To further test whether agrin deposition in axons depend on MT1-MMP activity, we have performed immunostaining experiments that show a significant reduction of agrin deposition by either pharmacological inhibition of MMP activity (Fig. 4A-D) or molecular manipulation of endogenous MT1- MMP expression (Fig. 5C-D). These results indicated the possible causal relationship between MMP-mediated ECM degradation and agrin deposition along the axons.

Additionally, we have also performed semi-quantitative reverse transcription PCR to examine the effects of MMP inhibition on agrin mRNA levels in young versus old cultured neurons (Fig. S3). We found that there is no significant difference in agrin transcript levels across different experimental conditions. These data suggested that time-dependent increase in agrin deposition along the neurites is regulated primarily by MMP-mediated ECM degradation (Fig. 1C-F), rather than developmental regulation of agrin expression between young and old neurons nor MMP-regulated agrin expression in neurons.

11. What are the targets of MT1-MMP in this system? Is agrin proteolytically cleaved by MT1-MMP as it is e.g. by neurotrypsin? Or is Lrp4 proteolytically cleaved by MT1-MMP? And related to that- what is the source of Lrp4 ectodomain that seem to necessary for synaptogenesis?

In our working model (Fig. 8I), ubiquitously expressed ECM proteins and neuronal Lrp4 are both the targets of MT1-MMP for the induction of presynaptic differentiation at developing NMJs. It is noteworthy that MMP-3 activity can proteolytically degrade and remove agrin from synaptic basal lamina at adult NMJs (VanSaun and Werle, 2000). In the present study, we have also investigated the possible action of neuronal MMPs in agrin degradation (Fig. 4E-F). By incubating 1-day-old cultured neurons with the MMP inhibitor, BB-94, for 24 hours, the pattern and intensity of agrin deposition along the neurites are comparable between BB-94-treated and control groups. This result ruled out that agrin is a target of neuronal MMP-mediated proteolytic activity at developing NMJs. We have further clarified this point in the revised manuscript, lines 263-273.

Intriguingly, Lrp4 is expressed in both motor neurons and skeletal muscles (Wu et al., 2012). In this revision, we have further determined the source of soluble Lrp4 ectodomain that regulates presynaptic differentiation (Fig. S8). Specifically, we found that morpholino-mediated knockdown of either neuronal or muscle Lrp4 significantly suppresses presynaptic and

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postsynaptic differentiation in nerve-muscle co-cultures, and this inhibitory effect can be reversed by ecto-Lrp4 treatment. This new data suggested that MT1-MMP-mediated proteolytic activation of Lrp4 in spinal neurons and muscle cells are both required for NMJ development. We have clarified this point in lines 514-517.

12. Inhibition of MT-MMP1 (MT1-MMP) is preventing synaptogenesis, which can rescued by application of MT-MMP1 (MT1-MMP). This suggests Lrp4 to be target of the protease (see point 4). Is therefore neuronal, transmembrane Lrp4 inhibiting synapse formation?

We thank the reviewer for this question. Previous studies showed that exogenous expression of Lrp4 in HEK293 cells (Wu et al., 2012) or NIH 3T3 cells (Yumoto et al., 2012) promotes presynaptic development in contacting axons. Since these cell lines have minimal or no expression of endogenous MT1-MMP (Chevalier et al., 2016; Fisher et al., 2006), it is unlikely that overexpression of full-length transmembrane Lrp4 proteins inhibits synapse formation.

13. Figure 1 demonstrates the accumulation of agrin along the axon, which seems to quite homogenous in 1-2 days old cultures. This in mind I wonder how presynaptic vesicles can be clustered at specific sites or whether MT1-MMP vesicles are directed to adhesion sites (maybe here artificially induced via agrin and bFGF beads) and this may be the main (and no less interesting) mechanism.

In the revision, we have also included synapsin I immunostaining that shows a close spatial correlation with agrin deposition and gelatin degradation along the neurites in cultured neurons (Fig. 1C). In Fig. 1D, line profiles of fluorescent gelatin, agrin, and synapsin I signals along the neurites further indicated the coupling events between MMP-mediated ECM degradation and presynaptic differentiation in different segments of the same neurite.

To further test whether SVs are simply clustered at adhesion sites induced by bead stimulation, we coated the beads with a ubiquitously expressed ECM protein, laminin-111, and found that they are incapable of inducing SV clustering at the bead-neurite contacts (Fig. 1 of this response letter). Interestingly, integrin β1 immunostaining indicated that neither agrin- nor laminin-coated beads can induce the formation of focal adhesion at the bead- neurite contacts. In contrast to a previous study using other cell types revealing that MT1- MMP vesicles are directed to focal adhesion sites (Stehbens et al., 2014), our results therefore suggested that presynaptic differentiation, together with directed MT1-MMP vesicular trafficking and surface insertion, are specifically regulated by the intracellular signaling pathways induced by agrin, but not by the secondary effects of integrin-mediated matrix adhesion sites induced by the physical bead contact *per se*.

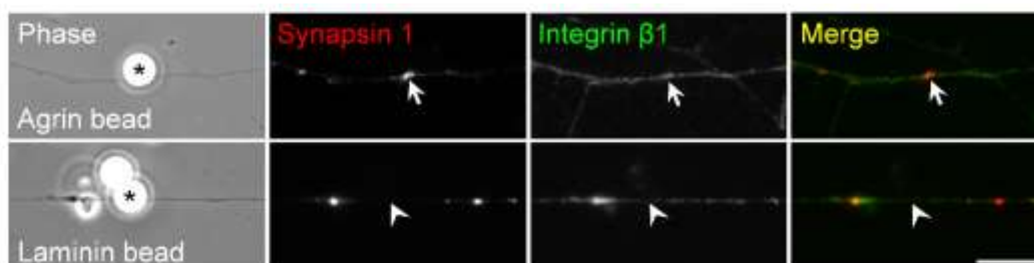

Fig. 1. Integrin-mediated matrix adhesion is not detected at bead-neurite contacts. Agrin-coated bead induces synapsin I, but not integrin β1, clustering at the bead-neurite contact (asterisk). In contrast, laminin- coated beads are not able to induce neither synapsin I nor integrin β1 localization at the bead-neurite contact (asterisk). Arrows point to the sites of SV clusters induced by agrin beads. Arrowheads point to the absence of SV or integrin β1 clusters at laminin bead-neurite contacts. Scale bar represents 10 µm.

14. In some figures labeling of the axes are not intuitive or misleading. E.g Figure 1b: Normalized extension can not have a label in um but probably in %.

We thank the reviewer for pointing out the mistakes. We have now corrected the labeling of the axes in Fig. 1B and 2C, as suggested.

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Second decision letter

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