# Local protein synthesis of neuronal MT1-MMP is required for agrin-induced presynaptic development

Jun Yu, Marilyn Janice Oentaryo and Chi Wai Lee
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**Original submission**

First decision letter

MS ID#: DEVELOP/2020/199000

MS TITLE: Local Protein Synthesis of Neuronal MT1-MMP for Agrin-Induced Presynaptic Development

AUTHORS: Jun Yu, Marilyn Janice Oentaryo, and Chi Wai Lee

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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.

Please attend to all of the reviewers' comments and 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. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

In this study by Yu and colleagues, the authors test whether Agrin-mediated pre-synaptic *changes* in motor neurons require local protein synthesis, which is a follow-up from a recent study published by this group in the Journal of Cell Science. Using isolated Xenopus spinal neuron axons (severed and using microfluidics), the authors show that pre-synaptic changes (mitochondria clustering, F-actin and SV2 staining) require new protein synthesis as changes in response to Agrin beads are blocked with cycloheximide. Similar findings are shown with rapamycin, an mTOR inhibitor. They also show that newly synthesized proteins concentrate at bead contact sites using O-propargyl-puromycin (OPP) labeling. Next they show that active mTOR and 4E-BP1 (phosphorylation changes) occur locally at the site of bead contact. They also show that RNP granules (labeled with Cy3-UTP) become immobilized at bead sites and suggest that MT1-MMP is likely locally transcribed using a 3-UTR Kaede photo-conversion experiment. Using ICC, they also show that concentration of endogenous MT1-MMP at bead contact sites also required protein synthesis and mTOR.

*Comments for the author*

This is a short paper that provides some interesting data, but it is not clear to me that they have provided enough new information to warrant publication in Development. The authors here focus on MT1-MMP, as their previous work suggested that MT1-MMP activity is necessary upstream of Agrin to promote pre-synaptic maturation. Here they show that local synthesis of MT1-MMP is also necessary for pre-synaptic maturation. Most of the data is convincing, but they did not explain the experiments fully. For example, what is the time course of the various treatments? They state that CHX was applied for 1 hr prior to Agrin stimulation but not how long Agrin beads were applied before fixation? Other details of protocol should be included, as this can help to understand the proposed mechanism. The proposed mechanism could be discussed, in a discussion section which I did not find? For example, how much MT1-MMP protein is present prior to Agrin stimulation and why is it not sufficient to simply redistribute existing MT1-MMP?

I can think of many additional experiments that would make this short paper stronger.

1. Use in situ hybridization (FISH) to show where MT1-MMP distributes with and without Agrin

2. They have a morpholino (previous paper), so could show that rapid/local MT1 expression reduced with KD. Crispr would be preferred.

3. I also wonder if the proposed requirement of new protein synthesis of MT1-MMP may also be exploited in a time course assay? For example, in experiments where they dynamically track RNPs (Cy3-UTP labelled), or mitochondria or SV2 (not done here, but possible to do), you would predict that if new MT1-MMP protein synthesis was necessary, there would be some delay after bead contact before stabilization of pre-synaptic markers. This delay should be lengthened by inhibiting protein synthesis.

4.Any experiment performed in vivo to show this is not just a tissue culture phenomenon. It should be noted that since they use spinal cord explants, the authors can not be certain they are looking at MNs in vitro, whereas in vivo this is clear.

Minor:
The first sentence of the abstract has improper grammar. I suggest: “Upon stimulation with extracellular cues, a significant number of proteins are synthesized distally along axons.” Other sentences could use some editing.

Reviewer 2

*Advance summary and potential significance to field*

This paper describes local protein synthesis at points of contact between cultured primary xenopus axons and agrin-coated beads. The authors show that presynaptic components localize to these sites, along with mitochondria and RNP-granules. These effects are present in both chamber cultures and in severed axons supporting the assertion that the protein synthesis is occurring locally in the axon. The effects are also blocked by cycloheximide, a general inhibitor of protein synthesis, and rapamycin, an inhibitor of the mTOR pathway. Among the locally synthesized proteins is MT1-MMP, which this group has previously shown to be important for agrin-mediated presynaptic specialization.

The role of agrin in presynaptic differentiation remains unclear, although previous studies support the idea that agrin's effect in the postsynaptic cell mediate a retrograde signal that includes, at least in part, LRP4 to induce presynaptic differentiation. The contribution of MT1-MMP to that process is an important prior result.
Therefore, the finding presented here that agrin induces local protein synthesis in axons and that MT1-MMP is one of the proteins translated is a potentially interesting and important finding. In general, the data is clear and well-presented and supports the conclusions drawn.

*Comments for the author*

Some comments to improve the manuscript and some concerns with data presented in Figure 4 are detailed below.

The manuscript is well-written overall, but would benefit from some proofreading for grammar. Most notably in the abstract, "Upon the stimulation of extracellular cues, a significant amount of proteins is known to be synthesized distally along the axon." Should read "Upon stimulation of extracellular cues, a significant number of proteins are known to be synthesized distally along the axon."

The supplemental figure shows a general effect of agrin on the axon, but does not distinguish if the newly synthesized proteins are from the cell body or locally translated in the axon. This result establishes the basis for subsequent experiments and could be a main figure if formatting allows five figures.

In figure 4, both A and B appear to be anecdotal one-off images that are not quantified. They are described as "representative images," but the number of times such results were observed is not stated and not attempts at quantification were made. These findings should at the very least be better described to clarify how frequently they were observed, and ideally, some quantification, particularly of multiple FRAP experiments should be provided.

Similarly, the RT-PCR experiment in panel C is not quantitative and it is unclear if the experiment was repeated. The methods section states that material from 160 chambers was used, but if this constitutes a single technical replicate, more is needed. Ideally, results should be Q-RT-PCR from at least three technical replicas. The data presented look promising, but need to be reproduced.

Throughout, it may be an overstatement to call the agrin-bead-associated specializations presynaptic differentiation, given that release of neurotransmitter etc. is not shown. The authors should address this in the introduction noting that the points of bead contact contain markers that are considered hallmarks of presynaptic differentiation, and that they are referred to throughout the paper thereafter as something like "sites of presynaptic specialization" or similar.

**First revision**

Author response to reviewers' comments

**Responses to reviewers' comments (DEVELOP/2020/199000)**

We sincerely thank the reviewers for their critical evaluation and constructive comments on our work. We are encouraged by their overall positive view of this study, including "***provides some interesting data***", "***most of the data are convincing***" from Reviewer #1, and "***potentially interesting and important finding***", "***the data is clear and well-presented and supports the conclusions drawn***" from Reviewer #2. Both reviewers, however, raised some important points on the study. Over the past 2-3 months, we have performed several sets of new experiments to address key issues raised by the reviewers, which are summarized below in the first section. 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 in this revision. We hope that the reviewers find our new findings, revision, and responses to be satisfactory. With the constructive comments from the reviewers, we are delighted to see that our work has been significantly strengthened by the new data and substantial revision.

**A list of new data in the revision:**

**1. Localization of MT1-MMP mRNA revealed by single-molecule fluorescence *in situ* hybridization (smFISH).** Our new smFISH experiments showed the spatial localization of MT1-MMP mRNA along the neurites upon agrin bead stimulation. We have also validated the specificity of MT1-MMP smFISH signals by the treatment of RNase A and the addition of BSA beads, which largely abolished the localized signals at bead-neurite contact sites. The new data is now presented in **Figure 4A-B**. (Also see Reviewer #1, Point #4; Reviewer #2, Point #4).

**2. Temporal correlation between Cy3-UTP localization and mitochondrial clustering induced by agrin beads.** Our new experiments indicated that agrin-induced Cy3-UTP localization and mitochondrial clustering are tightly coupled temporal events with very little time delay. This new data is now presented in **Figure S2**. (Also see Reviewer #1, Point #6).

**3. Additional quantitative analyses on Cy3-UTP-labelled RNP granules.** We have now provided new quantitative analyses on the axonal trafficking of RNP granules along neurites in response to agrin bead stimulation. Results of these analyses are now presented in **Figure 3B** and **lines 151-154**. (Also see Reviewer #2, Point #3).

**4. Suppression of MT1-MMP protein synthesis by antisense morpholino oligonucleotide (MO).** By using our previously characterized antisense MO sequence that specifically interferes the translation initiation of *Xenopus* MT1-MMP mRNA, our new experiment further showed that the localization of endogenous MT1-MMP proteins at agrin bead-neurite contacts was significantly reduced in MT1-MMP MO neurons. This new data is now presented in **Figure S3**. (Also see Reviewer #1, Point #5).

**Point-to-point responses to reviewers' comments:**

**Reviewer #1:**

*1. This is a short paper that provides some interesting data, but it is not clear to me that they have provided enough new information to warrant publication in Development.*

We thank the reviewer for praising our work that provides interesting data. Given that the intracellular trafficking and surface delivery of MT1-MMP have been well documented in many different cellular events, our study provides a previously unappreciated mechanism underlying the regulation of MT1-MMP protein expression via local translation during agrin- induced presynaptic development. With the addition of new experiments in response to the constructive comments from both reviewers, we hope that the reviewer finds our revised manuscript now presenting enough new information to convincingly support the novel idea of local MT1-MMP protein synthesis, which, we think, is of conceptual advance and significance and of wide

interest for the cell and developmental biology community that warrants publication in ***Development***.

*2. Most of the data is convincing, but they did not explain the experiments fully. For example, what is the time course of the various treatments? They state that CHX was applied for 1 hr prior to Agrin stimulation, but not how long Agrin beads were applied before fixation?*
*Other details of protocol should be included, as this can help to understand the proposed mechanism.*

We apologize that the details of experimental procedures were insufficient to understand the proposed mechanism in the first submission. We have now further elaborated the details of several experimental procedures in the Materials and Methods section of the revised manuscript. In particular, we have further clarified the details of our bead experiments in **lines 256-259**, "*In most experiments, cells were fixed after 4-hour bead stimulation, which has previously been demonstrated to induce the assembly of presynaptic specializations to the plateau level (Lee and Peng, 2006; Lee and Peng, 2008).*".

*3. The proposed mechanism could be discussed, in a discussion section, which I did not find? For example, how much MT1-MMP protein is present prior to Agrin stimulation and why is it not sufficient to simply redistribute existing MT1-MMP?*

Our recent study has suggested that MT1-MMP serves as a molecular switch from axonal outgrowth to synaptogenic phase in neuronal development (Oentaryo et al., 2020). In young cultured neurons, extracellular matrix (ECM)-mediated neuronal outgrowth is dominant due to the low surface expression of MT1-MMP proteins. While the level of surface MT1-MMP increases over time, the proteolytic activity of MT1-MMP mediates ECM degradation and LRP4 cleavage for agrin deposition and signaling, respectively. Therefore, it is hypothesized that a positive feedback mechanism underlying MT1-MMP expression and agrin deposition during the transition from neuronal outgrowth to synaptogenic phase. That explains why the redistribution of the existing MT1-MMP proteins (low surface expression level) prior to agrin stimulation may not be sufficient, and highlights the essential requirement of MT1-MMP local protein synthesis for agrin-induced presynaptic differentiation. We have now further elaborated these points in **lines 72-76**, "*Therefore, the temporally delayed expression of surface MT1-MMP is believed to serve as a molecular switch from axonal outgrowth to synaptogenic phase in mature neurons. Considering the inefficiency of transporting MT1-MMP proteins synthesized from the soma of highly polarized mature neurons, it is of interest to determine whether MT1-MMP can be locally translated at the presynaptic terminals*".

*4. I can think of many additional experiments that would make this short paper stronger. Use in situ hybridization (FISH) to show where MT1-MMP distributes with and without Agrin.*

We thank the reviewer for this excellent suggestion. We have now performed smFISH experiments that showed the spatial localization of MT1-MMP mRNA along the neurites upon agrin bead stimulation. In addition, we have validated the specificity of MT1-MMP smFISH signals by the treatment of RNase A and the addition of BSA beads, which largely abolished the localized signals at bead-neurite contact sites. The new data is now presented in **Figure 4A-B**.

*5. They have a morpholino (previous paper), so could show that rapid/local MT1 expression reduced with KD. Crispr would be preferred.*

We have now performed the suggested experiment to show that MT1-MMP antisense morpholino significantly reduces the localization of MT1-MMP proteins at agrin bead-neurite contacts. This new data is now presented in **Figure S3**.

In this study, we think that the use of antisense morpholino is preferrable to Crispr/Cas approach because of the following reasons: (1) The sequence of MT1-MMP antisense morpholino has already been fully validated in our previous studies (Chan et al., 2020; Oentaryo et al., 2020), which demonstrate its effectiveness in knocking down the endogenous MT1-MMP protein levels in *Xenopus* tissues by Western blot and immunocytochemistry approaches; (2) The phenotypic effects of MT1-MMP antisense morpholino could be fully rescued by overexpressing exogenous

MT1-MMP proteins, indicating that the non-specific binding of MT1-MMP morpholino to unintended targets is unlikely; (3) More importantly, as Crispr/Cas system affects genomic DNA, rather than mRNA transcripts, it might not be a suitable approach to study the local translation of MT1-MMP mRNA.

*I also wonder if the proposed requirement of new protein synthesis of MT1-MMP may also be exploited in a time course assay? For example, in experiments where they dynamically track RNPs (Cy3-UTP labelled), or mitochondria or SV2 (not done here, but possible to do), you would predict that if new MT1-MMP protein synthesis was necessary, there would be some delay after bead contact before stabilization of pre-synaptic markers. This delay should be lengthened by inhibiting protein synthesis.*

We thank the reviewer for this suggestion. In the revision, we have investigated the temporal correlation between Cy3-UTP localization and mitochondrial clustering at 2 different time points (30-min and 4-hour) after agrin bead stimulation. As early as 30 minutes, we found that 58.29% bead-neurite contacts exhibited both localized Cy3-UTP signals and mitochondrial clusters, which further increased to 67.81% after 4-hour agrin bead stimulation. Both Cy3-UTP localization and mitochondrial clustering were greatly inhibited by cycloheximide treatment. By contrast, we only observed 8.13% and 1.33% bead-neurite contacts with only localized Cy3-UTP signals at 30-min and 4-hour time points, respectively. Our results therefore indicate that agrin- induced Cy3-UTP localization and mitochondrial clustering are tightly coupled temporal events with very little time delay. This new data is now presented in **Figure S2**.

It is important to note that agrin beads were added into the neuronal cultures inside a laminar flow cabinet to avoid contamination. Prior to imaging experiments, the cultures were kept untouched for minimum 20 minutes to allow the beads to settle and make stable attachment with the neurites on the culture substrate. Therefore, we do not rule out the idea suggested by the reviewer that there would be some time delay, albeit rather brief, between the localization of Cy3-UTP-labelled RNP granules and the clustering of presynaptic markers. We have now discussed these points in **lines 162-165**, "*Our results therefore indicate that agrin-induced Cy3- UTP localization and mitochondrial clustering are temporally coupled events with very little time delay. Nevertheless, it is still plausible that RNP granule localization may be detected preceding the clustering of presynaptic markers shortly after agrin stimulation*".

*6. Any experiment performed in vivo to show this is not just a tissue culture phenomenon. It should be noted that since they use spinal cord explants, the authors cannot be certain they are looking at MNs in vitro, whereas in vivo this is clear.*

We thank the reviewer for this comment. Regarding the mixed neuronal types in spinal cord cultures, 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 the contact sites between spinal neurons and muscle cells (Peng et al., 2003). These studies therefore indicate that the major neuronal type in *Xenopus* spinal neuronal cultures is indeed the motor neurons. We have now clarified these points in **lines 111-113**, "*It is important noting that the motor neuron is the major neuronal type in Xenopus cultures, as postsynaptic acetylcholine receptor clustering is detected at up to 90% of contacts between spinal neurons and muscle cells (Peng et al., 2003)*".

We agree with the reviewer regarding the advantages of using *in vivo* models to further demonstrate the novel contribution of local MT1-MMP synthesis in presynaptic development. Although recent studies provide compelling evidence demonstrating local protein synthesis in axonal branching and maintenance of *Xenopus* retinal ganglion cells *in vivo* (Wong et al., 2017; Yoon et al., 2012), we would like to emphasize that the demonstration of local protein synthesis at *Xenopus* neuromuscular junctions (NMJs) *in vivo* is still considered to be experimentally challenging. As MT1-MMP is both pre- and post-synaptically expressed (Chan et al., 2020; Oentaryo et al., 2020), it would be difficult to differentiate the contribution of newly synthesized MT1-MMP proteins not only between the axonal and the somal compartments, but also between the presynaptic sites in spinal neurons and the postsynaptic sites in muscle fibres.

Nevertheless, we have attempted to address this reviewer's concern by investigating the localization of fluorescent MT1-MMP mRNA molecules in presynaptic terminals of developing *Xenopus* NMJs *in vivo*, using our recently established microinjection protocol to manipulate

specific blastomeres of 32-cell stage embryos that primarily give rise to spinal cord tissues in early *Xenopus* embryos (Oentaryo et al., 2020). Unfortunately, the signals of microinjected fluorescent MT1-MMP mRNA molecules were too weak to be detected in embryonic *Xenopus* spinal motor neurons *in vivo*. We hope that emerging technologies to be developed in the field of local protein synthesis will allow us to fully address this concern in our future study. We have now discussed these points in the revised manuscript - **lines 209-214**, "*While recent studies provide compelling evidence demonstrating local protein synthesis in axonal branching and maintenance of Xenopus retinal ganglion cells in vivo (Wong et al., 2017; Yoon et al., 2012), technological advances in enhancing mRNA probe sensitivity and its specific targeting to the presynaptic motor neurons are required in the future to allow us further understanding the regulation of MT1-MMP mRNA localization and translation at developing NMJs in vivo*".

*The first sentence of the abstract has improper grammar. I suggest: "Upon stimulation with extracellular cues, a significant number of proteins are synthesized distally along axons." Other sentences could use some editing.*

We thank the reviewer for pointing out this mistake. The sentence has been revised as suggested in the abstract. In addition, we have extensively revised our manuscript to correct other grammatical errors, improve the clarity, and reduce the word count to meet the requirements of this publication format.

**Reviewer #2:**

*The manuscript is well-written overall, but would benefit from some proofreading for grammar. Most notably, in the abstract, "Upon the stimulation of extracellular cues, a significant amount of proteins is known to be synthesized distally along the axon." Should read "Upon stimulation of extracellular cues, a significant number of proteins are known to be synthesized distally along the axon."*

We thank the reviewer for pointing out this mistake. This sentence has been revised to "*Upon the stimulation of extracellular cues, a significant number of proteins are synthesized distally along the axon*" in **lines 37-38**, as suggested by Reviewer #1. In addition, we have extensively revised our manuscript to correct other grammatical errors, improve the clarity, and reduce the word count to meet the requirements of this publication format.

*The supplemental figure shows a general effect of agrin on the axon, but does not distinguish if the newly synthesized proteins are from the cell body or locally translated in the axon. This result establishes the basis for subsequent experiments and could be a main figure if formatting allows five figures.*

We thank the reviewer for this suggestion. As the "Research Reports" format can be accompanied by up to four figures, we have included this piece of data as the supplementary figure. Nevertheless, we are flexible and agree if this data is presented in the main figure at the discretion of the editors on the total number of figures allowed.

*In figure 4, both A and B appear to be anecdotal one-off images that are not quantified. They are described as "representative images," but the number of times such results were observed is not stated and not attempts at quantification were made. These findings should at the very least be better described to clarify how frequently they were observed, and ideally, some quantification, particularly of multiple FRAP experiments should be provided.*

We thank the reviewer for this suggestion. Our representative images were chosen from a pool of data collected from at least 3 independent experiments. In the revised manuscript, we have now added new quantitative analyses that shows the percentage of bead-neurite contacts with increased Cy3-UTP signals, as well as the fluorescence intensities of Cy3-UTP signals along the neurites at the bead versus non-bead regions across different experimental groups in the new **Figure 3B**.

Regarding the FRAP experiments, we have now provided a quantitative analysis on 7 bead-neurite contacts from 3 independent experiments, in which we observed local capturing of the first moving Cy3-UTP granule by agrin beads in 11.88 ± 5.33 (SD) seconds after photobleaching.

This result is now presented in **lines 151-154**.

*1. Similarly, the RT-PCR experiment in panel C is not quantitative and it is unclear if the experiment was repeated. The methods section states that material from 160 chambers was used, but if this constitutes a single technical replicate, more is needed. Ideally, results should be Q-RT-PCR from at least three technical replicas. The data presented look promising, but need to be reproduced.*

We would like to clarify that our RT-PCR experiment (**Figure 4C**), which aims to demonstrate the presence of MT1-MMP mRNA in the axonal compartment, was performed using axonal and somal materials collected from a total of 160 microfluidic chambers pooled from 4 independent experiments. Therefore, our findings showing the presence of MT1-MMP mRNA, together with the absence of histone H4 mRNA, in the pooled axonal compartment samples convincingly indicated that MT1-MMP mRNA can be detected along the axons, rather than the possible contamination of the somal materials from any of the 4 independent experiments.

Although it is not our intention of using RT-PCR to quantitatively measure MT1-MMP mRNA level in relation to other mRNA levels, we agree with the reviewer that more technical replicas would certainly strengthen our conclusion regarding the presence of endogenous MT1- MMP mRNA along the neurites. After considering the extensive amount of work required for this experiment (160 microfluidic chambers, and neural tube tissues from 5 embryos per chamber), we have decided to perform another parallel experiment to further support our conclusion instead. Our new smFISH experiments showed the spatial localization of MT1-MMP mRNA along the neurites upon agrin bead stimulation. In addition, we have validated the specificity of MT1-MMP smFISH signals by the treatment of RNase A and the addition of BSA beads, which largely abolished the localized signals at bead-neurite contact sites. The new data is now presented in **Figure 4A-B**. With this new data, we hope that the reviewer is now convinced about the presence of MT1-MMP mRNA in the axonal compartment.

*2. Throughout, it may be an overstatement to call the agrin-bead-associated specializations presynaptic differentiation, given that release of neurotransmitter etc. is not shown. The authors should address this in the introduction noting that the points of bead contact contain markers that are considered hallmarks of presynaptic differentiation, and that they are referred to throughout the paper thereafter as something like "sites of presynaptic specialization" or similar.*

We thank the reviewer for raising this concern. In our recent study (Oentaryo et al., 2020), we have demonstrated that agrin beads locally induce presynaptic differentiation in cultured *Xenopus* spinal neurons, as reflected by the clustering of mitochondria, synaptic vesicle markers (synaptotagmin and synapsin I), and active zone markers (bassoon and piccolo). In addition, FM1-43 loading and unloading experiments further demonstrated that agrin bead- induced presynaptic specializations are functionally active that undergo synaptic vesicle recycling in response to high-potassium stimulation. Therefore, we feel that the use of "agrin bead-induced presynaptic differentiation" is justified. We have further clarified this point in **lines 91-93**, "*Our recent study reported that agrin-coated beads induce structural and functional synaptic specializations along the neurites in a spatiotemporally controllable manner (Oentaryo et al., 2020)*".

In the meantime, we have also toned down several sentences by using the suggested phrase - "sites of agrin-induced presynaptic specializations" in the revised manuscript, where it is more appropriate (**lines 78, 96, 110, 117, and 258**).

**References:**

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Second decision letter

MS ID#: DEVELOP/2020/199000

MS TITLE: Local Protein Synthesis of Neuronal MT1-MMP for Agrin-Induced Presynaptic Development

AUTHORS: Jun Yu, Marilyn Janice Oentaryo, and Chi Wai Lee
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks and some text modifications as indicated by reviewer 2.

Reviewer 1

*Advance summary and potential significance to field*

The authors show that Agrin-mediated pre-synaptic changes in motor neurons require local protein synthesis of MT1-MMP.

*Comments for the author*

The authors did a good job providing experimental details and added some new experiments, which support their findings. I approve of publication in Development now.

Reviewer 2

*Advance summary and potential significance to field*

My previous concerns regarding the rigor of some experiments, particularly those of figure 4 are addressed in this revised manuscript. This work is a significant advance over those studies, demonstrating the need for local translation and identifying MT1-MMP as an important product

*Comments for the author*

I do think that two points should be made in the introduction and/or discussion. First, Campagna and Bixby showed a role for agrin in the presynaptic differentiation of ciliary ganglion neurons in the 1990s. The prior work on Agrin in presynaptic differentiation should be cited. Second, and

conversely, Scheiffele et al. in their now classic Cell paper from 2000 did test Agrin for its ability to induce presynaptic differentiation in cerebellar climbing fibers and mossy fiber terminals and found no apparent activity, particularly in comparison to neurligins. Thus, the possible cell-specificity of Agrin in the presynaptic differentiation of peripheral motor and autonomic neurons should be noted.