Developmental arrest of *Drosophila* larvae elicits presynaptic homeostatic depression and enables prolonged studies of neurodegeneration

Sarah Perry, Pragya Goel, Nancy L. Tran, Cristian Pinales, Christopher Buser, Daniel L. Miller, Barry Ganetzky and Dion Dickman
DOI: 10.1242/dev.186312

Editor: Francois Guillemot

Review timeline

Original submission:	13 November 2019
Editorial decision:	2 January 2020
First revision received:	20 March 2020
Editorial decision:	14 April 2020
Second revision received:	16 April 2020
Accepted:	18 April 2020

Original submission

First decision letter

MS ID#: DEVELOP/2019/186312

MS TITLE: Presynaptic depression maintains stable synaptic strength in developmentally arrested Drosophila larvae

AUTHORS: Dion Dickman, barry ganetzky, Daniel Miller, Sarah Perry, and Pragya Goel

I have now received the reports of three referees on your manuscript and I have reached a decision. The reports are appended below and 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, all the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, referee 1 suggests that you analyse the movements of SmoxKD larvae and referee 2 requests that you analyse the size of synaptic vesicles at the NMJ in these larvae. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

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

Previous to this paper, it has been impossible to study synaptic homeostasis over long term periods of time in the Drosophila NMJ, a well-characterized model for synaptic plasticity. This study manipulates pupation-inducing insulin and ecdysone production by RNAi knockdown of smox (dSmad2) to extend the 3rd instar larval stage to over 30 days whereas wild type larva pupate at 5 days AEL. This allows the authors to study how the synapse changes over longer periods of time and with changes in size. Stable neurotransmission is maintained throughout the extended lifespan through a reduction in presynaptic neurotransmitter release despite growth of synapses-apparent using both presynaptic and postsynaptic markers. This technique will also allow researchers in the field to examine other modifications to synapses over longer periods of time.

The paper is written well, such that it is easy to follow.

Comments for the author

I have two concerns with the work. The first is that the reduction of dSmad2 (Smox) is not well-characterized or referenced in this paper. Smox RNAi could impact far more than the two targets (insulin and ecdysone) the authors are aiming to affect. For example, while the muscle doesn't look different in size, it looks qualitatively different in the ATI5 vs WT5 in Figure 1.
Maybe other possible impacts of smoxKD are not important for this study, but the possibility that there could be some impacts outside of what the authors reveal should be acknowledged.
The paper reads almost as though we are looking at WT animals with an extended larval period, but clearly smox is an important transcription factor and has other targets. Simple text changes would address this concern. Please include references to what is known and acknowledge what is not known about how smox affects a synapse and potentially muscle.

The second concern is that this paper does not take into account any potential changes in behavior between ATI animals and WT. For example, differences in movement behavior
(muscle use) likely impact synaptic homeostasis. The paper could be strengthened with an assessment of larval movement behavior in smoxKD vs WT at a similar age and then also smoxKD over time (day 5 vs. d17 and d33 for example. This would address if muscle/body size vs. muscle use impacts synaptic strength at the NMJ.

Minor changes:
Figure 1E and F-- please add labels (WT5ATI5, 17, and 33) to the EPSP traces and to the x axis of the bar graph. The matching colors help, but labels clarify and there is ample space for them. Similarly, add labels to supplemental figure 4-1.

I would also appreciate an N.S. or an * to indicate significant change from WT at day5 in Figure 2B, C, and D. I realize that the significance is indicated in the supplement, but it is a bit of a pain to search for that as a reader.

Reviewer 2

Advance summary and potential significance to field

This is an interesting study looking at the effect of arresting larval development on the stability of neuromuscular function in the third instar larvae. The authors use a genetic manipulation that ablates the production of ecdysone, which is required to transition from the larval to the pupal stage of development. Under these unique conditions, development is arrested at the larval stage for an extended period (~30 days) but the larvae continue to grow resulting in a larger than normal larvae prior to death. The continued growth of the muscle allowed the authors to investigate how the function of the neuromuscular function changes in the face of this abnormal larval growth program. The authors report that during this arrested development, neurotransmission at the NMJ is maintained stable despite the changes in the size of the muscle. Further analysis demonstrates that the size of the response of the muscle to the release of a single vesicle of neurotransmitter,

referred to as quantal size, increases during the period and the synapse reduces the total number of vesicles released (a.k.a. quantal content) to compensate referred to as presynaptic homeostatic depression (PHD), a form of plasticity that these authors have previously studied. Although this is an interesting observation, the results are somewhat preliminary and the normal context for this plasticity isn't clear. Importantly for this review, it is unclear, as presented, how these results inform the reader about normal developmental processes related to the development of the nervous system. At this point, the paper requires substantial revisions before it is appropriate for publication in Development.

Comments for the author

Major critiques

1-The authors show that quantal size increases during ATI and state that this is due to increased GluR function based solely on immunocytochemistry. Although the authors show an increase in GluR staining, this analysis does not rule out a presynaptic contribution to Q (i.e. synaptic vesicle size). Because previous studies have shown that increased post-synaptic sensitivity to neurotransmitter via manipulation of GluR abundance have been shown to increase both mEJPs and EJPs (for example see DiAntonio et al., 1998), in contrast to what is shown during ATI, it is important to rule out a presynaptic contribution to quantal size. The authors should address vesicle size during ATI using EM. Although this is a difficult analyses, it is key to understanding the contribution of post-synaptic glutamatergic signaling to the reduction in quantal content observed during ATI. This is especially important is determining if there is a transynaptic component to the signaling required for this form of PHD.

2-The authors show that synaptic growth continues including Brp puncta and conclude that the number of active zones that participate in release (N) is increasing. It is already established at this synapse that not all active zones participate in release so the characterization of the number of release sites using immuncytochemisty is inaccurate. To truly determine the number of active zones participating in release the authors should utilize genetically encoded calcium indicators that allow the resolution of participating active zones during release under these novel developmental conditions. This approach will also allow the direct measuring of the probability of release at each release site.

Minor comments

1-Error bars are missing from Figure 2.

2-From the imaging data presented it looks like there is no change in GluR subunit stoichiometry. This would be supported by some wave-form analysis of the mEPSPs such as decay or half-width. This analysis would also potentially support the contention that the change in quantal size is solely a post-synaptic event.

3-The effects of prolonged larval development on muscle integrity and function are not investigated. Isn't it possible that muscle contraction/function could also feed into the regulation of neurotransmission at the NMJ during ATI? Are there changes in fiber number, muscle nuclei, sarcomeres, etc. Related to this, what happens to larval mobility/crawling during ATI?

4-Since satellite cells have not been observed in the Drosophila muscle, it might be expected that the number of fibers might diminish during ATI due to use-related damage. Would this change the interpretation of the data?

5-Line 294 is missing "that"

Reviewer 3

Advance summary and potential significance to field

Presynaptic depression maintains stable synaptic strength in developmentally arrested Drosophila larvae by Perry et al.
This is a very interesting and rigorous manuscript that describes a novel genetic strategy that extends the experimental utility of the Drosophila larval NMJ. The approach rest on stalling larval development at the third instar stage for up to a staggering ~30 days. This is achieved by selectively abrogating the expression of transcription factors necessary for ecdysone secretion from the prothoracic gland. The fundamental and novel observation is that despite the great increase in synapse boutons that exceed the growth of the innervated muscle, the evoked neurotransmission remains steady. The authors explore the electrophysiological mechanisms of this adaptive response that maintains evoked neurotransmission to hone in on a novel presynaptic depression mechanism. This process is expressed in the form of decrease probability of release as indicated by failure analysis.
The paper is carefully executed, discussed, and conclusions are well supported by findings. While the molecular mechanisms of the new form of presynaptic adaptive depression are not explored, this paper provides a solid base where to begin. Arrested larval development preparation plus the new form of adaptive presynaptic depression are of sufficient novelty and excitement. However, the authors add another aspect that creates both additional excitement yet at the cost of a change in the focus of the paper. The authors realize that this novel arrested preparation offers a ground for additional experimentation assessing the impact of genetic manipulations that induce neuropathology. This is explored with RNAi targeting stathmin.

Comments for the author

The new stathmin data are quite exciting but are lost in the context of the title and focus of the paper. There is a clear asymmetry between the efforts to identify the mechanisms that account for presynaptic depression versus those devoted to explore the usefulness of the preparation to study neuropathology.
I would like to suggest two avenues to correct this: 1) the title should be more encompassing of the stathmin findings. 2) There could be additional efforts to expand on the experimental efforts to establish the arrested NMJ as a sensitive and quantitative model for neuropathology. For example, the polyglutamine (polyQ) stretch within the Huntingtin (Htt) protein produces pathology in a manner sensitive to the length of the stretch at the NMJ (DOI: https://doi.org/10.1523/JNEUROSCI.2752-16.2017). Since there are already transgenes that encompass different length of the polyQ repeat, the penetrance of phenotypes could be explored in the arrested NMJ preparation. I will leave to the authors to decide the extent to consider this second option.

There some additional points that I would like the authors to clarify/address:
1) Line 367 “In contrast, there is no evidence for changes in synaptic vesicle size at ATI NMJs, as the enhanced postsynaptic glutamate receptor levels observed are sufficient to explain the increased quantal size (Fig. 3). ”
I concur with the authors. I would like to suggest that they add EM studies to complement the studies, if they are already available.
2) Line 385 “Although the ATI model does not appear to exhibit the features described at aging mammalian NMJs”
It is not clear whether the authors believe the arrested larval NMJ is or not a model for aging studies. This need some additional elaboration.

First revision

Author response to reviewers' comments

RESPONSE TO REVIEWERS

We thank the three Reviewers for their time and efforts in providing constructive comments for our manuscript. We are encouraged by the overall positive assessments from all three Reviewers and appreciate the valid and helpful suggestions raised to improve the clarity and impact of our study. The central comments revolved around **1)** examining larval movement across the lifespan of ATI larvae, and **2)** characterizing synaptic vesicle size using electron microscopy to determine whether changes in vesicle size contributes to the adaptive plasticity we describe. We have focused on addressing these and other outstanding issues in this revised manuscript.

First, we have performed a new and extensive analysis of synaptic vesicle size across ATI NMJs using EM. This has found no significant differences in the mean synaptic vesicle diameter between the WT and ATI NMJs; this data is now presented an an entirely new Figure 4. In addition, we have performed a larval locomotion assay and have included this data below for the Reviewers to assess.

Finally, we have provided extensive additional controls, analyses, and textual revisions to improve the clarity as suggested by the Reviewers. These results and new data are included in additions to Figures 1, 2, and 4 as well as Supplemental Figure S1.

Together, these efforts have greatly improved the manuscript, and we hope the Reviewers now deem this revision to be appropriate for publication in *Development*.

RESPONSE TO REVIEWER 1

Reviewer 1 states: Previous to this paper, it has been impossible to study synaptic homeostasis over long term periods of time in the Drosophila NMJ, a well-characterized model for synaptic plasticity. This study manipulates pupation-inducing insulin and ecdysone production by RNAi knockdown of smox (dSmad2) to extend the 3rd instar larval stage to over 30 days whereas wild type larva pupate at 5 days AEL. This allows the authors to study how the synapse changes over longer periods of time and with changes in size. Stable neurotransmission is maintained throughout the extended lifespan through a reduction in presynaptic neurotransmitter release despite growth of synapses-apparent using both presynaptic and postsynaptic markers. This technique will also allow researchers in the field to examine other modifications to synapses over longer periods of time.

The paper is written well, such that it is easy to follow.

1. ***I have two concerns with the work. The first is that the reduction of dSmad2 (Smox) is not well-characterized or referenced in this paper. Smox RNAi could impact far more than the two targets (insulin and ecdysone) the authors are aiming to affect. For example, while the muscle doesn't look different in size, it looks qualitatively different in the ATI5 vs WT5 in Figure 1. Maybe other possible impacts of smoxKD are not important for this study, but the possibility that there could be some impacts outside of what the authors reveal should be acknowledged. The paper reads almost as though we are looking at WT animals with an extended larval period, but clearly smox is an important transcription factor and has other targets. Simple text changes would address this concern. Please include references to what is known and acknowledge what is not known about how smox affects a synapse and potentially muscle.***

We thank the Reviewer for highlighting the significance of this study, and understand the points raised regarding what is known about Smox-RNAi and possible non-specific effects. We would like to make two points in response to these concerns. First, it is important to emphasize that our ATI manipulation only targets knock down of Smox to the prothoracic gland using *phantom-Gal4* (*phm-Gal4*; crossed to *UAS-Smox-RNAi*). Thus, while we cannot rule out possible secondary effects of Smox-RNAi within the prothoracic gland, the ATI manipulation should not impact Smox or any other factors in tissues outside of the prothoracic gland. We have now clarified this point in the revised manuscript (**lines 152-159**).

Second, it is important to note that the stable synaptic strength in extended larval stages we report in this manuscript does not rely exclusively on Smox knock down in the prothoracic gland. We have also found similar properties of prolonged larval growth in extended third instars (ETI) at 5 days AEL and for up to 9 days AEL using Torso-RNAi crossed to *phm-Gal4* as extended third instars (Miller et al., *J. Neurosci* 2012). We have found no significant differences in NMJ structure or function between WT.5, ETI.5, ATI.5, nor between ETI.9 and ATI.9. Furthermore, during the initial stages of this study, we used three independent RNAi lines targeting different genes in the prothoracic gland but that all led to arrested third instars (generiously provided by Naori Yamanaka at UC Riverside, who was involved in the original ATI screens in Mike O'Connor's lab at U of Minnesota). We chose Smox because it led to the most robust arrest, but similar phenotypes were observed in these other genes knocked down in the PG that induce ATI (Danielson et al., 2016). This was obliquely discussed in the original manuscript, and we have added more details to underscore the point that independent manipulations that arrest the larval stage exhibit similar NMJ phenotypes (**lines 159-162)**.

2. ***The second concern is that this paper does not take into account any potential changes in behavior between ATI animals and WT. For example, differences in movement behavior (muscle use) likely impact synaptic homeostasis. The paper could be strengthened with an assessment of larval movement behavior in smoxKD vs WT at a similar age and then also smoxKD over time (day 5 vs. d17 and d33 for example). This would address if muscle/body size vs. muscle use impacts synaptic strength at the NMJ.***

We understand the Reviewer's point that examining larval movement behavior may be of interest. As suggested by the reviewer, we have assessed larval movement using a larval mobility and locomotion assay (as described in Batlevi et al., 2010). In this approach, single wandering third-instar larvae are placed on a plain 1% agarose plate (100 mm) above 5 mm grid paper. The number of gridlines crossed within two minutes are scored for at least 10 animals per genotype. Larvae are allowed to acclimate on the plate briefly before the assay. We find similar movement between WT.5 and ATI.5 larvae, as expected, but a gradual reduction in mobility across the ATI lifespan, with ATI.33 larvae barely moving at all. We have provided a Reviewer Figure below for consideration (see Reviewer Figure 1). This data can be included as a supplementary figure in the manuscript if the reviewers insist.

NOTE*: We have removed unpublished data that had been provided for the referees in confidence.*

However, we have concerns about whether these results are interpretable, and in fact may be misleading to readers. Larval locomotion is not simply the result of the NMJ synaptic physiology that we have investigated in this manuscript. There are a variety of complex factors that contribute to movement (metabolism, hormonal changes, pre-motor activity, central pattern generators, etc), any or all of which could have changed as the ATI larvae adapted to the new developmental state. It is poorly understood how these factors are integrated and contribute to larval locomotion and NMJ circuit plasticity. It is therefore difficult to interpret, or even speculate, about the cause and effect of reduced movement on NMJ structure/function in ATI larvae. Indeed, the sluggishness observed in later stages of ATI larvae may not even be an accurate reflection of muscle use. For example, the body wall muscles might actually be working harder than normal without being able to move the bloated larval body very successfully. If muscle use or strength were to be accurately determined, an assay other than locomotion would be necessary (i.e. direct measurement of contraction strength in response to depolarization).

Together, we fear that presenting this locomotor data without being able to properly interpret what it means may be misleading to readers. We therefore respectfully propose not to include this behavioral data in the final manuscript.

Response to Minor Comments

1. ***Figure 1E and F-- please add labels (WT5ATI5, 17, and 33) to the EPSP traces and to the x axis of the bar graph. The matching colors help, but labels clarify and there is ample space for them. Similarly, add labels to supplemental figure 4-1.***

We agree and thank the Reviewer for the suggestion. We have now added the labels to Figures 1E, 1F, 3E-G, as well as to Supplemental Figure S1.

2. ***I would also appreciate an N.S. or an * to indicate significant change from WT at day5 in Figure 2B, C, and D. I realize that the significance is indicated in the supplement, but it is a bit of a pain to search for that as a reader.***

We agree and have now added significance labels to Figure 2B, 2C, and 2D.

RESPONSE TO REVIEWER 2

Reviewer 2 states: This is an interesting study looking at the effect of arresting larval development on the stability of neuromuscular function in the third instar larvae. The authors use a genetic manipulation that ablates the production of ecdysone, which is required to transition from the larval to the pupal stage of development. Under these unique conditions, development is arrested at the larval stage for an extended period (~30 days) but the larvae continue to grow resulting in a larger than normal larvae prior to death. The continued growth of the muscle allowed the authors to investigate how the function of the neuromuscular function changes in the face of this abnormal larval growth program. The authors report that during this arrested development, neurotransmission at the NMJ is maintained stable despite the changes in the size of the muscle. Further analysis demonstrates that the size of the response of the muscle to the release of a single vesicle of neurotransmitter, referred to as quantal size, increases during the period and the synapse reduces the total number of vesicles released (a.k.a. quantal content) to compensate referred to as presynaptic homeostatic depression (PHD), a form of plasticity that these authors have previously studied. Although this is an interesting observation, the results are somewhat preliminary and the normal context for this plasticity isn't clear. Importantly for this review, it is unclear, as presented, how these results inform the reader about normal developmental processes related to the development of the nervous system. At this point, the paper requires substantial revisions before it is appropriate for publication in Development.

1. ***The authors show that quantal size increases during ATI and state that this is due to increased GluR function based solely on immunocytochemistry. Although the authors show an increase in GluR staining, this analysis does not rule out a presynaptic contribution to Q (i.e. synaptic vesicle size). Because previous studies have shown that increased post-synaptic sensitivity to neurotransmitter via manipulation of GluR abundance have been shown to increase both mEJPs and EJPs (for example see DiAntonio et al., 1998), in contrast to what is shown during ATI, it is important to rule out a presynaptic contribution to quantal size. The authors should address vesicle size during ATI using EM. Although this is a difficult analysis, it is key to understanding the contribution of post-synaptic glutamatergic signaling to the reduction in quantal content observed during ATI. This is especially important as determining if there is a transynaptic component to the signaling required for this form of PHD.***

We agree that measuring synaptic vesicle size by EM would be of interest. As the Reviewer notes, there is precedence for increases in vesicle size increasing quantal size. However, the only known mechanism for increasing vesicle size requires either overexpression of the vesicular glutamate transporter or defects in synaptic vesicle endocytosis (Daniels et al., 2004; Dickman et al., 2005; Goel et al., 2019a; Verstreken et al., 2002), and there is no evidence for either of these processes at ATI NMJs. More importantly, the observed increase in GluR abundance assessed by immunostaining is sufficient, in principle, to fully explain the enhanced quantal size, so we saw no compelling reason to invoke additional factors that may serve to enhance quantal size. Finally, an increase in synaptic vesicle size would only compound the factors that appear to enhance synaptic strength at ATI NMJs, and would not serve to explain why, instead, synaptic strength remains stable.

Given the reasons stated above, this Reviewer may have been suggesting that, in fact, synaptic vesicle size *decreased* to perhaps compensate for NMJ growth and/or GluR abundance? This would indeed be interesting, although this would not only be unexpected from the ATI extended third instar stage but would also be unprecedented, as there are no known examples of vesicle size being

reduced at the fly NMJ.

Nevertheless, we have now performed a full EM characterization of WT.5, ATI.5, ATI.17, and ATI.25 NMJs. Our blinded measurements revealed no significant difference in synaptic vesicle diameter between NMJs of WT and any of the ATI time points analyzed. This data is now presented in an entirely new **Figure 4** along with significant revisions to the text (**lines 257-263**). We the thank this reviewer for encouraging this ultrastructural analysis, which has improved our study.

2. ***The authors show that synaptic growth continues including Brp puncta and conclude that the number of active zones that participate in release (N) is increasing. It is already established at this synapse that not all active zones participate in release so the characterization of the number of release sites using immuncytochemisty is inaccurate. To truly determine the number of active zones participating in release the authors should utilize genetically encoded calcium indicators that allow the resolution of participating active zones during release under these novel developmental conditions. This approach will also allow the direct measuring of the probability of release at each release site.***

We apologize for this point not being clearly presented in the original manuscript. The Reviewer is certainly correct that not all release sites participate in neurotransmission at the NMJ, and we obviously have not performed the experiments necessary to determine the fraction and properties of which release sites participate in transmission at ATI NMJs and compare this to wild type NMJs. To do so would require quantal imaging at NMJs, as the reviewer noted, correlated with individual release sites as well as additional electrophysiological experiments such as mean/variance analysis. While this information would certainly be of interest, an entire manuscript dedicated to these questions would be required and may be the subject of a future study.

However, we would like to respectfully underscore that the question of how many active zones participate in transmission is not of central interest to the current manuscript. Rather, the motivation and interpretation for examining the anatomical number and intensities of active zones in the current manuscript is focued on two points. First, a reduction in the anatomical number of active zones could, in principle, contribute to the homeostatic reduction in quantal content observed at ATI NMJs. For example, while presynaptic growth and bouton numbers increase at ATI NMJs, perhaps the total number of anatomical active zones do not change compared to WT.5. Indeed, there is evidence for such homeostatic adapations in anatomical active zone density (Graf et al., *Neuron* 2009; Goel et al., *J. Neurosci*, 2019a). However, we observe a concommicant increase in Brp puncta number with increasing bouton number (Figure 2). Our immunostaining and quantification of Brp puncta therefore rules this possibility out. This point is now more clearly made in the revised manuscript (**lines 207-217**).

Second, reductions in active zone size and/or intensity have recently been shown to homeostatically compensate for synaptic overgrowth and stabilize transmission at non-ATI NMJs (Goel et al., *J. Neurosci,* 2019a; Goel et al., *JCB*, 2019b). Thus, we considered whether a reduction in Brp puncta size or intensity may have occurred at ATI NMJs that similarly served to compensate for synaptic overgrowth. However, we found that Brp puncta size and intensity increased at ATI NMJs along with growth, consistent with recent work suggesting that the age of active zones determines their relative size and intensity (and, in the case of neurotransmission, P_r; Akbergenova et al., 2018). We have now made these points clear in the revised manuscript and are careful not to imply any statements about functional release changes at ATI active zones based on immunostaining. These revisions are presented in **lines 218-227** in the revised manuscript.

Response to Minor Points

1. ***Error bars are missing from Figure 2.***

We apologize for not including error labels in Figure 2 - they were presented in the Supplemental Table S1 so as not to overly complicate the graphs in Figures 2B-2D. Reviewer 1 shared a similar comment. We have now included error labels in Figure 2 as suggested.

2. ***From the imaging data presented it looks like there is no change in GluR subunit stoichiometry. This would be supported by some wave-form analysis of the mEPSPs such as***

decay or half-width. This analysis would also potentially support the contention that the change in quantal size is solely a post-synaptic event.

This is a very astute point and we fully agree. We have now analyzed mEPSP rise and decay time constants in WT and ATI NMJs. This analysis shows similar decay time constants for mEPSPs between WT and ATI NMJs. As the Reviewer notes, this is consistent with the immunostaining suggesting similar enhancements and relative shoiciometry of GluRIIA- and GluRIIB-containing GluRs. This data is now presented in a new panel of Figure 3 (Figure 3G) and discussed on **lines 253-254** in the revised manuscript. We thank the reviewer for suggesting this additional analysis.

3. ***The effects of prolonged larval development on muscle integrity and function are not investigated. Isn't it possible that muscle contraction/function could also feed into the regulation of neurotransmission at the NMJ during ATI? Are there changes in fiber number, muscle nuclei, sarcomeres, etc. Related to this, what happens to larval mobility/crawling during ATI?***

Some of these questions were also raised by Reviewer 1. First, there are indeed changes to the passive properties of ATI muscles, presented in Figure 1D and in the Supplemental Table S1. We have not observed any other major changes in muscle nuclei or muscle segments throughout our analysis. However, we have assessed larval movement as detailed in our response to Reviewer 1 above, which demonstrates a reduction in movement as the ATI lifespan progresses (Reviewer Figure 1). The reason(s) and impacts of this are unclear, as discussed in our response to Reviewer 1.

4. ***Since satellite cells have not been observed in the Drosophila muscle, it might be expected that the number of fibers might diminish during ATI due to use-related damage. Would this change the interpretation of the data?***

This is an interesting point. We searched for any evidence of age-related dimishment of NMJ integrity and muscle function (Figure 6). Beyond what we have presented in the manuscript and Supplemental data, we do not find any evidence for reductions in the number of muscle fibers across the ATI lifespan, at least at the segments we have studied (abdominal segments A2, A3, A4, and A5; muscles 6,7,12, 13, and 4).

5. ***Line 294 is missing "that".***

Thank you for catching this typo. We have made the correction.

RESPONSE TO REVIEWER 3

Reviewer 3 states: This is a very interesting and rigorous manuscript that describes a novel genetic strategy that extends the experimental utility of the Drosophila larval NMJ. The approach rest on stalling larval development at the third instar stage for up to a staggering ~30 days. This is achieved by selectively abrogating the expression of transcription factors necessary for ecdysone secretion from the prothoracic gland. The fundamental and novel observation is that despite the great increase in synapse boutons that exceed the growth of the innervated muscle, the evoked neurotransmission remains steady. The authors explore the electrophysiological mechanisms of this adaptive response that maintains evoked neurotransmission to hone in on a novel presynaptic depression mechanism. This process is expressed in the form of decrease probability of release as indicated by failure analysis. The paper is carefully executed, discussed, and conclusions are well supported by findings. While the molecular mechanisms of the new form of presynaptic adaptive depression are not explored, this paper provides a solid base where to begin. Arrested larval development preparation plus the new form of adaptive presynaptic depression are of sufficient novelty and excitement. However, the authors add another aspect that creates both additional excitement yet at the cost of a change in the focus of the paper. The authors realize that this novel arrested preparation offers a ground for additional experimentation assessing the impact of genetic manipulations that induce neuropathology. This is explored with RNAi targeting stathmin.

1. ***The new stathmin data are quite exciting but are lost in the context of the title and focus of the paper. There is a clear asymmetry between the efforts to identify the mechanisms that account for presynaptic depression versus those devoted to explore the usefulness of the preparation to study neuropathology.***

I would like to suggest two avenues to correct this: 1) the title should be more encompassing of the stathmin findings. 2) There could be additional efforts to expand on the experimental efforts to establish the arrested NMJ as a sensitive and quantitative model for neuropathology. For example, the polyglutamine (polyQ) stretch within the Huntingtin (Htt) protein produces pathology in a manner sensitive to the length of the stretch at the NMJ (DOI: https://doi.org/10.1523/JNEUROSCI.2752-16.2017). Since there are already transgenes that encompass different length of the polyQ repeat, the penetrance of phenotypes could be explored in the arrested NMJ preparation. I will leave to the authors to decide the extent to consider this second option.

We thank this Reviewer for finding our manuscript of significant interest, importance, and rigor. We agree that there is an imbalance between the space devoted to NMJ plasticity relative to the *stathmin* data. Our intent in this study was to **1)** define NMJ structure and function in the context of the ATI lifespan; **2)** characterize any plasticity that is induced by the ATI manipulation; and **3)** demonstrate the utility of this system for studies of neurodegeneration. Points 1 and 2 necessarily received the majority of the focus of the current manuscript, while point 3 established a foundation for future studies to probe NMJs over longer time scales in mutations of interest, which includes that of the Htt protein. In fact, we are currently examining NMJ function and degeneration not only in the polyQ Htt transgenes referenced by this Reviewer, but also in Drosophila models of other neurodegenerative conditions, including SMA (fly SMN1 alleles), ALS (SOD1, C9orf73, GR dipeptide repeats), and Wallerian/Sarm1-mediated degeneration. These studies are at preliminary stages and require more time than the 90 days suggested for the revision of this manuscript. We hope to dedicate an entirely new manuscript to the study of other models of neurodegeneration in the ATI manipulation in a future manuscript, with the analysis of *stathmin* mutants in the ATI background establishing the foundation.

In terms of revising the title, we agree that incorporating the stathmin findings would be beneficial. We have now changed the title to "Developmental arrest of *Drosophila* larvae elicits presynaptic depression and enables prolonged studies of neurodegeneration". We thank this Reviewer for encouraging the change in title.

2. ***Line 367 "In contrast, there is no evidence for changes in synaptic vesicle size at ATI NMJs, as the enhanced postsynaptic glutamate receptor levels observed are sufficient to explain the increased quantal size (Fig. 3). "***

I concur with the authors. I would like to suggest that they add EM studies to complement the studies, if they are already available.

We appreciate this point made by Reviewer 3, which was shared by Reviewer 2. EM studies would indeed greatly complement the manuscript. We have now included a characterization of synaptic vesicle size using EM at NMJs of WT.5, ATI.5, ATI.17, and ATI.25. These findings are detailed in the Response to Reviewer 2 Point 1 above and as an entirely new **Figure 4** in the revised manuscript.

3. ***Line 385 "Although the ATI model does not appear to exhibit the features described at aging mammalian NMJs".***

It is not clear whether the authors believe the arrested larval NMJ is or is not a model for aging studies. This needs some additional elaboration.

We apologize this statement was not more clear. As described also in our Response to Reviewer 2 Point 4 above, we spent considerable time searching for hallmarks of NMJ aging, including denervation, retractions/footprints, fragmentation, and presynaptic destabilization (by analyzing the number of boutons with Futch filaments). We were surprised at how robust NMJ structure remained, even at terminal ATI.33 stages, where we were unable to find significant signs of NMJ

aging. We have elaborated on this in the revised manuscript (**lines 308-325**) to more clearly make the point that ATI NMJs do not exhibit classical features of an aging neuromuscular junction.

Second decision letter

MS ID#: DEVELOP/2019/186312

MS TITLE: Developmental arrest of Drosophila larvae elicits presynaptic homeostatic depression and enables prolonged studies of neurodegeneration

AUTHORS: Dion Dickman, Nancy L Tran, barry ganetzky, Daniel Miller, Cristian Pinales, Christopher Buser, Sarah Perry, and Pragya Goel

I have now received the reports of two of the three referees who reviewed the earlier version of your manuscript and I 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.

The reviewers' evaluation is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestion of referee 2. Please attend to this comment in your revised manuscript and in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Previous to this paper, it has been impossible to study synaptic homeostasis over long term periods of time in the Drosophila NMJ, a well-characterized model for synaptic plasticity. This study manipulates pupation-inducing insulin and ecdysone production by RNAi knockdown of smox (dSmad2) to extend the 3rd instar larval stage to over 30 days whereas wild type larva pupate at 5 days AEL. This allows the authors to study how the synapse changes over longer periods of time and with changes in size. Stable neurotransmission is maintained throughout the extended lifespan through a reduction in presynaptic neurotransmitter release despite growth of synapses-apparent using both presynaptic and postsynaptic markers. This technique will also allow researchers in the field to examine other modifications to synapses over longer periods of time.

Comments for the author

This revision satisfies all of my concerns. The work will substantially contribute to the field and is appropriate for publication in Development.

Reviewer 2

Advance summary and potential significance to field

I believe that the authors have addressed my concerns and that the manuscript is now ready for publication in Development. I have only one minor critique (see below)

Comments for the author

Minor critique: for the new EM analysis, I'm assuming that the n refers to micrographs analyzed. Please include the number of animals per condition as well.

Second revision

Author response to reviewers' comments

RESPONSE TO REVIEWERS

We thank the Reviewers for finding our revised manuscript of significant interest and to be appropriate for publication in Development.

RESPONSE TO REVIEWER 1

Reviewer 1 states: Reviewer 1 Advance summary and potential significance to field
Previous to this paper, it has been impossible to study synaptic homeostasis over long term periods of time in the Drosophila NMJ, a well-characterized model for synaptic plasticity. This study manipulates pupation-inducing insulin and ecdysone production by RNAi knockdown of smox (dSmad2) to extend the 3rd instar larval stage to over 30 days whereas wild type larva pupate at 5 days AEL. This allows the authors to study how the synapse changes over longer periods of time and with changes in size. Stable neurotransmission is maintained throughout the extended lifespan through a reduction in presynaptic neurotransmitter release despite growth of synapses-apparent using both presynaptic and postsynaptic markers. This technique will also allow researchers in the field to examine other modifications to synapses over longer periods of time.

Reviewer 1 Comments for the author
This revision satisfies all of my concerns. The work will substantially contribute to the field and is appropriate for publication in Development.

We thank the Reviewer for highlighting the significance of this study and judging the manuscript now appropriate for publication in Development.

RESPONSE TO REVIEWER 2

Reviewer 2 states: Reviewer 2 Advance summary and potential significance to field
I believe that the authors have addressed my concerns and that the manuscript is now ready for publication in Development. I have only one minor critique (see below).

We thank the reviewer for the constructive comments which has improved the final manuscript.

Response to Minor Point

1. Reviewer 2 Comments for the author Minor critique: for the new EM analysis, I'm assuming that the n refers to micrographs analyzed. Please include the number of animals per condition as well.

We apologize for not including the number of animals per condition for the EM experiments in Figure 4. We have now included this information in the Figure 4 legend – all samples were obtained from three animals for each data set.

Third decision letter

MS ID#: DEVELOP/2019/186312

MS TITLE: Developmental arrest of Drosophila larvae elicits presynaptic homeostatic depression and enables prolonged studies of neurodegeneration

AUTHORS: Dion Dickman, Nancy L Tran, barry ganetzky, Daniel Miller, Cristian Pinales, Christopher Buser, Sarah Perry, and Pragya Goel
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.