Calcium waves facilitate and coordinate the contraction of endfeet actin stress fibers in *Drosophila* interommatidial cells

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MS ID#: DEVELOP/2021/199700

MS TITLE: Calcium waves facilitate and coordinate the contraction of endfeet actin stress fibers in Drosophila interommatidial cells

AUTHORS: Henry Chiu-Yu Chang and Don F Ready

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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.

Reviewer 1

Advance summary and potential significance to field

During development, contractile forces must be coordinated across tissues to elicit morphological changes.

One mechanism that regulates contractile force is the second messenger, Ca2+, which is known to regulate the cytoskeleton and a wide number of other processes (Paudel et al., Int. J. Mol. Sci. 2018; Berridge et al., 2003). While we know mechanisms by which Ca2+ regulates both conventional and unconventional myosin (Heissler and Sellers, JMB, 2016), how this is coordinated across tissues in distinct contexts is often unknown.

In this manuscript, the authors examine the coordination of Ca2+ waves across the Drosophila pupal ommatidial epithelium. The authors characterize Ca2+ waves that propagate across the eye tissue. They show that these waves require IP3 Receptor, but not phototransduction. Using an IP3R mutant they show that IP3 Receptor is required for stress fiber organization and contraction, as well as a defect in basement membrane organization/levels.

Comments for the author

Major comments:
1) Ca2+ regulates many processes, which includes, but is not limited to the cytoskeleton (Paudel et al., 2018; Berridge et al., 2003). For this reason I think it would strengthen the paper to make the most direct connection between Ca2+ release and stress fiber behavior. For example: Do stress fibers contract in a wave? What is spatial/temporal correlation between Ca2+ release and stress fiber contraction? How many Ca2+ waves does it take to fully contract stress fibers?

2) There is both a defect in cytoskeletal organization and basement membrane deposition, making it difficult to interpret which causes the other. The authors conclude that “tensile forces from the stress fibers shape the basement membrane in connection with endfeet contraction.” But, one could also interpret this result as basement membrane defects causing defects in stress fiber organization.

Minor
1) Ca2+ ‘spike’ and ‘wave’ are used interchangeably. I suggest using consistent terminology.

2) A main conclusion of the paper is that Ca2+ regulates stress fiber organization/contraction. However this data is qualitative (e.g. Fig. 7-9). I suggest that the authors quantify stress fiber length and phospho-myosin levels to compare IP3R-/- and wild-type.

3) Statistical comparisons were only done for one of the graphs in the paper (Fig. 7F). Recommend doing statistical comparisons for other analyses where statements of difference/similarity are being made.

4) Why is the phospho-myosin not in stress fibers? I realize it is a projection, can you see phospho-myosin in proximal sections?

Reviewer 2

Advance summary and potential significance to field

This manuscript reports a novel developmental phenomenon: Retinal calcium waves in interommatidial cells (IOCs) during pupal development and adult stages in Drosophila. Using a genetically encoded calcium sensor, the authors describe calcium waves occurring in non-neuronal retinal cells, including primary secondary and tertiary pigment cells, but not in cone cells. They also show that the waves are independent of the phototransduction process and are thus spontaneous. They further determine that the IP3 receptor (IP3R) is cell-autonomously required for the waves, suggesting that calcium activity comes from ER calcium storage. They also report that IP3R mutant clones have defective retinal structure, observed as problems in the basal endfeet of IOCs. The defects are consistent with the defective contraction of endfeet stress fibers. Finally they suggest a model that the calcium waves are required for the structural development of the fly eye via the coordinated activity of the calmodulin-dependent myosin light chain kinase (MLCK) pathway.

As a summary, the manuscript reports the discovery and characterization of a novel and exciting developmental phenomenon - retinal calcium waves. While correlated calcium activity in developing tissue has been found in multiple systems including recently in the fly optic lobe by the Zipursky lab, this is still a significant finding in an important model system, the developing fly eye that was pioneered by the author many year ago and represents a very powerful system to understand the mechanisms of this phenomenon. The experiments are well designed and performed with proper control.

Comments for the author

There are concerns that should be addressed as follows.

Major concerns.

- While the data clearly support the model that IP3R is required for the retinal structure, the causality between IOC calcium waves and actin cytoskeletal is not well demonstrated by the current data. Regardless of the insignificant role of IP3R in the phototransduction cascade and for cell specification in the developing fly retina, the IP3R (and ER calcium storage/release) have myriads of significant cellular and developmental roles. However, the author seems to claim that the developmental phenotype of IP3R mutants is entirely mediated by IOC calcium waves.
To address this point, the authors should show that a similar phenotype is observed when the retinal waves are inhibited in an IP3R- (and ER release machinery)-independent way. For example, in the discussion, the authors suggest inx2 as a related mechanism. If this were to be the case, inx2 mutants should have a related retinal phenotype.
- The authors use only a single condition of IP3R mutants that removes IP3R function throughout the entire developmental time of IOCs. However, IOC calcium waves specifically occur in a narrow time window of a later developmental stage.
Thus, genetic manipulations with better temporal controls such as temperature sensitive Gal80 would support the argument that the calcium waves are required for retinal development and for the phenotypes described.

Minor points.
- The authors emphasize the 'coordinative role of the wave' throughout the manuscript and the title, but this is an overstatement. The reported data depend solely on IP3R receptor mutants and all reported phenotypes are cell-autonomous.
This does not support a correlative function. While the waves themselves are a coordinated phenomenon, they might be required for cell-autonomous calcium activity in the context of retinal structural development. This is very important and rigorously discussed issue in the studies of mammalian retinal waves (i.e. permissive vs. instructive role of activity), and the authors should not claim the correlative function without any direct evidence.
- LongGMR-Gal4 should be expressed in every retinal cell, including photoreceptors. This means that the reported waves in IOCs should overlap with the recently reported PSINA in photoreceptors (Akin et al. 2019) at 55%. This strongly suggests that photoreceptors should also exhibit activity during these experiments. However, the authors do not mention photoreceptor activity while they describe all other retinal cell types.
- Does the surge of the calcium signal observed in the control eye after 30sec of illumination disappear in norpA mutant eyes?

Reviewer 3

Advance summary and potential significance to field

New features and potential roles of Ca2+ signaling related to epithelial morphogenesis and mechanics are described in the manuscript.

In the manuscript, “Calcium waves facilitate and coordinate the contraction of endfeet actin stress fibers in Drosophila interommatidial cells,” the authors provide support for a role of IP3R in coordinating the contraction of actin stress fibers in the endfeet of interommatidial cells (IOCs) in

the Drosophila retinal epithelium. The authors have also performed a systematic and quantitative characterization of dramatic Ca2+ wave activity in Drosophila eyes.

Key findings include:
- IOC Ca2+ waves do not depend on phototransduction but require IP3R
- IP3R removal leads to defects in the stress fibers in IOC endfeet and leads to an increased basal retinal surface. Loss of IP3R disrupts the collagen IV basal network.
- Of note in the report are findings of persistent waves (not associated with phototransduction) into the adult stages and a connection of calcium dynamics to the phenotypes of IP3R to endfoot contraction.

Comments for the author

There are a few concerns regarding the conclusions and interpretations drawn from the study:
pMyosin phenotype noted in the ip3r knockdown experiments could also be due to the interactions between IP3R and Myosin and not just due to Ca2+ regulation of myosin. No specific evidence or cross-correlation analysis definitely shows that it is the Ca2+ (as opposed to IP3R – myosin binding activity such as Walker Denise S., Sung Ly, Katherine C. Lockwood, and Howard A. Baylis. Current Biology 12, no. 11 (2002): 951-956) that is important for the phenotype.
It is unclear why upstream stimuli of PLC21C or Galpha q, which are active in other epithelial systems and important for stimulating Ca2+ release via IP3R was not characterized to confirm the phenotype analysis. Does inhibition of Plc21C or Galphaq impact pMyosin in the endfeet?
It is strange that experiments that perturb GJ were not performed to verify whether coordination of Ca2+ (and other second messengers) strictly require gap junctions or not. If the intercellular calcium dynamics itself are important for regulation and contraction of actin stress fibers, why is the myosin and collagen phenotypes largely clone autonomous? Further discussion of this result seems warranted.
Is the late stages of Ca2+ transients required to maintain pMyo in adult eyes?

Specific comments:
There were no methods associated with imaging the larval eye disc. These are interesting but impact on pMyosin in the larval stages could be more carefully described. Is there any discernible cross-correlation between Ca2+ activity and Myosin dynamics seen?
The cells mentioned in legends were not adequately marked in several figures
p. 16 It is not clear what is meant by “their profiles”
p. 23 shape tissue(s) or shape a tissue
Figure 1 : Include scale bars Fig. 1C Numbers 2, 3, 2, in C not described in caption. Should it be 2 deg, etc.
as in B? The slices in C should be marked in B more clearly.
The SI figure showing the cross-sectional view was helpful and seemed to be important to include in Figure 1.

Fig. 2: Not clear what is C23, C24, D12, etc. Cell labels in A are not clear.
Fig. 2B appears somewhat confusing.
Fig. 3B E3p, E4a, etc are not clearly marked in A
It is not clear which cells correspond to cell numbers in legends in subsequent plots either.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the author

Major comments:
1)Ca2+ regulates many processes, which includes, but is not limited to the cytoskeleton (Paudel et al., 2018; Berridge et al., 2003). For this reason I think it would strengthen the paper to make the most direct connection between Ca2+ release and stress fiber behavior. For example: Do stress

fibers contract in a wave? What is spatial/temporal correlation between Ca2+ release and stress fiber contraction? How many Ca2+ waves does it take to fully contract stress fibers?

To see the stress fiber contractions in real time, we've tried imaging zipper-GFP in vivo with 2-photon microscopy but find the laser power required to visualize the floor creates photodamage. In addition, the movements in adult IOC endfeet will likely be small since the ridge network resists contraction.

To establish a direct functional link between Ca2+ increase and floor reduction, we've also tried rescuing the stress fiber defects in IP3R- eyes with heat pulses to LGMR > TRPA1. However, in pilot experiments, we find the TRPA1 response is slow relative to IOC Ca2+ spikes and we cannot replicate the dynamics.

2)There is both a defect in cytoskeletal organization and basement membrane deposition, making it difficult to interpret which causes the other. The authors conclude that “tensile forces from the stress fibers shape the basement membrane in connection with endfeet contraction.” But, one could also interpret this result as basement membrane defects causing defects in stress fiber organization.

To test whether IP3R affects vkg synthesis, we have performed Western on head lysates from IP3R- eyes (generated by EGUF; FRT82B, GMR-hid) showing vkg::GFP level is not affected. This result is shown in Supplemental Fig 5 of the revised manuscript. While this observation doesn’t exclude the possibilities of IP3R directly regulating ECM organization through remodeling or other post-translational modifications, it suggests the basement membrane defect in IP3R clones does not depend on vkg synthesis.

Formally, it is possible that vkg organization is the lead actor with stress fiber contraction following in tow, but we view this as a cooperative synergism of active actomyosin force building counterforce into the ECM. The interplay of tension and stress fiber organization is a large and active area of molecular cell biology and in-depth, quantitative analysis of tension and structure in the eye will require development of methods well beyond this initial report.

Minor
1)Ca2+ ‘spike’ and ‘wave’ are used interchangeably. I suggest using consistent terminology.

We thank reviewer for the suggestion and have modified the text to be more consistent with our word usage. We have adhered to the usage of “waves” when referring to intercellular propagation of Ca2+ increases. We retained the usage of “spikes” when referring to Ca2+ increases in a given cell.

2)A main conclusion of the paper is that Ca2+ regulates stress fiber organization/contraction. However, this data is qualitative (e.g. Fig. 7-9). I suggest that the authors quantify stress fiber length and phospho-myosin levels to compare IP3R-/- and wild-type.

While our images of stress fibers are qualitative, we are confident with the conclusion that stress fiber morphology/organization is disrupted in IP3R- cells (we performed this comparison of mutant and wild type phenotypes with side-by-side clones in mosaic eyes). During our analysis, we have noticed that the severity of stress fiber defects is influenced by the clone size and clone location, indicating that a meaningful quantitative analysis needs to take into account of additional parameters. Furthermore, as the retina is not flat, a measurement of stress fiber length needs to adjust for those obliquely imaged. Thus, our goal in this initial report is outline the broad strokes of the phenomenon. We will increase the quantitative nature of our analysis as we improve the mastery of these assays.

As for phosphor-myosin, we have compared the mean intensities of p-MLC staining in wild type and IP3R- tissues with ImageJ. The staining intensity is decreased by 37% in IP3R- clones, and this result is included in revised Fig 9.

3)Statistical comparisons were only done for one of the graphs in the paper (Fig. 7F). Recommend doing statistical comparisons for other analyses where statements of difference/similarity are being made.

We have quantified two parameters: floor area (Fig 7F) and grommet size (Fig 8E). In the original text, the statistical analysis for grommet size was only mentioned in the Legend (p value is 0.003). We have modified the Fig 8E panel to make this point more obvious.

4)Why is the phospho-myosin not in stress fibers? I realize it is a projection, can you see phospho-myosin in proximal sections?

Phospho-myosin does overlap with stress fibers. Phalloidin staining of this image was not included to better illustrate the p-MRLC intensity difference between IP3R and wild type clones.

Reviewer 2 Comments for the author
There are concerns that should be addressed as follows.

Major concerns.
- While the data clearly support the model that IP3R is required for the retinal structure, the causality between IOC calcium waves and actin cytoskeletal is not well demonstrated by the current data. Regardless of the insignificant role of IP3R in the phototransduction cascade and for cell specification in the developing fly retina, the IP3R (and ER calcium storage/release) have myriads of significant cellular and developmental roles. However, the author seems to claim that the developmental phenotype of IP3R mutants is entirely mediated by IOC calcium waves. To address this point, the authors should show that a similar phenotype is observed when the retinal waves are inhibited in an IP3R- (and ER release machinery)-independent way. For example, in the discussion, the authors suggest inx2 as a related mechanism. If this were to be the case, inx2 mutants should have a related retinal phenotype.

We agree that a demonstration of causality between Ca2+ waves and actomyosin network would strengthen this paper. To this end, we've tried rescuing IP3R- eyes with heat pulses to LGMR > TRPA1 to establish a direct functional link between Ca2+ increase and stress fiber contraction. However, in pilot experiments, we find the TRPA1 response is slow relative to IOC Ca2+ spikes. To replicate the dynamics, we are exploring whether UAS-ChR2 might provide a better match.

As for Inx2, we have tried monitoring GCaMP6 activity in Inx2B mosaic eyes. Consistent with the previous report that Inx2 affects dpp signaling in determining eye size (Richard and Hoch, 2015), Inx2B mosaic eyes are noticeably smaller and rough. This disruption of eye architecture and patterning complicates the analysis and interpretation of Inx2's role in Ca2+ wave propagation.

- The authors use only a single condition of IP3R mutants that removes IP3R function throughout the entire developmental time of IOCs. However, IOC calcium waves specifically occur in a narrow time window of a later developmental stage. Thus, genetic manipulations with better temporal controls such as temperature sensitive Gal80 would support the argument that the calcium waves are required for retinal development and for the phenotypes described.

We thank the reviewer for the suggestion. Using Gal80ts with GAL4 should, in principle, allow us to pinpoint the temporal requirement for IP3R function with thermally controlled RNAi-mediated knockdown or rescue. However, these experiments are not feasible for the following technical reasons. For the knockdown, we obtained two Bloomington RNAi lines against IP3R (TRiP.JF01957 and TRiP.HMC03351) and found that JF01957 is more effective of the two (determined by IF intensity with the silencing of GMR > GFP::IP3R in the presence of Dcr2). Still, JF01957, when driven by lGMR-GAL4 in the presence of Dcr2, does not eliminate Ca2+ waves, most likely due to an incomplete removal of IP3R proteins (the IF showed high residual GFP::IP3R signal as well). This is expected since we choose a moderate driver for this work to avoid disrupting eye architecture. For the rescue, we obtained the UAS-ltpr.V line (donated by Gaiti Hasan) from Bloomington, which contains a X-linked insertion expressing an embryonic IP3R isoform. Our pilot experiment suggested that this line, when expressed with lGMR-GAL4, is not capable of rescuing. Given that these GAL4-dependent knockdown and rescue were unsuccessful, we did not pursue further with Gal80ts.

Minor points.
- The authors emphasize the 'coordinative role of the wave' throughout the manuscript and the title, but this is an overstatement. The reported data depend solely on IP3R receptor mutants and all reported phenotypes are cell-autonomous. This does not support a correlative function. While the waves themselves are a coordinated phenomenon, they might be required for cell-autonomous calcium activity in the context of retinal structural development. This is very important and rigorously discussed issue in the studies of mammalian retinal waves (i.e. permissive vs. instructive role of activity), and the authors should not claim the correlative function without any direct evidence.

A major challenge for late retinal morphogenesis is establishing the accurate pitch of the photoreceptor array across the eye; for optimal eye function, ommatidia must “point” in tightly specified directions, accurately, and smoothly, across the eye’s curvature. We propose waves effect this smooth distribution, sequentially and repetitively visiting neighborhoods and “tightening up” IOC feet in concert with the local mechanical environment. As tissue-wide events, waves are positioned to harmonize, or coordinate, the precise, smooth dome that establishes photoreceptor pitch across the entire eye.

Investigating whether waves are instructive or permissive will require an ability to experimentally manipulate waves and, noted elsewhere, our efforts to do so to date have been unsatisfying. So far, we find TRPA1 dynamics in response to temperature shift do not resemble endogenous waves. More sophisticated methods than are currently available to us will need to be developed for future experiments.

While it is well documented that correlated spontaneous activity in the developing vertebrate visual system contributes to the refinement of retinotopic maps, IOC calcium waves described here, commencing as non-synaptic cellular connectivity of a non-neuronal network is complete and expressed in IOC stress fiber contraction, would appear to be a substantially different biological process. As noted by Choi, Chen and Desplan (2021), PSINA in the developing Drosophila visual system may provide a closer parallel to the vertebrate instance and we have included a citation for readers wishing to pursue the topic in more depth.

- LongGMR-Gal4 should be expressed in every retinal cell, including photoreceptors. This means that the reported waves in IOCs should overlap with the recently reported PSINA in photoreceptors (Akin et al. 2019) at 55%. This strongly suggests that photoreceptors should also exhibit activity during these experiments. However, the authors do not mention photoreceptor activity while they describe all other retinal cell types.

The PSINA described in Akin et al. (2019) includes Periodic and Turbulent Stages, which take place during ~55-70 and ~70-95 hAPF (hours after pupae formation) respectively. IOC waves are unlikely to be related to these stimulus independent Ca2+ events, as IOC waves emerge much later at P12 (approximately 140 hAPF, Bainbridge and Bownes, 1981). Rh1 expression starts before IOC wave emergence (Rh1 begins at 78% of pupal life; Kumar and Ready, 1995), suggesting that Ca2+ events in photoreceptors observed at this time are likely stimulus dependent. Since we focus on Ca2+ waves in IOCs, which are independent of phototransduction, we did not monitor GCaMP6 in photoreceptors.

- Does the surge of the calcium signal observed in the control eye after 30sec of illumination disappear in norpA mutant eyes?

Yes, the initial surge is still present in NorpA eyes, suggesting that this surge is independent of phototransduction.

Reviewer 3 Comments for the author
There are a few concerns regarding the conclusions and interpretations drawn from the study: pMyosin phenotype noted in the ip3r knockdown experiments could also be due to the interactions between IP3R and Myosin and not just due to Ca2+ regulation of myosin. No specific evidence or cross-correlation analysis definitely shows that it is the Ca2+ (as opposed to IP3R – myosin binding activity such as Walker, Denise S., Sung Ly, Katherine C. Lockwood, and Howard A. Baylis. Current Biology 12, no. 11 (2002): 951-956) that is important for the phenotype.

Our simple model is based on the observations that 1) both IOC waves and actin stress fibers are disrupted by IP3R-, 2) both waves and stress fiber contractions occur specifically in IOCs during the late pupal phase, and 3) phospho-MRLC is reduced in IP3R- cells. Walker et al. (2002) and other papers (e.g. Turvey et al., 2005; Hours and Mery, 2010) have suggested that actin cytoskeletal elements, via direct interaction, could influence the distribution of IP3R proteins and Ca2+ signaling. While it is possible that actomyosin network could influence IP3R localization in Drosophila eye and IP3R could impact stress fibers via alternative mechanisms, our results are more consistent with a scenario, in which IP3R functions upstream to regulate IOC waves and endfeet stress fiber contraction. In any case, we have modified the Discussion to be more circumspect.

It is unclear why upstream stimuli of PLC21C or Galpha q, which are active in other epithelial systems and important for stimulating Ca2+ release via IP3R was not characterized to confirm the phenotype analysis. Does inhibition of Plc21C or Galphaq impact pMyosin in the endfeet?

Drosophila has three phospholipase C orthologs: norpA, sl (small wing), and Plc21C. We have already shown that a complete removal of norpA does not affect IOC waves. To ask whether Plc21C is required for IOC waves, we first obtained two Plc21C RNAi lines, TRiP.HMS00436 and TRiP.JF01210. Knockdown using these lines with lGMR-GAL4 shows no inhibition of the IOC waves. However, these results are not meaningful, as we do not know the efficacy of the knockdown. We have also obtained four independent Plc21C alleles, namely Plc21Cc00245, Plc21CKG05991, Plc21CKG08451, and Plc21CA246 (BDSC, Indiana), monitored the GCaMP6 signals in seven allelic combinations, and observed IOC waves in all.

We chose not to include these results in the revision because without ascertaining the strength of these alleles, they are inconclusive, suffering the same pitfall as the RNAi approach. To visualize the stress fibers at IOC endfeet, additional crosses need to be performed to render these Plc21C alleles pigment-less. Because IOC waves were still present, we did not pursue this.

To definitively address the role of Plc21C in IOC waves, we have requested Plc21C1, a CRISPR/Cas9-generated null allele, from Dr. Mariana Wolfner (Cornell University, USA). We would like to recombine this allele onto a FRT chromosome and monitor the GCaMP6 signals and stress fiber morphology in Plc21C1 mutant cells with a side-by-side internal control. Currently the allele is unavailable, and this approach is time consuming; we are unable to complete it in time for the revision.

It is strange that experiments that perturb GJ were not performed to verify whether coordination of Ca2+ (and other second messengers) strictly require gap junctions or not. If the intercellular calcium dynamics itself are important for regulation and contraction of actin stress fibers, why is the myosin and collagen phenotypes largely clone autonomous? Further discussion of this result seems warranted.

Regarding gap junction, we have monitored GCaMP6 activity in Inx2B mosaic eyes. In our hands, Inx2B mosaic eyes are smaller and rough, consistent with the previous report that Inx2 affects dpp signaling in determining eye size (Richard and Hoch, 2015). The fact that patterning is disrupted in Inx2 could complicate the interpretation of its role in Ca2+ wave propagation.

As for cell autonomy of these phenotypes, our view is that the regulation of stress fiber contraction at IOC endfeet by Ca2+ increases in a given cell is autonomous. The wave nature of these Ca2+ increases coordinate the reduction of basal surface sequentially and repetitively (see Reviewer 2 Minor point 1).

Is the late stages of Ca2+ transients required to maintain pMyo in adult eyes?

We show that the level of pMyo in pupal eyes depends on IP3R. To specifically show that the late stages of Ca2+ increases are responsible for adult pMyo level, we would need to conditionally inactivate IP3R. Our attempt to do this has been unsuccessful (please see Reviewer 2 point 2).

Specific comments:
There were no methods associated with imaging the larval eye disc. These are interesting but impact on pMyosin in the larval stages could be more carefully described. Is there any discernible cross-correlation between Ca2+ activity and Myosin dynamics seen?
We have appended the method for the eye disc GCaMP6 imaging in the Materials and Methods section. Because removal of IP3R does not disrupt retinal cell specification and patterning, we did not look at phospho-MRLC in eye discs.

The cells mentioned in legends were not adequately marked in several figures

We have modified the figures to better mark the cells.

p. 16 It is not clear what is meant by “their profiles”

We meant “their sizes” and have changed the text.

p. 23 shape tissue(s) or shape a tissue

This is fixed.

Figure 1: Include scale bars

This is fixed.

Fig. 1C Numbers 2, 3, 2, in C not described in caption. Should it be 2 deg, etc. as in B? The slices in C should be marked in B more clearly.

We have modified the Figure to make the labeling clearer.

The SI figure showing the cross-sectional view was helpful and seemed to be important to include in Figure 1.

We have replaced Fig 1A with a new 3D rendering image including vkg::GFP. This image provides a better view of the ECM and actomyosin network at the retinal basement.

Fig. 2: Not clear what is C23, C24, D12, etc. Cell labels in A are not clear.

C23, C24, and D12 are merely cell labeling, and we have modified Fig 2A to make this clearer. We chose not to change these labeling for the revision because doing so will require altering the cell labeling for all subsequent data collection and analysis. The map of labelled cells is shown in Figure S1A.

Fig. 2B appears somewhat confusing.

The goal of Fig. 2B is to show that waves can originate from any part of the eye. We have removed the temporal reference of wave initiation, which should make the panel clearer.

Fig. 3B E3p, E4a, etc are not clearly marked in A

We have modified Fig 3A to make the labelling clearer.

It is not clear which cells correspond to cell numbers in legends in subsequent plots either.

We have modified the legends to better explain the labeling.

Second decision letter

MS ID#: DEVELOP/2021/199700

MS TITLE: Calcium waves facilitate and coordinate the contraction of endfeet actin stress fibers in Drosophila interommatidial cells

AUTHORS: Henry Chiu-Yu Chang and Don F Ready

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.

The overall evaluation is positive and we would like to publish your manuscript in Development, following addressing the few remaining comments from one of the reviewers.

Reviewer 1

Advance summary and potential significance to field

While we know mechanisms by which Ca2+ regulates both conventional and unconventional myosin (Heissler and Sellers, JMB, 2016), how this is coordinated across tissues in distinct contexts is often unknown. In this manuscript, the authors examine the coordination of Ca2+ waves across the Drosophila pupal ommatidial epithelium. The authors characterize Ca2+ waves that propagate across the eye tissue. They show that these waves require IP3 Receptor, but not phototransduction. Using an IP3R mutant they show that IP3 Receptor is required for stress fiber organization and contraction, as well as a defect in basement membrane organization/levels.

Comments for the author

I appreciate how the authors have improved this manuscript. While they have addressed most of my criticisms, there needs to be more rigor with regards to statistical reporting.

Major:
1) Fig. 6B – There is improper reporting on statistics. First, n is not defined (i.e. cells or whole retinas analyzed). Second, it is not described in figure or figure legend how many experimental replicates were reported for each condition. This is also the case for data presented in Fig. 3-5. Third, error bars are not defined. Fourth, there are no statistical comparisons presented for the data in this panel or bar graphs in prior figures. I encourage the authors to think of a better way to present the experimental data present in the paper, because bar graphs provide minimal information. See Lord et al., JCB, Vol. 219, No. 6, (2020) for recommendations in this area.
2) Fig. 7F and 8E – n and error bars are not defined.

Minor:
1) p. 11, top paragraph – “primary pigment cells resembled to those observed in the secondary and tertiary pigment cells” à has an extra “to”

Reviewer 2

Advance summary and potential significance to field

The observation of waves of activity is an important discovery

Comments for the author

While we fully appreciate the attempts made by the authors to address the comments from the reviewers, they have not managed to figure out precise answers to many of the major points raised

by them. The authors provide some valid technical reasons for failing to experimentally test the function of the waves.
However, the main observation remain well documented and represents a striking feature, but the functional part is still weak.
Because of the importance of the observation, I still support publication of the paper as is.

Reviewer 3

Advance summary and potential significance to field

The principal advance in this paper is the description and discovery of Ca2+
waves in retinal epithelium, specifically the interommatidial cells. The authors also report an interesting phenotype downstream of IP3R signaling , which regulates stress fibers in IOC endfeet. This suggests that IP3R signaling is important for regulating stress fiber contraction and floor morphogenesis.

Comments for the author

The authors made an authentic attempt to address concerns raised in the first round. However, not all of the experiments were completed or points were addressed due to technical issues. This is balanced by the interesting findings of how IP3R is important for organizing the Collagen IV network under the IOC endfeet. There are other alternative interpretations that cannot be fully ruled out at this stage, but the role of calcium regulated by IP3R is a reasonable interpretation.
Note that it was difficult to confirm that all reported changes in the rebuttal letter were implemented in the revised manuscript. It would have been much easier to work with a manuscript that included tracked or highlighted changes.
p. 18: last sentence, replace modulating with a stronger word (like increasing).
p. 23: consider moving up the nice definition of Grommets and Ridges to the introduction
Consider modifying the title to focus on calcium /ip3R signaling (not the waves) coordinating contraction of the actin stress fibers.

Second revision

Author response to reviewers' comments

Reviewer 1

Reviewer 1 Comments for the Author:
I appreciate how the authors have improved this manuscript. While they have addressed most of my criticisms, there needs to be more rigor with regards to statistical reporting.

Major:
1)Fig. 6B – There is improper reporting on statistics. First, n is not defined (i.e. cells or whole retinas analyzed). Second, it is not described in figure or figure legend how many experimental replicates were reported for each condition. This is also the case for data presented in Fig. 3-5. Third, error bars are not defined. Fourth, there are no statistical comparisons presented for the data in this panel or bar graphs in prior figures. I encourage the authors to think of a better way to present the experimental data present in the paper, because bar graphs provide minimal information. See Lord et al., JCB, Vol. 219, No. 6, (2020) for recommendations in this area. 2)Fig. 7F and 8E – n and error bars are not defined.

We thank reviewer for the suggestion and have made significant modifications in this regard.

For Fig 6B, we have replaced the panel with a Superplot of Ca^{2+} characteristics from two wild type and two *norpA* mutant retinas. The numbers from individual cells of different retinas are represented by color coded dots. The averages from IOCs of each retina are represented by circles

of corresponding colors, and the averages from IOCs of the same genotypes are indicated by bars. These scatter plots should give a better representation of the data distribution from independent recordings. Student's t-test, with n=2 (n is the number of retinas analyzed for each genotype), suggests that IOC waves in wild type and *norpA* retinas are not significantly different. These results are included in the revised Fig 6 legend.

Fig 3C compares the spike characteristics of IOC and primary pigment cells from the same retina. The revised panel now presents these data in scatter plots to better illustrate the distributions of measurements. We have used this format to present data in revised Supplemental Fig 1, 2, and 3.

For Fig 5G, we have added the analysis of a second retina for each age group to the comparison of IOC waves in aging retinas. The results are illustrated in a Superplot format and explained in revised Fig 5 legend.

For Fig 7F, the original panel had three comparisons of hexagonal regions defined in Fig 7D and E. The first one compared the area size of 3 wild type and 6 *IP3R* hexagons from one single *IP3R* mosaic eye at the cornea level, showing that the apical area is not affected by *IP3R*. The second one compared the areas of 2 and 4 wild type hexagons from one mosaic eye, showing that the basement area is not influenced by mitotic recombination. These two comparisons are now presented in scatter plot format to better illustrate the distribution of measurements.

The third comparison in the previous version aggregated the area measurements of 24 wild type and 32 *IP3R* hexagonal regions from 9 independent mosaic retinas. To better illustrate the distribution of data points, we choose to present the measurements of 14 wild type and 18 *IP3R* hexagons from 4 mosaic retinas in a Superplot format in this revision. Individual measurements and averages from different mosaic retinas are represented by color-coded dots and circles, respectively.

For Fig 8E, the original panel presented area measurements of 16 wild type and 29 *IP3R* grommets from one single mosaic eye. We have now added the grommet size measurements from an independent mosaic eye (15 wild type and 34 *IP3R*) and presented these results in a Superplot. The t-test with n=2 (2 eyes) has a p-value of 0.008, suggesting the reduction of grommet size in IP3R mutant clones is significant.

Minor:

1)p. 11, top paragraph – "primary pigment cells resembled to those observed in the secondary and tertiary pigment cells" à has an extra "to"

Fixed.

Reviewer 2

Reviewer 2 Comments for the Author:
While we fully appreciate the attempts made by the authors to address the comments from the reviewers, they have not managed to figure out precise answers to many of the major points raised by them. The authors provide some valid technical reasons for failing to experimentally test the function of the waves. However, the main observation remains well documented and represents a striking feature, but the functional part is still weak. Because of the importance of the observation, I still support publication of the paper as is.

Reviewer 3
Reviewer 3 Comments for the Author:
The authors made an authentic attempt to address concerns raised in the first round. However, not all of the experiments were completed or points were addressed due to technical issues. This is balanced by the interesting findings of how IP3R is important for organizing the Collagen IV network under the IOC endfeet. There are other alternative interpretations that cannot be fully ruled out at this stage, but the role of calcium regulated by IP3R is a reasonable interpretation.

Note that it was difficult to confirm that all reported changes in the rebuttal letter were implemented in the revised manuscript. It would have been much easier to work with a manuscript that included tracked or highlighted changes.

p. 18: last sentence, replace modulating with a stronger word (like increasing).

Thanks for the suggestion. Fixed

p. 23: consider moving up the nice definition of Grommets and Ridges to the introduction

We have added one sentence in the Introduction (bottom of page 3) defining these structures.

Consider modifying the title to focus on calcium /ip3R signaling (not the waves) coordinating contraction of the actin stress fibers.

We prefer to leave the title unchanged, as *IP3R* mutation disrupts both the waves and actin stress fiber morphology. Modifying the title at this juncture will require significant rewriting.

Third decision letter

MS ID#: DEVELOP/2021/199700

MS TITLE: Calcium waves facilitate and coordinate the contraction of endfeet actin stress fibers in Drosophila interommatidial cells

AUTHORS: Henry Chiu-Yu Chang and Don F Ready
ARTICLE TYPE: Research Article

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