Polyglutamine repeat proteins disrupt actin structure in Drosophila photoreceptors.

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Abstract
Expansions of polyglutamine-encoding stretches in several genes cause neurodegenerative disorders including Huntington's Disease and Spinocerebellar Ataxia type 3. Expression of the human disease alleles in Drosophila melanogaster neurons recapitulates cellular features of these disorders, and has therefore been used to model the cell biology of these diseases. Here, we show that polyglutamine disease alleles expressed in Drosophila photoreceptors disrupt actin structure at rhabdomeres, as other groups have shown they do in Drosophila and mammalian dendrites. We show this actin regulatory pathway works through the small G protein Rac and the actin nucleating protein Form3. We also find that Form3 has additional functions in photoreceptors, and that loss of Form3 results in the specification of extra photoreceptors in the eye.

Keywords
Actin, Drosophila, Formin, Huntington's disease, Photoreceptor, Polyglutamine

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ABSTRACT

Expansions of polyglutamine-encoding stretches in several genes cause neurodegenerative disorders including Huntington's Disease and Spinocerebellar Ataxia type 3. Expression of the human disease alleles in Drosophila melanogaster neurons recapitulates cellular features of these disorders, and has therefore been used to model the cell biology of these diseases. Here, we show that polyglutamine disease alleles expressed in Drosophila photoreceptors disrupt actin structure at rhabdomeres, as other groups have shown they do in Drosophila and mammalian dendrites. We show this actin regulatory pathway works through the small G protein Rac and the actin nucleating protein Form3. We also find that Form3 has additional functions in photoreceptors, and that loss of Form3 results in the specification of extra photoreceptors in the eye.

1. Introduction

Polyglutamine (polyQ) disorders are dominant neurodegenerative diseases caused by expanded CAG nucleotide tracts within protein coding genes (Orr and Zoghbi, 2007). There are nine known polyQ diseases, including Huntington's Disease (HD) and Spinocerebellar Ataxia type 3 (SCA3), each caused by CAG expansions encoding long stretches of glutamine within a different protein (HTT and ATXN3, respectively). In each case, having a small number of CAG repeats allows for wild type function of the encoded protein, while having a larger number of repeats causes a pathogenic gain of function and protein aggregation.

The fruit fly Drosophila melanogaster has been a powerful model system for investigating the cellular and molecular mechanisms of these diseases (McGurk et al., 2015). Researchers have expressed expanded, pathogenic alleles of polyQ genes in Drosophila neurons to study their cellular responses, while expressing short, wild type alleles as a control. Expression of HTT or ATXN3 disease alleles in Drosophila dendritic arborization neurons recapitulated these dendritic defects, which were similar to those seen in the corresponding disease (Jackson et al., 1998; McGurk et al., 2015; Warrick et al., 1998).

One feature common to polyQ diseases is reduced dendritic complexity (Clark et al., 1997; Graveland et al., 1985; Guidetti et al., 2001). Expression of HTT or ATXN3 disease alleles in Drosophila dendritic arborization neurons recapitulated these dendritic defects, which were caused by disruption of the actin cytoskeleton (Lee et al., 2011). Dendritic structure in these cells could be rescued by coexpression of an activated form of Rho-family GTPase Rac (Rac.V12), which regulates actin structure. Further understanding of interactions between polyQ proteins and the actin cytoskeleton would illuminate a poorly understood aspect of polyQ diseases.

Rho-family GTPases regulate actin filaments through direct interaction with Formins, actin nucleating proteins that promote the formation of linear actin filaments (Liu et al., 2010). Extensive studies of the formin diaphenous show that it exists in an auto-inhibited form until bound by activated Rho (Higgs, 2005). Drosophila melanogaster has six formin genes, any of which might be regulated by Rac in dendrites (Liu et al., 2010).

We have identified interactions between polyQ proteins and the actin cytoskeleton in Drosophila melanogaster photoreceptor neurons. These neurons are not required for life in a laboratory setting, allowing us to analyze cellular responses to polyQ proteins over time. Here we report that HTT and ATXN3 disease alleles induce massive disruptions of the actin cytoskeleton in photoreceptors, with severity that increases with age. Coexpression of Rac.V12 rescues these actin defects, demonstrating that the mechanism that patterns dendrites is also active in photoreceptors. We further show that formin Form3 also regulates these actin structures, and may function downstream of Rac. We conclude that photoreceptors can be used to model dendritic actin regulation in response to polyQ proteins, as regulation of actin in photoreceptor rhabdomeres uses the same pathway employed in dendrites.

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2. Materials and methods

2.1. Fly genetics

Flies were raised at 25 °C in alternating 12 h light and 12 h dark on Nutri-fly BF media (Genesee Scientific, San Diego, CA). For all experiments, flies contained one copy of GMR-Gal4 and one copy of any UAS-regulated transgene. Fly strains GMR-Gal4 (Song et al., 2000), UAS-HTT.16Q, UAS-HTT.128Q (expressing full-length HTT) (Song et al., 2000), UAS-ATXN3.27Q, UAS-ATXN3.84Q (expressing full-length ATXN3) (Warrick et al., 2005), UAS-Rac.V12 (Luo et al., 1994), UAS-fhos RNAi (P{TRiP.HMS00308}), UAS-capu RNAi (P{TRiP.HMS00712}), UAS-fos RNAi (P{TRiP.JF01606}), UAS-fhl RNAi (P{TRiP.HMS00445}), UAS-DAAM RNAi (P{TRiP.HMS01978}), UAS-form3 RNAi (P{TRiP.HMS00393}) (Ni et al., 2011) were obtained from the Bloomington Drosophila Stock Center.

2.2. Eye staining

Flies were collected once a day, and either dissected immediately (1 day old) or aged for 1 week before dissection (8 days old). Eyes were dissected, fixed, and stained as previously described (Williamson and Hiesinger, 2010). Eyes were stained with Texas Red-phalloidin (Sigma-Aldrich), phalloidin-CF555 (Biotium Inc., Fremont, CA), anti-Rh1 (4C5, Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-mouse-Alexa 488 (VWR International, Radnor, PA) and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were collected on a Nikon A1R confocal microscope (Nikon Instruments, Melville, NY) and processed with Fiji (Rueden et al., 2017; Schindelin et al., 2012) and Photoshop CC 2015 (Adobe Systems Inc., San Jose, CA).

2.3. Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.0. Rhabdomere areas from multiple genotypes were compared using one way ANOVA with Bonferroni multiple comparison procedures. 10 rhabdomeres from at least three different ommatidia were measured for each condition, using the polygon selection tool in Fiji to measure areas. Pairwise comparisons were performed using a two tailed t-test.

3. Results

3.1. Polyglutamine-containing proteins disrupt photoreceptor actin structure

Each facet, or ommatidium, of a Drosophila melanogaster eye contains 8 photoreceptor neurons (R1-R8), seven of which can be seen in a cross-section (Pellikka et al., 2002) (Fig. 1A). Outer photoreceptors (R1-R6) are arranged in a stereotypical trapezoid pattern. Inner photoreceptors (R7 & R8) are smaller and are stacked on one another vertically. All photoreceptors position a rhabdomere near the center of the ommatidium. The rhabdomere is made of tightly stacked plasma membrane containing high levels of the light-sensing transmembrane protein Rhodopsin. The rhabdomere also contains a high level of F-actin. Phalloidin staining reveals the structure of the rhabdomeres, as well as cortical actin underlying the rest of the plasma membrane (Fig. 1A, purple). We expressed full-length human polyQ proteins in photoreceptor cells using GMR-Gal4, and analyzed actin structure by staining eyes with phalloidin. Eyes of eight day old flies expressing
short, wild type forms of Huntingtin (HTT.16Q) or Ataxin 3
(ATXN3.27Q) in photoreceptors look wild type when stained with
phalloidin (Fig. 1B, D), showing high levels of staining at rhododemes
and lower levels at the cortex. The staining pattern was indistinguish-
able from that of eyes with GMR-Gal4 only (data not shown). Eyes
expressing expanded, disease alleles of these genes (HTT.128Q and
ATXN3.84Q) dramatically change actin localization (Fig. 1C, E). Actin
accumulates at some rhododemes, but these rhododemes are much
smaller than wild type. Cortical accumulation of actin is enhanced and
irregular, high in some regions and undetectable in others. In some
ommatidia, rhododemes are not fully differentiated from the rest of
the plasma membrane, and actin seems to accumulate equally on all of
these membranes.

Since HD and SCA3 are degenerative disorders, whose severity in-
creases over time, we wanted to see if polyQ-induced actin redistribu-
tion was also progressive. At 1 day of age, SCA3.84Q and HTT.128Q
expressing eyes had more rhododemes and a more typical structure
than at 8 days (Fig. 1F–H). SCA.84Q eyes had a mean of 6.5 rhodod-
emes/ommatidium at day 1 compared to 2.0 at day 8 (t-test,
p < 0.0001). HTT.128Q changed less dramatically, from 6.0 rhododo-
emes/ommatidium at day 1 to 3.8 at day 8 (t-test, p < 0.0001). Rhododemes at day 1 were small, but clearly distinct from other
membranes. F-actin also accumulated evenly at the cortex, though more
abundantly than in wild type. We conclude that expanded polyQ pro-
teins induce actin rearrangement, which becomes more severe and ir-
regular over time.

3.2. Polyglutamine-induced photoreceptor defects are partially rescued by
Rac signaling

To better characterize rhododeme defects induced by long HTT and
ATXN3 alleles, we stained eyes with both phalloidin and an antibody to
Rhodopsin (Rh1). Rh1 is the light detecting protein in outer photo-
receptors, but is absent from inner photoreceptors. In eyes expressing
HTT.16Q or ATXN3.27Q, Rh1 accumulates in its wild type pattern at
the rhododeme membrane (Fig. 2A, G). In eyes expressing HTT.128Q
or ATXN3.84Q, Rh1 staining is mostly lost (Fig. 2C, I). Puncta of Rh1
still present in these eyes tend to colocalize with phalloidin puncta in
remaining rhododemes.

PolyQ-induced actin mislocalization in dendritic arborization neu-
rons was rescued by expression of a constitutively active form of the
Rho-family GTPase Rac (Rac.V12) (Lee et al., 2011). To see if Rac also
regulated actin in photoreceptors, we coexpressed expanded polyQ al-
leles and Rac.V12. Rac.V12 signifi-
cantly rescued rhabdomeric accu-
mulation of actin caused by both HTT.128Q and ATXN3.84Q, though
the eyes were still disorganized (Fig. 2F, L, M). Interestingly, Rac.V12
rescued Rh1 accumulation in ATXN3.84Q-expressing photoreceptors,
but not in HTT.128Q photoreceptors (Fig. 2E, K).

3.3. Form3 regulates actin accumulation in photoreceptors

Small G proteins can regulate actin structure through formin pro-
teins, which nucleate new actin filaments. Drosophila melanogaster
has six formin orthologs: dia, capu, fhos, frl, DAAM, and form3 (Liu et al.,
2010). To see which formins might function downstream of Rac in
photoreceptors, we used RNAi to knock down each formin gene.
Knockdown of most formin genes displayed only mild effects on eye
development or actin structure in 1 day old eyes (Fig. 3A–E). Notably,
frl knockdown caused ommatidial rotation defects, as previously re-
ported (Fig. 3C) (Dollar et al., 2016). Only knockdown of fhos or form3
casted a reduction of rhododeme size (ANOVA: f = 42.62, df = (6,63),
p < 0.0001) (Fig. 3E–G). Form3 knockdown also induced diffuse ac-
cumulation of f-actin throughout the photoreceptor cytoplasm. There-
fore, form3 appears to be a primary regulator of actin nucleation in

![Fig. 2. Actin mislocation is partially rescued by co-expression of activated Rac.V12. In 1 day old flies, Rh1 localizes to the six outer rhododemes in eyes expressing wild type alleles of ATXN3 (A, B) and HTT (G, H). Rhabdomeric actin and Rh1 accumulation are lost in eyes expressing diseases alleles of ATXN3 (C, D) and HTT (I, J). Co-expression of Rac.V12 restores actin localization to rhododemes for both disease alleles (F, L), but only restores Rh1 localization for ATXN3 (E, K). Mean number of actin-containing rhododemes and standard deviations are shown in M. * indicates p < 0.05. ** indicates p < 0.0001.](image)
photoreceptor neurons, with actin forming aberrant structures in the absence of form3.

To see if form3 RNAi caused photoreceptor degeneration, we analyzed eyes of eight day old flies. Surprisingly, rhabdomeric and cortical actin was restored in older flies (Fig. 4B). Rhabdomere size was restored to wild type levels (ANOVA: $f = 105.5$, df = (2,7), $p < 0.0001$) (Fig. 4G). No sign of degeneration was observed.

If form3 functions downstream of Rac in photoreceptors, Rac.V12 should not rescue the form3 RNAi actin defects. As predicted, form3 RNAi flies co-expressing Rac.V12 were not significantly larger than form3 RNAi rhabdomeres and had diffuse cytoplasmic actin filaments (Fig. 4E, G).

3.4. Form3 regulates photoreceptor cell fate

Eye development is regulated by a network of signaling pathways which ensure each ommatidium has 6 outer photoreceptors and 2 stacked inner photoreceptors. However, many ommatidia in form3 RNAi eyes had actin accumulations that looked like extra outer and inner photoreceptor rhabdomeres (Fig. 4B). Costaining with Rh1 antibodies showed that these extra outer rhabdomeres strongly accumulated Rh1 in a typical rhabdomere pattern (Fig. 4A–C). Inner photoreceptor rhabdomeres also had weak accumulations of Rh1, which is not seen in wild type. Quantitation of this defect revealed that form3 RNAi ommatidia had an average of 7.7 Rh1-positive rhabdomeres compared to 6 in wild type (t-test, $p < 0.0001$).

Extra rhabdomeres are also apparent in form3 RNAi, Rac.V12 eyes (Fig. 4D–F). All apparent outer photoreceptor rhabdomeres were positive for Rh1. Inner photoreceptor rhabdomeres did not accumulate Rh1, in contrast to eyes expressing form3 RNAi alone. Therefore, Rac.V12 rescued some, but not all, of the defects caused by form3 RNAi.
4. Discussion

4.1. Actin structure is disrupted by disease-associated polyQ proteins

We have identified an actin regulatory pathway that is disrupted by disease-associated polyQ proteins in Drosophila neurons. While photoreceptors have rhabdomeres instead of the dendrites found in most neurons, both contain complex actin structures with analogous functions. Actin filaments are threaded though the membrane folds of the rhabdomere, providing structural support, and also form a web at the rhabdomere base (Arikawa et al., 1990) (Chang and Ready, 2000). Actin filaments also support dendritic structure, with actin disorganization resulting in reductions of dendritic size and complexity (Konietzny et al., 2017).

Therefore, we propose that rhabdomeric and dendritic defects caused by polyQ proteins are analogous, and that studies in photoreceptors can shed light on dendritic phenotypes. We report that activated Rac.V12 rescues rhabdomeric actin defects in photoreceptors, just as it rescues actin and dendrite defects in dendritic arborization neurons (Lee et al., 2011) (Fig. 2). This idea is supported by a recent yeast two-hybrid screen that found HTT physically interacted with multiple small G protein regulatory proteins. Follow-up studies in mouse striatal cells found that Rac RNAi reduced the toxicity of expanded HTT proteins (Tourette et al., 2014). Thus the regulatory systems identified in fly photoreceptors may be relevant to mammalian disease processes.

We propose that actin accumulates at rhabdomeres and the cell cortex in a Form3-dependent manner, but that only rhabdomeric actin is regulated by Rac (Fig. 5A). Disease associated polyQ proteins disrupt the Rac-dependent pathway, resulting in less Form3 recruitment to rhabdomeres and leaving more Form3 to associate with the cortex (Fig. 5B). This would result in the reduction of rhabdomeric actin and accumulation of cortical actin caused by polyQ proteins (Fig. 1). The partial rescue by Rac.V12 indicates that Rac-dependent signaling is only one of many processes disrupted by polyQ proteins (Fig. 2).

Disease associated polyQ proteins disrupt actin structures and dendrite morphology in many systems. In Drosophila dendritic arborization neurons, expanded polyQ alleles of HTT, ATXN1, and ATXN3 all reduce dendritic actin accumulation and cause a reduction in the size of the dendritic arbor (Lee et al., 2011). Expanded polyQ alleles of ATXN2 cause a loss of Purkinje cell dendritic arbor that precedes cell death (Pulst et al., 2000). Drosophila ATXN2 mutants have missing or disorganized actin structures in nurse cells, bristles, and developing eyes (Satterfield et al., 2002).

Loss of rhabdomeric actin increased as flies aged (Fig. 1). This progressive phenotype is reminiscent of the age-related progression of Huntington’s Disease and Spinocerebellar Ataxia. However, the early, persistent expression of polyQ transgenes in fly eyes could also cause developmental defects that might alter this phenotype. In these studies, we are unable to separate any potential developmental defects from the progressive defects.

4.2. Form3 regulates neuronal actin filaments

Form3 regulates both rhabdomeric and cortical actin structures (Fig. 5C). We find that form3 RNAi causes reduced rhabdomeric actin and little detectable cortical actin in 1 day old photoreceptors (Fig. 3F).
Instead, actin filaments assemble in the photoreceptor cytoplasm, presumably nucleated by other formins or other actin regulators. Other formins do function in photoreceptors, FrI is required for planar cell polarity in developing eyes, a requirement recapitulated in our studies (Dollar et al., 2016) (Fig. 3D). Double mutants of DAAM and FrI have stronger photoreceptor phenotypes than either mutant alone, indicating that DAAM is also active in these cells (Dollar et al., 2016). We found that fhos RNAi also affected rhabdomere size (Fig. 3E). Fhos has been shown to regulate actin in sarcomere formation and autophagic cell death, but has not previously been studied in the eye (Anhezini et al., 2012; Shwartz et al., 2016).

**Form3** has not been extensively studied, but several reports indicate that it functions in *Drosophila* neurons. In developing embryos, *form3* is only expressed in tracheal and CNS cells (Tanaka et al., 2004). *Form3*
RNAs were isolated from fly heads, but not from non-neuronal S2 cells (Ashwal-Fluss et al., 2014). Mutation of tdp-43 caused increased transcription of form3 in the larval CNS (Hazelett et al., 2012). RNAi of form3 impaired the performance of flies in an olfactory assay (Walkinshaw et al., 2015). Thus, expression and functional data indicate that form3 functions in many types of neurons.

4.3. Form3 is required for eye development

In addition to disrupting actin structures, form3 RNAi altered eye development. Many ommatidia had extra outer and inner photoreceptors (Fig. 4B). Extra outer photoreceptors are also seen in fat facets (faf) mutants, which fail to downregulate Notch signaling in developing eye discs (Overtstreet et al., 2004). These extra photoreceptors are cells that would otherwise have developed as cone cells. Like faf mutants, form3 RNAi causes a rough eye phenotype (data not shown). It is unclear if Notch signaling is involved in the form3 RNAi phenotype.

Extra inner photoreceptor might also be due to changes in cell fate, or might be due to a change in inner photoreceptor morphology. Normally, the two inner photoreceptors are stacked and only one can be seen in a focal plane. A change in the growth or organization of these cells might cause them to lie next to one another, so that both could be seen in a single image. Notably, we never detected more than two inner photoreceptors in an ommatidium.

Form3 RNAi inner photoreceptors also expressed low levels of Rh1, which does not occur in wild type inner photoreceptors (Fig. 4C). This change in gene expression is surprising, as there is no clear mechanism for a formin to impact gene expression. Coexpression of Rac.V12 with form3 RNAi rescues this Rh1 phenotype without impacting the actin phenotype, suggesting that Rh1 expression is not just responding to the change in actin regulation (Fig. 4F). Therefore, form3 may have functions beyond actin regulation in photoreceptor development.

5. Conclusions

We have found that expanded PolyQ protein disrupts a neuronal actin regulatory pathway involving Rac and Form3. While our work was performed in Drosophila photoreceptor neurons, it is in agreement with studies in other neurons and other species. Given the practical advantages of photoreceptors for studies of neurodegeneration, we propose that photoreceptors will be a useful system for further studies of this pathway. We have also identified Form3 as an actin regulator in Drosophila neurons, and shown that it is required for proper eye development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcn.2018.08.005.

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