Neurodegeneration Caused by Polyglutamine Expansion Is Regulated by P-Glycoprotein in *Drosophila melanogaster*

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ABSTRACT Trinucleotide CAG repeat disorders are caused by expansion of polyglutamine (polyQ) domains in certain proteins leading to fatal neurodegenerative disorders and are characterized by accumulation of inclusion bodies in the neurons. Clearance of these inclusion bodies holds the key to improve the disease phenotypes, which affects basic cellular processes such as transcription, protein degradation and cell signaling. In the present study, we show that P-glycoprotein (P-gp), originally identified as a causative agent of multidrug-resistant cancer cells, plays an important role in ameliorating the disease phenotype. Using a *Drosophila* transgenic strain that expresses a stretch of 127 glutamine repeats, we demonstrate that enhancing P-gp levels reduces eye degeneration caused by expression of polyQ, whereas reducing it increases the severity of the disease. Increase in polyQ inclusion bodies represses the expression of *mdr* genes, suggesting a functional link between P-gp and polyQ. P-gp up-regulation restores the defects in the actin organization and precise array of the neuronal connections caused by inclusion bodies. β -Catenin homolog, Armadillo, also interacts with P-gp and regulates the accumulation of inclusion bodies. These results thus show that P-gp and polyQ interact with each other, and changing P-gp levels can directly affect neurodegeneration.

THE polyglutamine (polyQ) expansion neurodegenerative diseases are characterized by the increase in CAG trinucleotide repeats in diseased genes. Nine diseases, including Huntington's and several spinocerebellar ataxias, have been identified so far, which result from accumulation of insoluble mutant proteins, making the neurons vulnerable to degeneration in specific regions of the brain (Mattson and Magnus 2006). It is still not known why only a specific population of neurons is affected, though these aggregates are widely expressed in the central nervous system (Welch and Diamond 2001; Michalik and Broeckhoven 2003). The onset of disease is by the accumulation of intracellular inclusion bodies formed due to gain of function of misfolded protein aggregates (Ross and Poirier 2004). The mutant polyQ peptide monomers are soluble in nature but they have a natural tendency to associate with each other and form insoluble aggregates either in neurons or in extracellular regions

(Di Figlia *et al.* 1997; Ross and Poirier 2004; Bossy *et al.* 2008; Takahashi *et al.* 2008). The onset and severity of the disease is directly proportional to the length of polyQ tracts (Legleiter *et al.* 2010).

The expanded polyQ proteins exhibit more stability and evade degradation by proteasome machinery and the disease progression is due to imbalance between accumulation and clearance of the aggregates (Verhoef *et al.* 2002; Matus *et al.* 2008). However in neurodegenerative diseases, stress caused by misfolded proteins, damage the ubiquitin–proteasome system, which leads to defects in clearance of inclusion bodies (Matus *et al.* 2008; Riederer *et al.* 2011). The pathogenic conditions could also be because of overproduction of aggregates (Benjamin 2012), which further recruits other proteins known as aggregate interacting proteins (AIPs) (Mitsui *et al.* 2002). AIPs include chaperones such as heat shock cognate 70 (HSC70), human DNA J-1 and J-2 (HDJ-1 and HDJ-2), heat shock protein 84, translational elongation factor-1 (EF-1), and 20S proteasome protein (Mitsui *et al.* 2002). Apart from cytoplasmic inclusions, the mutant proteins have an inherent tendency to get translocated into the nucleus, forming intranuclear inclusions (Davies *et al.* 1997). Nuclear aggregates were found

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to colocalize with transcription factors such as cAMP-responsive element-binding protein (CREB)-binding protein (CBP), TATA-binding protein (TBP), and TBP-associated factors, thus affecting the transcriptional state of the cell (Perutz *et al.* 1994; Zhai *et al.* 2005). In Huntington's disease, the mutant huntingtin (htt) protein aggregates interfere with organellar trafficking and proteins at synaptic vesicles (Trushina *et al.* 2012). Such interactions result in loss of normal functions and ultimately neuronal death by obstructing the axonal transport (Cruz *et al.* 2005). Aggregation of mutant polyQ peptides depends on polar zipper formation of polyQ molecules by hydrogen bonds that are similar to β -amyloid proteins, the causative agent of Alzheimer's disease (Esposito *et al.* 2008). It has been shown that a membrane transporter, P-glycoprotein (P-gp), interacts with β -amyloid protein aggregates and is involved in the movement of β -amyloid proteins from brain to blood (Cirrito *et al.* 2005).

P-gps are plasma membrane glycoproteins of ~170 kDa, belonging to the super family of ATP-binding cassette (ABC) transporters, also called traffic ATPases (Labialle *et al.* 2002). P-gp came into notice when several multidrug-resistant cancer cell lines were found to have an increased expression of P-gp and multiple drug-resistant-associated proteins (MRPs) (Simon and Schindlert 1994). The ABC family represents one of the largest families of proteins that serve as fundamental transport system and regulate the trafficking of diverse molecules across biological membranes, thus playing a central role in cellular physiology. It is present in cell membranes and in membranes of intracellular organelles, transporting various structurally unrelated hydrophobic substrates across the membranes. The basal expression level of P-gp in human body is low; however, few cell types in kidney, liver, pancreas, jejunum, adrenal glands, and biliary canaliculi show enhanced P-gp expression (Thiebaut *et al.* 1987). The human genome carries 49 ABC genes, arranged in seven subfamilies, designated from A to G (Vasiliou *et al.* 2009). The ABC subfamily B includes *MDR1* in humans and *mdr1a* and *mdr1b* in rodents. There are three genes encoding P-gp in *Drosophila melanogaster* and they are named according to their cytological positions as *mdr49*, *mdr50*, and *mdr65* (Wu *et al.* 1991). These genes have 50% identity to mammalian homologs and 53% homology among themselves at the nucleotide level.

The present study was aimed at determining the role of P-gp in polyQ-mediated pathogenesis in *Drosophila*. We used fly eye as a model system to express polyQ aggregates resulting in eye degeneration. We show that P-gp regulates the pathogenesis caused by expanded polyQ aggregates. Increase or decrease in polyQ aggregates by P-gp consequently affects arrangement of rhabdomeres, actin organization, and the neuronal connections. Adherens junction complex component, Armadillo, also seems to be regulated by altered levels of P-gp. *mdr* gene transcription was repressed by mutant polyQ, suggesting that there is a functional link between P-gp and polyQ that could be mediated through Armadillo.

Table 1 Categories of eye degeneration in *GMR-GAL4UAS-127Q/CyO*; +/- flies

Categories of eye degeneration	Phenotype
Mild	Less roughening in eye (with lesions only at the periphery)
Moderate	In between mild and severe (with degeneration and necrotic patches in either of the eyes)
Severe	With severe degeneration in both the eyes (eye size smaller than mild eyes)

Materials and Methods

Fly strains and culture conditions

Flies were reared in a humidified, temperature-controlled incubator at 24.5°. All larvae were reared in standard density culture on standard laboratory fly medium (10% yeast, 2% agar, 10% sucrose, 10% autolyzed yeast, 3% nipagin, and 0.3% propionic acid). For colchicine food, colchicine (Sigma-Aldrich, no. C9754) was dissolved in triple-distilled water and added to the molten media at 60° to a final concentration of 10 μ M and 20 μ M, with constant stirring while being poured. Similarly, verapamil (Sigma-Aldrich, no. V-106) was dissolved in ethanol and added to the molten media to a final concentration of 20 μ M. First instar larvae were kept in 50-ml plastic food plates containing 25 ml of either food or food + drug and were transferred at pupal stage to vials. Similar numbers of first instar larvae were transferred on food plates to avoid crowding. The fly stocks for the studies were obtained from the Bloomington Stock Center, except where mentioned. *Oregon R*⁺ was used as wild-type control. *GMR-GAL4* driver was used to drive the expression of UAS constructs in the eye imaginal discs. The fly stocks are as follows:

1. *w¹¹¹⁸; UAS-127Q* (Kazemi-Esfarjani and Benzer 2000) A transgenic line in which a 127-CAG trinucleotide repeat unit, flanked by a HA tag, is placed in *cis* to the UAS promoter.
2. *UAS-mdr49RNAi* Bloomington Stock Center, expresses dsRNA for RNAi of *mdr49* (FBgn0004512) under UAS control, Transgenic RNAi Project (Trip).
3. *UAS-mdr50RNAi* Bloomington Stock Center, expresses dsRNA for RNAi of *mdr50* (FBgn0010241) under UAS control, Trip.
4. *UAS-mdr65RNAi* Bloomington Stock Center, expresses dsRNA for RNAi of *mdr65* (FBgn0004513) under UAS control, Trip.

Proper crosses were set to get the stocks of the following genotypes: *GMR-GAL4 UAS-127Q/CyO*, *GMR-GAL4 UAS-127Q/+*; *UAS-mdr49RNAi/+*, *GMR-GAL4 UAS-127Q/+*; *UAS-mdr50RNAi/+*, and *GMR-GAL4 UAS-127Q/+*; *UAS-mdr65RNAi/+*.

Antibodies and immunocytochemistry

Eye imaginal discs from third instar (120 hr) larvae were dissected in 1× PBS, fixed in 4% paraformaldehyde for

Figure 1 Eye degeneration in *GMR-GAL4 UAS-127Q/CyO* flies. The degeneration was categorized as mild (B), moderate (C), and severe (D) in comparison to normal in wild type (A). The eyes of wild-type adult flies had normal pigmentation without any degeneration. *GMR-GAL4 UAS-127Q/CyO* flies in the mild category had fewer necrotic patches and less ommatidial degeneration in either of the eyes. Flies in the moderate category, had more patches in both the eyes. The severe category showed increased degeneration and necrosis in both of the eyes. Nail polish imprints of wild-type eyes (E) showed proper ommatidial organization. *GMR-GAL4 UAS-127Q/CyO* mild eye phenotype showed less fused ommatidia (F) in comparison to the moderate (G) and severe (H) types.

20 min at room temperature, rinsed in PBST (1× PBS, 0.1% Triton X-100), blocked in blocking solution (0.1% Triton X-100, 0.1% BSA, 10% FCS, 0.1% deoxycholate, 0.02% thiomersol) for 2 hr at room temperature. Tissues were incubated in primary antibody at 4° overnight. After 0.1% PBST (3 × 20 min each) washing, tissues were blocked for 2 hr and incubated in the secondary antibody. Tissues were rinsed in 0.1% PBST and counterstained with DAPI (1 µg/ml, Molecular Probe) for 15 min at room temperature. They were again washed in 1× PBS and mounted in antifadant, DABCO (Sigma). Primary antibodies used were anti-HA (1:40), anti-ELAV (1:100), anti-Armadillo (1:100), and anti-mab22c10 (1:100). Stains used were rhodamine-123 (1 µg/ml), phalloidin (1 µg/ml), and DAPI (1 µg/ml). All preparations were analyzed under a Zeiss LSM 510 Meta Confocal microscope and images were processed with Adobe Photoshop7.

Reverse transcription-PCR

Eye imaginal discs from third instar larvae of different genotypes and after different treatments were dissected out

and poly(A) RNA was extracted (Trizol method), followed by reverse transcription (RT) with Super-Script Plus (Invitrogen). PCR cycle conditions were as follows: 94° (3 min), 30 cycles of 94° (30 sec), primer annealing was done at 56° for *mdr65*, 60° for *mdr50*, and 54° for *mdr49* (30 sec each), 72° (30 sec), and final extension at 72° for 10 min. Primers used in the present study had the following sequences:

mdr49 (forward/reverse)
CTTCCCAGTGCCAATAGAGC
AGCCGTGATTCTCCTTCTT;
mdr50 (forward/reverse)
CTGGGTCGTGAGGAAATGTT
CGGTGAATCACACACCATGT;
mdr65 (forward/reverse)
CGTTAGGTGGAGAATCGAAA
CGCTCAAGCGGTTACAATCT; and
G3PDH (forward/reverse)
CCACTGCCGAGGAGGTCAACTA
GCTCAGGGTGATTGCGTATGCA.

Gel images were analyzed in Gelvue UV Transluminator Gel Documentation and Analysis system (Syngene). For all RT-PCR analysis, band intensities were measured by two methods: Alpha imager software and Histogram tool of Adobe photoshop 7.0. Each experiment was done six times and mean was taken considering even the slightest variation in controls. Mean ratio of band intensity of experimental and control was calculated, and bars were drawn taking the GPDH value as one.

Rhodamine efflux assay

Eye discs were dissected out in 1× PBS at room temperature and incubated in rhodamine-123 (1 µg/ml) for 15 min at 37°, followed by immediate chilling. The tissues were then incubated in 1× PBS at 37° so as to allow the efflux of the dye for 15 min. The eye discs were then mounted in 1× PBS and scanned.

Results

Altering P-gp levels affects 127-Q mediated neurodegeneration

To study the role of P-gp on polyQ-mediated pathogenesis, *GMR-GAL4 UAS-127Q/CyO* transgenic stock, based on the *UAS-GAL4* system (Phelps and Brand 1998) was used. The polyQ allele containing 127 CAG repeats was expressed in the eye imaginal discs, using *GMR-GAL4*. The flies developed degenerated eyes, but the mutation exhibited variable expression. The phenotypes included loss of pigmentation, formation of necrotic patches, and collapsed eyes. The degeneration was categorized into mild, moderate, and severe (Table 1) based on the extent of degeneration, as described by Sanokawa-Akakura *et al.* 2010.

The eyes of wild-type flies showed normal pigmentation and absence of degeneration (Figure 1A). In *GMR-GAL4*

Figure 2 P-gp function in the eye is affected by colchicine and verapamil feeding. Eye imaginal discs from wild-type larvae grown on normal food, showed a moderate level of rhodamine-123 in the eye discs (A), while eye discs from 10 μ M colchicine-fed larvae, did not allow the dye to accumulate (B), indicating an up-regulation in the function of P-gp. However, 20 μ M verapamil feeding resulted in high rhodamine accumulation (C), suggesting that its efflux is inhibited after verapamil treatment. Mean fluorescence intensity was calculated and represented in the form of a graph showing significant reduction in accumulation of rhodamine-123 after colchicine feeding (D, 2) and increase after verapamil feeding (D, 3). Kruskal–Wallis one-way analysis of variance on ranks was applied and differences were found to be significant at P -value <0.05. Bar, 100 μ m.

UAS-127Q/CyO flies, the regular arrangement was disrupted progressively from mild (Figure 1B) to moderate (Figure 1C) and severe (Figure 1D). The flies with eyes in the mild category had fewer necrotic patches and less ommatidial degeneration in one of the eyes, whereas flies in the moderate category had eyes with more patches in both eyes, and in the severe category, flies showed increased degeneration and necrosis in both eyes. In a few cases, the size of the eyes expressing polyQ aggregates was smaller than the wild-type control (data not shown).

To understand the changes in eye phenotype, the arrangement of ommatidia was studied using the nail polish imprint technique (Arya and Lakhota 2008), which reflects their arrangement. Each ommatidia consists of closely packed stacks of microvilli, called “rhabdomeres,” projecting into interommatidial space. These are highly amplified and structured apical cell membranes just like outer segments of vertebrate rods and cones (Kumar and Ready 1995). The wild-type adult eyes (Figure 1E) showed a normal array of hexagonal lattice, whereas the adult eyes with polyQ mis-expression exhibited loss of ommatidial clusters and regular spacing due to multiple fusions. The mild eye phenotype had less fused ommatidia (Figure 1F) in comparison to moderate (Figure 1G) and severe types (Figure 1H). To exclude the role of *CyO* balancer in eye degeneration, we also examined the degeneration in *GMR-GAL4/UAS-127Q* flies and obtained identical results (Supporting Information, Figure S1). To avoid setting crosses every time, we continued using *GMR-GAL4 UAS-127Q/CyO* flies.

Before examining the effect of chemicals on P-gp levels and consequent effect on eye degeneration, we examined

the effect of colchicine and verapamil on wild-type eye discs. The morphology of eye imaginal discs from wild-type larvae grown on 10 μ M colchicine and 20 μ M verapamil were checked by staining with phalloidin and DAPI. It was found that none of the chemical treatments affected the morphology of eye discs as compared to control larval eye discs, suggesting that these chemicals do not affect the cells adversely if used in appropriate concentrations (Figure S2).

We also confirmed whether these chemicals altered the function of ABC transporters in the eye discs of wild-type larvae using rhodamine-123, a P-gp substrate (Altenberg *et al.* 1994). It was found that in larvae fed on 10 μ M colchicine, there was an increased efflux of rhodamine-123, with no staining in eye discs, suggesting an enhanced expression of P-gp by colchicine (Figure 2B). On the other hand, treatment with 20 μ M verapamil, which is a P-gp inhibitor (Callaghan and Denny 2002; Summers *et al.* 2004), resulted in a significantly increased accumulation of rhodamine-123 (Figure 2C) as compared to wild type (Figure 2A), suggesting a loss of P-gp function. We also measured the fluorescence intensity using the Histogram tool of Zeiss LSM Meta 510 software and found statistically significant decrease in fluorescence after colchicine treatment and increase after verapamil treatment (Figure 2D).

Having confirmed that these drugs do not have undesired effects on eye discs and affect P-gp function, we looked at the changes in eye phenotype after 10 μ M colchicine treatment. After feeding first instar larvae of *GMR-GAL4 UAS-127Q/CyO* flies on food containing 10 μ M colchicine, a significant increase in the number of flies having mild eye phenotype was observed as compared to larvae fed on

normal food (Figure 3). In normal food, the mean percentage of mild eye flies was $30.36 \pm 0.2\%$, which increased significantly up to $54.12 \pm 0.1\%$ after 10 μM colchicine feeding. Consequently there was a significant decrease in the moderate and severe eye phenotype after colchicine feeding. To find out if a further increase in colchicine concentration improved the eye degeneration, the larvae were fed on 20 μM colchicine. Contrary to the expected results, it was found that 20 μM colchicine concentration increased the percentage of flies with severe eye phenotype and decreased the mild eye phenotype. This unexpected observation could be because of additional toxicity caused by increased levels of colchicine. Thereafter, only 10 μM colchicine was used for further experiments. On feeding larvae with 20 μM verapamil, it was observed that there was a significant decrease in the number of flies with mild and moderate eye phenotype (Figure 3) and a significant increase in the number of flies with severe eye phenotypes. Thus it can be said that colchicine and verapamil induced contrasting changes in the eye phenotype and, since both of them affected P-gp, it can be said that P-gp plays a role in degeneration caused by expanded polyQ peptides. These results demonstrated a functional link between P-gp and polyQ.

P-gp alters the levels of polyQ aggregates and rhabdomere arrangement

Having observed the changes in eye degeneration after feeding *GMR-GAL4 UAS-127Q/CyO* first instar larvae on colchicine and verapamil, we looked at the inclusion bodies and rhabdomere organization. Eye imaginal discs from third instar larvae of *GMR-GAL4 UAS-127Q/CyO* and wild type were immunostained with anti-HA antibody and anti-ELAV antibody to identify polyQ aggregates and rhabdomeres, respectively. In the wild type, there were no polyQ inclusion bodies and therefore no staining with anti-HA antibody was observed (Figure 4, A and E), however in *GMR-GAL4 UAS-127Q/CyO* larvae fed on normal food, distinct inclusion bodies were observed (Figure 4B). A marked decrease in the level of polyQ inclusion bodies was observed when larvae were fed on 10 μM colchicine (Figure 4C) compared to larvae grown on normal food (Figure 4B). An opposite effect was observed when these larvae were fed on verapamil food, where the number of polyQ inclusion bodies was greatly enhanced (Figure 4D). At a higher magnification, it was observed that the size as well as number of inclusion bodies were reduced after 10 μM colchicine feeding (Figure 4G) as compared to those fed on normal food (Figure 4F). On the other hand, the inclusion bodies appeared to be very large and increased in number after verapamil feeding (Figure 4H). It was also checked whether these results were a direct effect of P-gp on polyQ aggregates or because of the effect of colchicine or verapamil on the normal function of the UAS-GAL4 system. To confirm this, first instar larvae from *GMR-GAL4/UAS-GFP* were fed on normal food and on food containing 10 μM colchicine and 20 μM verapamil, and third instar larval eye discs were observed for any

Figure 3 Colchicine and verapamil feeding alters the eye degeneration phenotypes. *GMR-GAL4 UAS-127Q/CyO* when grown on normal food had a maximum number of flies in the moderate category. On 10 μM colchicine feeding, a significant increase in mild eye phenotype and a decrease in moderate eye phenotype were observed. The least number of flies was observed in the severe eye category. Treatment with 20 μM colchicine significantly decreased the percentage of flies with mild eye phenotype and enhanced moderate and severe eye phenotypes. On feeding with 20 μM verapamil, a significant increase in the percentage of flies in the severe eye category and a decrease in the mild and moderate eye categories were observed. The experiment was done in replicates of five with 410–450 flies in each group; data were pooled and mean percentage of flies was subjected to multiple comparisons vs. control by using the Holm–Sidak method and the differences were found to be statistically significant. * $P \leq 0.05$, statistically significant.

changes in the level of GFP expression. It was observed that GFP levels were not altered after the chemical treatments (Figure S3), which clearly showed that P-gp affected polyQ aggregate formation. On observing the rhabdomeres, it was found that they were regularly arranged in wild type (Figure 4I), which was lost in *GMR-GAL4 UAS-127Q/CyO* eye imaginal discs when fed on normal food (Figure 4J). However after colchicine feeding (Figure 4K), the arrangement of rhabdomeres improved and was very much like wild type. On the other hand, verapamil-fed larvae had severely degenerated rhabdomere arrangement (Figure 4L). The increase or decrease in the fluorescence intensities of polyQ aggregates in eye imaginal discs was quantified by measuring the mean fluorescence intensity using the Histogram tool of Zeiss LSM Meta 510 software (Figure 4M). It was found that the mean intensity of polyQ aggregates was significantly low in 10 μM colchicine-fed larvae and increased after 20 μM verapamil feeding when compared to larvae fed on normal food. These results clearly correlated the extent of degeneration to the amount of polyQ inclusion bodies present.

P-gp knockdown aggravates 127-Q mediated degeneration in adult eyes

To validate the results obtained by colchicine and verapamil feeding, we next depleted P-gp levels genetically. The three *mdr* genes, *mdr49*, *mdr50*, and *mdr65* were down-regulated by expressing their respective RNAi constructs.

Figure 4 Alteration in the polyQ inclusion bodies and arrangement of rhabdomeres following changes in P-gp. Eye imaginal discs immunostained with antibodies against polyQ showed that polyQ is not expressed in wild type (A and E). Eye discs from *GMR-GAL4 UAS-127Q/CyO* larvae grown on normal food showed the presence of polyQ aggregates (B), which decreased when larvae were fed on 10 μ M colchicine (C) and increased after 20 μ M verapamil treatment (D). At high magnification, the changes in inclusion bodies were very distinct (F–H). Normal arrangement of rhabdomeres was observed in wild-type larvae (I) after immunostaining with anti-ELAV antibody. Eye discs from *GMR-GAL4 UAS-127Q/CyO* showed fused, degenerated rhabdomeres (J). Improvement in rhabdomere arrangement was observed after 10 μ M colchicine feeding (K) and severe degeneration was observed after feeding on 20 μ M verapamil (L). All images are projections of optical sections obtained by confocal microscope. Bar, 50 μ m, except in E–L, which is 5 μ m. Mean fluorescence intensity of polyQ aggregates showed a significant reduction after feeding on 10 μ M colchicine and an increase after feeding on 20 μ M verapamil when compared to the polyQ aggregates grown on normal food (M). Staining was done in replicates of six with 20 eye discs in each group. One-way ANOVA was applied using the Holm–Sidak method, where significance is represented as *** $P \leq 0.001$.

The down-regulation in expression of *mdr* genes after *mdr-RNAi* was confirmed via reverse transcription–PCR after driving them with *GMR-GAL4* (Figure S4). We coexpressed *mdr49-RNAi*, *mdr50-RNAi*, and *mdr65-RNAi* one at a time along with *GMR-GAL4 UAS-127Q/CyO* and observed the effects. Adult eyes were screened for degeneration and categorized as mild, moderate, or severe. It was observed that there was a significant reduction in mild eye phenotype when one copy of the *RNAi* of each *mdr* transgene was expressed. The mean percentage of mild eye phenotype flies declined from 29.4 ± 0.7 in *GMR-GAL4 UAS-127Q/CyO* to 10.6 ± 0.2 , 21.08 ± 1.0 , and 23.31 ± 1.0 when *mdr49-RNAi*, *mdr50-RNAi*, and *mdr65-RNAi* (Figure 5A), respectively, were

coexpressed. The decrease in mild eye phenotype shifted the graph toward the severe eye phenotype, which increased from 28.0 ± 0.6 in *GMR-GAL4 UAS-127Q/CyO* to 41.7 ± 2.3 , 35.3 ± 0.3 , and 29.73 ± 1.8 when *mdr49-RNAi*, *mdr50-RNAi*, and *mdr65-RNAi* (Figure 5C), respectively, were coexpressed. We did not, however, observe any significant difference in the moderate eye phenotypes (Figure 5B). Nail polish eye imprints of these flies indicated the increased destruction and loss of ommatidial arrays in *mdr-RNAi* background (data not shown). On comparing the effects between different *mdr-RNAi*, we did observe a variation in the extent of effect of each *mdr* gene in altering the polyQ phenotype. It was observed that *mdr49* had a maximum effect followed by

Figure 5 PolyQ-mediated degeneration is aggravated after *mdr*-RNAi. Coexpression of *mdr*-RNAi with UAS-127Q aggravated the degeneration. There was a significant reduction in mild eye phenotype (A) in *mdr49-RNAi* when compared to only UAS-127Q expressing alone. Reduction was also in *mdr50-RNAi* and *mdr65-RNAi* but to a lesser degree than *mdr49-RNAi*. The difference in moderate eye phenotypes after coexpression of polyQ was not significant as compared to expression of polyQ alone (B). Severe eye phenotype increased significantly after *mdr49-RNAi* compared to *mdr50-RNAi* and *mdr65-RNAi* (C). Scoring was done in replicates of three with 600 flies in each group. One-way ANOVA was applied using the Holm-Sidak method. Significance is represented as *** $P \leq 0.001$ and ** $P \leq 0.005$, respectively.

mdr50 and the least effect was by *mdr65*, suggesting that *mdr49* and *mdr50* could be having a more direct role in polyQ-mediated pathogenesis. This can also be related to the functional differences between these genes (Mayer *et al.* 2009; Ricardo and Lehmann 2009). On comparing the observations, it was found that *mdr65-RNAi* background showed a significant decrease in mild phenotype just like *mdr49-RNAi* and *mdr50-RNAi*. However, *mdr65-RNAi* background did not bring a significant increase in the number of severe eye phenotype as seen in the case of *mdr49-RNAi* and *mdr50-RNAi*, although the extent of the degeneration with *mdr65-RNAi* was more than the flies expressing polyQ aggregates alone. The variations in numbers of flies in different categories were subjected to one-way analysis of variance by the Holm-Sidak method, and they were found to be significant for mild and severe eye phenotypes, but not for moderate eye phenotype. We checked whether this phenotype is an RNAi artifact by expressing *UASbhl-RNAi* in conjunction with polyQ aggregates. We did not observe any difference from the control, suggesting that these findings are P-gp specific (Figure S5). These results suggested that P-gps do have a role in polyQ-mediated neurotoxicity.

***mdr*-RNAi affected the intensity and frequency of polyQ aggregates and rhabdomere arrangement**

Having observed that reduction in *mdr* genes affected the eye phenotypes, we examined the polyQ inclusion bodies and rhabdomere arrangement in the eyes. The inclusion bodies were not observed in wild type (Figure 6, A and F). Enhanced accumulation of inclusion bodies was observed in *mdr49-RNAi* (Figure 6C), *mdr50-RNAi* (Figure 6D), and *mdr65-RNAi* (Figure 6E) compared to *GMR-GAL4 UAS-127Q/CyO* (Figure 6B), suggesting that decreased P-gp

levels increased the number of inclusion bodies. At higher magnification, these results appeared more convincing and the inclusion bodies appeared more globular in *mdr49-RNAi* (Figure 6H) and less in *mdr50-RNAi* (Figure 6I) and *mdr65-RNAi* (Figure 6J), compared to only *GMR-GAL4 UAS-127Q/CyO* (Figure 6G). To reconfirm that *mdr* depletion affected polyQ aggregation and not colchicine feeding (as shown above), we genetically depleted the levels of each *mdr* via *mdr*-RNAi in the larvae expressing polyQ and fed them on 10 μ M colchicine. Eye discs from these larvae showed similar level of polyQ aggregates in the case of *mdr49-RNAi* and *mdr50-RNAi*. However in the case of *mdr65-RNAi*, 10 μ M colchicine feeding resulted in a low number of aggregates (Figure S6). The difference in the polyQ aggregation among different RNAi's were due to their functional differences (see Discussion).

When the organization of rhabdomeres was observed, it was found that in *GMR-GAL4 UAS-127Q/CyO* the rhabdomeres were disrupted (Figure 6L), compared to wild type (Figure 6K). Expressing each of the *mdr*-RNAi along with polyQ, disrupted the rhabdomere assembly further. Coexpression of *mdr49-RNAi* (Figure 6M) caused the rhabdomeres to be more fused and degenerated and similarly coexpression of *mdr50-RNAi* (Figure 6N) also resulted in disarrangement and loss of neurons but to a lesser extent than *mdr49-RNAi*. The least degeneration was observed in the case of *mdr65-RNAi* (Figure 6O). The difference in fluorescence intensities of polyQ alone or in conjunction with *mdr*-RNAi was measured using the LSM Image Examiner tool of the Zeiss confocal microscope (Figure 6P). It was observed that fluorescence intensities of polyQ aggregates increased when expressed in conjunction with *mdr*-RNAi for all the *mdr* genes. Apart from increased polyQ expression, it was also observed that expressing *mdr*-RNAi in polyQ background affected the shape of eye imaginal discs, making them either very rudimentary or larger than the eye discs expressing polyQ aggregates alone (data not shown). These results provided a direct correlation between polyQ and P-gp.

PolyQ reduces the transcription of *mdr* genes

To validate that phenotypic alterations in *GMR-GAL4 UAS-127Q/CyO* were due to altered P-gp levels, we checked the expression of *mdr* transcripts in *GMR-GAL4 UAS-127Q/CyO* under different conditions. Semi-quantitative RT-PCR was carried out in third instar (115–120 hr) larval eye discs for *mdr49*, *mdr50*, and *mdr65* genes (Figure 7). It was observed that expression levels of *mdr49*, *mdr50*, and *mdr65* were similar in *Oregon R*⁺ (lane A) and UAS-127Q undriven (lane B), which were used as controls; however, the expression of *mdr50* was highest in comparison to *mdr49* and *mdr65* in both the controls. In *GMR-GAL4 UAS-127Q/CyO* grown on normal food (lane C), it was observed that the expression of *mdr49* and *mdr50* was reduced significantly in comparison to *Oregon R*⁺ and UAS-127Q undriven. The decrease suggested that polyQ aggregates somehow controlled the transcriptional activity of P-gp genes and repressed transcription.

Figure 6 Expression of *mdr-RNAi* increased the polyQ inclusion bodies and disrupted rhabdomeres in eye discs. The number of inclusion bodies was greatly enhanced when polyQ was coexpressed with *mdr49-RNAi* (C), *mdr50-RNAi* (D), and *mdr65-RNAi* (E) in comparison to only polyQ expression (B). In wild type, no polyQ was observed (A and F). At higher magnification, the number and size of inclusion bodies was more in all the three *RNAi* lines (H–J) as compared to only polyQ (G). Degeneration and fusion of rhabdomeres was observed after coexpression of polyQ with *mdr49-RNAi* (M), *mdr50-RNAi* (N), and *mdr65-RNAi* (O) in comparison to eye discs expressing polyQ alone (L). The arrangement of rhabdomeres was normal in wild type (K). Inclusion bodies were observed by immunostaining with anti-HA antibody and rhabdomeres were observed by anti-ELAV antibody. Mean fluorescence intensities of polyQ aggregates (P) after down-regulation of P-gp via *mdr-RNAi* were calculated and a significant increase in fluorescence intensity was observed when UAS-127Q was coexpressed with *mdr49-RNAi*, *mdr50-RNAi*, and *mdr65-RNAi*. Staining was done in triplicate and number of eye discs scored was 35 in each group. Significant difference is represented as $P \leq 0.05$, using Kruskal–Wallis one-way analysis of variance on ranks.

Contrary to *mdr49* and *mdr50*, the level of *mdr65* transcripts in *GMR-GAL4 UAS-127Q/CyO* was more than *Oregon R⁺* and UAS-127Q undriven, suggesting that *mdr65* regulation was different from *mdr49* and *mdr50*. Upon 10 μ M colchicine feeding (lane D), the expression of *mdr49*, *mdr50*, and *mdr65* increased significantly, whereas no change was observed after 20 μ M verapamil (lane E) feeding. *Glyceraldehyde-3-phosphate dehydrogenase (GPDH)* was used as an internal control to ensure the integrity of RT-PCR and equal loading. RT-PCR results were quantified by plotting an intensity graph (Figure 7F), which showed that the basal levels of expression for each of the three genes were different under normal condition. Expression of *mdr50* was highest followed by *mdr49* and *mdr65* under normal conditions. However polyQ aggregates repressed the transcription of *mdr49* and *mdr50* but not *mdr65* genes. These results clearly demonstrated that polyQ affected the transcription of *mdr* genes.

Inhibition of P-gp via verapamil feeding or *mdr-RNAi* results in disrupted axonal connections

The eight photoreceptor cells of *Drosophila* compound eyes have different spectral sensitivity and neuronal connectivity (Newsome *et al.* 2000). The eight photoreceptor axons from these cells bundled together to form ommatidial fascicles, which extended to the optic stalk to reach the brain. Later they spread out on the lateral surface of the brain before turning to optic lobes. The projections of photoreceptor axons into the optic lobes reflect the differentiation of the photoreceptors in the eye disc (Newsome *et al.* 2000). Any inadequacy during earlier differentiation of photoreceptors leads to the connectivity defects in the axonal projections. We examined the axonal connections in *GMR-GAL4 UAS-127Q/CyO* larvae when fed on normal food (Figure 8B) and compared it with *Oregon R⁺* (Figure 8A). It was observed that the axonal connections were disrupted when

Figure 7 Expression of *mdr* genes in *GMR-GAL4 UAS-127Q/CyO* and after chemical treatments. RT-PCR analysis of *Oregon R*⁺ (A) and *UAS-127Q* undriven (B) showed almost similar levels of *mdr49*, *mdr50*, and *mdr65* genes, although the expression of *mdr50* was much higher than *mdr49* and *mdr65*. In *GMR-GAL4 UAS-127Q/CyO* larvae fed on normal food (C), there was a decrease in transcription level of *mdr49* and *mdr50* compared to both the controls, while there was an increase in *mdr65*. Feeding on 10 μ M colchicine enhanced the expression of all three genes (D), whereas feeding on 20 μ M verapamil did not have any effect (E). Quantitative analysis of expression showed significant changes in *mdr* genes after different treatments (F). Glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) is used as an internal control to ensure the integrity of RT-PCR. For statistical analysis, the Holm-Sidak method was used for multiple comparisons vs. control group. Significance is represented as *** $P \leq 0.001$ and ** $P \leq 0.005$, respectively.

polyQ was expressed in the eyes, as compared to *Oregon R*⁺, suggesting that increase in polyQ aggregates affected the axonal connections. However, after 10 μ M colchicine feeding, there was a remarkable improvement in the continuity of the axonal connections (Figure 8C) and severe degeneration of axonal bundles in the larvae fed on 20 μ M verapamil (Figure 8D). The disruption in axonal connections could be due to increase in inclusion bodies in axons, which may disrupt the cytoskeleton. Since actin filaments are responsible for polarized trafficking of axonal connections (Newsome *et al.* 2000), we checked the arrangement of actin filaments in the eye discs and observed that expression of polyQ aggregates caused discontinuity and misarrangement of actin filaments. They were disordered in *GMR-GAL4 UAS-127Q/CyO* larvae fed on normal food (Figure 8F), compared to regular organization in wild type (Figure 8E). This damage in actin assembly improved after 10 μ M colchicine feeding (Figure 8G), whereas 20 μ M verapamil feeding completely disorganized them (Figure 8H). We also observed the axonal connections after *mdr-RNAi* was coexpressed with *GMR-GAL4 UAS-127Q/CyO*. Maximum degeneration of axonal connections was observed with *mdr49* depletion (Figure 8I) compared to *mdr50* (Figure 8J) and *mdr65* depletion (Figure 8K). It was also checked whether expression of *mdr-RNAi* itself leads to degeneration in axonal connection and it was found that in *GMR-GAL4* driven *mdr-RNAi*, the arrangement of axons was normal and same as wild-type control, and it did not show any deterioration, suggesting that increase in axonal degeneration of polyQ-expressing eye discs after expression of *mdr-RNAi* was due to

interaction of polyQ and *mdr* (Figure S7 A–D). These results confirmed that polyQ aggregates disrupted axonal guidance and organization of actin filaments and that are affected by P-gp expression levels.

Verapamil-mediated increase in polyQ level destroys the arrangement of adherens junctional proteins

Armadillo is a core component of the adherens junction and very dynamic in nature. It is a *Drosophila* homolog for β -catenin, whose levels change during eye morphogenesis, depending on the need of cell–cell adhesion during cellular rearrangement. In eye development, these complexes are important for the alignment of rhabdomeres to the ommatidial optical axis and are mediated by cellular junctions containing adherens junction proteins (Menzel *et al.* 2007). A number of other reports have shown that β -catenin also interacts with polyQ aggregates (Godin *et al.* 2010), so it was of much interest to check the expression of Armadillo by immunostaining the eye discs with antibodies against Armadillo, under various conditions. In wild-type eyes, the interommatidial precursor cells made a precise pattern in interommatidial lattice (Figure 9A), which was disrupted in *GMR-GAL4 UAS-127Q/CyO* larvae grown on normal food (Figure 9B), and Armadillo appeared to be scattered in the intercellular matrix, indicating severe degeneration of the adherens junctions. However, 10 μ M colchicine feeding (Figure 9H) improved the arrangement of Armadillo and the interommatidial precursor cells were arranged more or less similar to that of wild type. Similarly, it was found that after inhibition of P-gp by verapamil treatment, there was an increased disarrangement

Figure 8 PolyQ aggregation leads to defects in neuronal connections and F-actin organization. Immunostaining of eye imaginal discs with mab-22c10 antibody showed regular arrangement of neuronal connections in wild type (A). Expression of polyQ aggregates in eye imaginal discs of *GMR-GAL4 UAS-127Q/CyO* larvae resulted in disruption of these axonal connections (B). Feeding *GMR-GAL4 UAS-127Q/CyO* larvae on 10 μ M colchicine improved the connections (C), which were similar in appearance to wild type, whereas after feeding on 20 μ M verapamil there was further degeneration of these connections (D). Observation of actin organization by phalloidin staining showed a regular pattern in wild type (E) and disruption in *GMR-GAL4 UAS-127Q/CyO* larvae (F). This organization improved after colchicine feeding (G) and deteriorated further after verapamil feeding (H). On expression of *mdr49-RNAi* (I), *mdr50-RNAi* (J), *mdr65-RNAi* (K), along with *UAS-127Q*, de-

teriorated the neuronal connections further. The images are single confocal sections. Bar, 5 μ m in A–K. Staining was done in triplicate and number of eye discs used in each group was 25 in each experiment.

in the coordinated assembly of adherens junctions, as well as an increase in the free form of Armadillo (Figure 9D). When P-gp was depleted via *mdr-RNAi*, severe disarrangement of interommatidial lattice and an enormous increase in free Armadillo proteins were observed. Maximum defects were seen in *mdr49-RNAi* (Figure 9E) followed by *mdr50-RNAi* (Figure 9F) and *mdr65-RNAi* (Figure 9G). We checked whether depletion of P-gp itself caused disarrangement of adherens junctions in *GMR-GAL4* driven *UASmdr-RNAi* larvae and found that there was no change in the arrangement of adherens junctions or in the level of free Armadillo, indicating that observed results were entirely due to expression of polyQ aggregates in conjunction with *mdr-RNAi* (Figure S7 E and F). These results suggested that the increased polyQ and decreased P-gp could be destabilizing the adherens junctions and releasing free Armadillo; as a result, increased Armadillo was observed in cells. This finding was similar to an earlier report that stated that a cytoplasmic, nonphosphorylated form of β -catenin increases after expression of polyQ tract in mutant huntingtin protein (Godin *et al.* 2010).

Discussion

The *Drosophila* visual system is a promising system for the genetic analysis of several biological processes and diseases.

The present study exploits the recent advances in ectopically induced neurodegeneration condition in eyes to correlate with the role of P-gp in polyQ-mediated pathogenesis. The evidence presented in this article points to increased P-gp being responsible for the decrease in the pathogenesis caused by polyQ aggregates, suggesting that polyQ aggregates could be substrates of P-gp. This is supported by the fact that polyQ aggregates and β -amyloid plaques both exhibit classical β -sheet-rich circular dichroism spectra (Chen *et al.* 2002) and that P-gp is directly responsible for the efflux of β -amyloid plaques (Lam *et al.* 2001). On the other hand, reduction in P-gp aggravated the diseased phenotype as observed by using verapamil and also at the genetic level. Down-regulation of *mdr* genes enhanced the toxicity caused by polyQ aggregates; however, the effects of all three *mdr* genes were not identical, with *mdr49-RNAi* in polyQ background exhibiting maximum degeneration and *mdr65-RNAi* causing the least. This differential effect may be attributed to the functional differences between the three genes. It has also been reported earlier that *mdr49* and *mdr65* differentially respond to colchicine, where *mdr49* is up-regulated but *mdr65* is not affected (Tapadia and Lakhotia 2005). DNA sequence alignment indicates that both *mdr49* and *mdr65* have 50 to 52% nucleic acid identity to the entire coding region of human *mdr1a* and *mdr1b* cDNAs (Wu *et al.*

Figure 9 Expanded polyQ aggregates disrupt the adherens junctions and Armadillo organization. Wild-type eye (*Oregon R+*) with no aggregates of polyQ showed distinct patterns of interommatidial lattice (A), but expression of polyQ aggregates in *GMR-GAL4 UAS-127Q/CyO* on normal food showed scattered Armadillo protein and disruption of proper arrangement (B). Flies fed on 10 μ M colchicine showed improved arrangement of adherens junction, and a very low level of Armadillo was found to be scattered in the intercellular spaces (C). On the other hand, verapamil treatment disarranged the assembly of adherens junctions as well as increased the free form of Armadillo (D). Expression of *mdr-RNAi* in polyQ background led to disrupted adherens

junction protein and increased free and unarranged Armadillo in *GMR-GAL4 UAS-127Q/+; mdr49-RNAi/+* (E), *GMR-GAL4 UAS-127Q/+; mdr50-RNAi/+* (F), and *GMR-GAL4 UAS-127Q/+; mdr65-RNAi/+* (G). The images are projections of optical sections taken by confocal microscope. Bar, 5 μ m. Staining was done in triplicate and number of eye discs used in each group was 25 in each experiment.

1991). However, functionally *mdr49* is more similar to human *mdr1* and mouse *mdr1* and *mdr3* genes, which are capable of conveying multidrug resistance (Wu *et al.* 1991). In our studies too, we found that *mdr49* is functionally more responsive to stresses than *mdr65*. Not many studies have been done on *mdr50* but from the present results, it seems that *mdr50* is functionally similar to *mdr49*. Under normal circumstances the inclusion bodies are present in the nucleus and cytoplasm; however, the expanded polyQ inclusion bodies tend to aggregate in the nucleus where they interfere with the transcriptional machinery (Lamark and Johansen 2012). In the present results, too, we find transcriptional repression of *mdr49* and *mdr50* following aggregation of polyQ in the nucleus, which is consistent with an earlier report of transcriptional repression of the *MDR1* gene by expanded *htt* construct in human cell lines (Steffan *et al.* 2000; Friedman *et al.* 2008). Under normal circumstances, p53 represses *MDR1*; however, repression by mutant *htt* was found to be independent of p53 (Thottassery *et al.* 1997), suggesting that expanded *htt* mediated transcriptional repression in a manner similar to p53. Thus it appears that neuronal intranuclear inclusions inhibit transcription of p53-regulated genes such as *MDR-1* (Steffan *et al.* 2000). The expression of *mdr65*, on the contrary, was enhanced, which could be responsible for reduced eye degeneration in *mdr65-RNAi* progeny. The increase in *mdr65* could be a compensatory mechanism that a cell employs when one of the genes is down-regulated. After 10 μ M colchicine feeding, the expression of all *mdr* genes gets enhanced and results in improvement in eye phenotypes. Similarly inhibition of P-gp by verapamil feeding mimicked the *mdr49-RNAi* and *mdr50-RNAi* phenotypes. Since verapamil interacts with

P-gp at the protein level and inhibits its activity, we do not observe a significant decrease in the transcription of *mdr* genes after verapamil treatment.

The development of a neuronal connectivity pattern that characterizes a mature nervous system is generated with great precision. The differentiating neurons send out axons that find their appropriate synaptic targets (Newsome *et al.* 2000) and this axonal integrity is essential for the function of the neurons. Axonal growth and guidance is governed by actin filaments and microtubules, and the correct path of the growing axons is ensured by the growth cone, which is maintained by the dynamic cytoskeleton. Normal axonal transport is mandatory for transport of neuronal cargoes responsible for viability and other functions (Stokin and Goldstein 2006). Earlier it has been shown that polyQ aggregates localize in the neuronal processes, such as axons and dendrites, which lead to axonal degeneration (Lee *et al.* 2004; Morfini *et al.* 2009). We speculate that disruption of axonal connection in *GMR-GAL4 UAS-127Q/CyO* larvae grown on normal food could be because of disorganization of actin filaments due to reduction in P-gp levels, which may destabilize actin organization. P-gp is known to interact with actin filaments via specialized molecules known as ezrin/radixin/moesin (ERM) proteins, which cross-link actin filaments with the plasma membrane and are involved in determining cell polarization (Luciani *et al.* 2002). Thus it is possible that in neurodegenerative diseases, low expression of *mdr* genes causes imbalance of P-gp–actin interaction, and destabilization of actin organization results in disrupted axonal connections. The present results also show that polyQ expression in eye discs leads to disruption of both actin filaments as well as axonal connections. Moreover

Figure 10 Model representing the role of P-gp and β -catenin in polyQ-mediated pathogenesis. In a neuron under normal circumstances, β -catenin regulates transcription of *mdr* genes, resulting in accumulation of enough P-gp on the membrane to interact with actin as well as β -catenin (A). However, in the presence of mutant polyQ, a nonactive form of β -catenin accumulates in the cytoplasm and polyQ aggregates enter the nucleus where it inhibits the transcription of *mdr* genes, resulting in a low level of P-gp in the membranes. The low level of P-gp in the membrane destabilizes the actin– β -catenin–cadherin complex; as a result, free β -catenin accumulates in the cell (B).

the expression of *mdr*-RNAi in conjunction with polyQ deteriorated the condition even more, suggesting the involvement of P-gp in polyQ-mediated pathogenesis.

Cell adhesion is essential for spatial organization of interommatidial lattice of *Drosophila* eyes (Hayashi and Carthew 2004) and the function of this interommatidial lattice is to organize and optically insulate the ommatidial arrays. Cell–cell adhesion is maintained by linking the cytoplasmic domain of cadherins, which are cell adhesion molecules, to actin cytoskeleton (Halbleib and Nelson 2006). Catenins, α - and β -, are characterized as important molecules that mediate this cross-linking (Orsolic *et al.* 1999). Later on, β -catenins were also identified to participate in transducing signals within the Wnt pathway (Caricasole *et al.* 2005). In the absence of Wnt signaling, cytoplasmic β -catenin is constantly degraded by axin complex, which includes adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3), resulting in phosphorylation and proteosomal degradation of β -catenin (He *et al.* 2004). Presence of Wnt signaling prevents degradation of β -catenin and allows its transportation inside the nucleus followed by transcription of Wnt responsive genes. Thus, β -catenin homeostasis is maintained in the cell by either being a part of the cell adherens junction or as a part of the Wnt signaling complex (MacDonald *et al.* 2009). The *Drosophila* homolog of β -catenin is Armadillo, which is present at cellular junctions to form a multiprotein complex involving cell adhesion and anchoring actin filaments (Oda

et al. 1993). Absence of Armadillo protein is shown to cause disruption of cell–cell adhesion and integrity of the actin cytoskeleton (Pai *et al.* 1996). Here we show that polyQ aggregation results in disintegration of adherens junctions and an increase in cytoplasmic Armadillo. However, this increase does not seem to correlate with enhanced P-gp transcript, although it has been shown that *MDR1* is one of the candidate genes of the Wnt pathway (Correa *et al.* 2012). Even in cancer cell lines, it has been shown that β -catenin up-regulates *mdr1* (Liu *et al.* 2010). Thus, it seems that although Armadillo levels increase, they are not correlated with an increase in transcriptional activity. Similar results have been obtained in Huntington's disease; mutant htt protein is shown to up-regulate the level of phosphorylated cytoplasmic content of β -catenin, which does not increase the transcription of downstream target genes (Godin *et al.* 2010). Wnt signaling controls the level of β -catenin by regulating its synthesis and degradation and maintains the free β -catenin level in the nucleus so as to transcribe the Wnt responsive genes (Correa *et al.* 2012). In relation to the polyQ expansion proteins, it has been shown that mutant htt protein interferes with the β -catenin destruction complex. This leads to toxic stabilization of β -catenin, which is lethal to the striatal neurons (Godin *et al.* 2010). We show that inhibition of P-gp leads to disruption of the adherens junction assembly and an enormous increase in free Armadillo/ β -catenin level, either by feeding on verapamil or by *mdr*-RNAi. P-gp is known to interact with actin and Armadillo

(Lim *et al.* 2006) and some groups have shown that increase in P-gp levels has a direct correlation with GSK3 inhibition followed by β -catenin nuclear localization (Lim *et al.* 2006). So the present study functionally links P-gp, polyQ aggregates, and Armadillo in the pathogenesis of neurodegeneration. From the present results, we hypothesize that impairment of P-gp levels reduces Armadillo at the adherens junctions and also disrupts actin filaments. Whether the dispersion of Armadillo is the cause or effect of actin disruption is still to be elucidated. Mutant polyQ proteins also contribute to cytoplasmic accumulation of Armadillo in two ways, first, by inhibiting its degradation and second, by preventing its nuclear translocation. Mutant polyQ also inhibits P-gp transcription either directly or by inhibiting Armadillo. Reduction of P-gp levels on the membranes could destabilize Armadillo as well as actin filaments, which may contribute to degeneration of rhabdomeres as well as neuronal connections.

On the basis of these results, we propose a model (Figure 10), which states that in a neuronal cell, under normal circumstances, β -catenin regulates transcription of P-gp, which results in accumulation of enough P-gp on the membrane to interact with actin as well as β -catenin. However, in the presence of mutant polyQ, a nonactive form of β -catenin accumulates in the cytoplasm and polyQ aggregates enter the nucleus where it inhibits the transcription of *mdr* genes (Friedman *et al.* 2008). This results in lowered P-gp transcription, which reduces P-gp in the membranes. The low level of P-gp in the membrane destabilizes the actin- β -catenin-cadherin complex; as a result, free β -catenin accumulates in the cell. Thus, altering P-gp levels can alter the extent of degeneration caused by accumulation of polyQ.

This study identifies a novel strategy to control neurodegenerative diseases by manipulating P-gp levels. It can be used as an effective therapeutic approach for ameliorating neurodegenerative disease.

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Supporting Information

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Neurodegeneration Caused by Polyglutamine Expansion Is Regulated by P-Glycoprotein in *Drosophila melanogaster*

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Figure S1 *CyO* balancer chromosome has no role in eye degeneration. Eye imaginal discs immunostained with antibodies against polyQ showed no difference in number of aggregates and arrangement pattern of polyQ aggregates of crosses *GMR-GAL4 UAS-127Q/CyO* (A) and *GMR-GAL4/UAS-127Q* (B), suggesting that *CyO* balancer does not cause eye degeneration. C and D represent the magnified view of *GMR-GAL4 UAS-127Q/CyO* and *GMR-GAL4/UAS-127Q* respectively. Arrangement of rhabdomeres was observed in *GMR-GAL4 UAS-127Q/CyO* (E) and *GMR-GAL4/UAS-127Q* (F) larvae and in both the cases same level of degeneration was observed. Scale bar = 50 μ m for A and B, 5 μ m for C to F.

Figure S2 Colchicine and verapamil do not affect the cellular morphology of eye discs. Eye imaginal discs from wild type (*Oregon R*⁺) 3rd instar larvae grown on normal food were stained with phalloidin (A and D) and counterstained with DAPI showed normal F-actin arrangement, (G) shows the merged image of phalloidin with DAPI. Eye discs of larvae grown on 10µM colchicine showed no difference in arrangement of actin filaments (B and E), (H) shows the merged image of phalloidin and DAPI. Similarly 20µM verapamil also did not affect the integrity and arrangement of eye discs as shown by actin arrangement (C and F) and DAPI staining in merged image (I). Scale bar = 5µm in A-C and 50 µm in D-I.

Figure S3 Colchicine and verapamil do not affect the normal function of UAS-GAL4 system. Eye discs from 3rd instar larva of *GMR-GAL4/UAS-GFP* showed expression of GFP below the morphogenetic furrow (A), counterstained with DAPI (D). Larvae grown on 10μM colchicine did not show any difference in GFP expression level or pattern when compared to wild type (B), counterstained with DAPI (E). Similarly larvae grown on 20μM verapamil also did not show any difference in GFP expression level or in pattern of expression (C), counterstained with DAPI (F). G, H, I show the merged images for the three different groups. Scale bar = 50μm.

Figure S4 Down regulation of genes *mdr49*, *mdr50* and *mdr65* after expression of *RNAi*. In *GMR-GAL4/+; UAS-mdr49RNAi/+* larvae (B), there was a decrease in transcription level of *mdr49* when compared to wild type control (A). In *GMR-GAL4/+; UAS-mdr50RNAi/+* larvae, a decrease in transcription level of *mdr50* gene (D) was observed when compared to wild type control (C). Similarly in *GMR-GAL4/+; UAS-mdr65RNAi/+* larvae there was a decrease in transcription level of *mdr65* gene (E) when compared to wild type control (F). Glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) was used as an internal control to ensure the integrity of RT-PCR.

Figure S5 Effect of *mdr-RNAi* in eye discs is not a RNAi artifact. External eye morphology of *GMR-GAL4 UAS-127Q/+; UAS-bnl-RNAi/+* flies showed same level of eye degeneration in different categories as compared to *GMR-GAL4 UAS-127Q/CyO* flies, suggesting that RNAi for any other gene has no effect on polyQ mediated degeneration and findings in result 5 are specific to *mdr-RNAi*.

Figure S6 Colchicine does not reduce polyQ in *mdr-RNAi* expressing eye discs. Larvae expressing polyQ aggregates in the background of *mdr-RNAi* were treated with 10µM colchicine. It was found that in case of *mdr49-RNAi* and *mdr50-RNAi*, number as well as intensity of polyQ aggregates remain similar to normal food larvae expressing polyQ aggregates (B and C). However in case of *mdr65-RNAi*, the aggregate's number was found to be low (D). Suggesting that effect of colchicine on polyQ aggregates is mediated via *mdr* transporters. Scale bar= 5µm.

Figure S7 Expression of *mdr-RNAi* does not lead to neuronal degeneration or disruption of adherens junction complex. Immunostaining of eye imaginal discs with mab-22c10 antibody showed regular arrangement of neuronal connections in wild type (A) and in *GMR-GAL4* driven RNAi of *mdr49*, *mdr50* and *mdr65* genes suggesting that expression of *mdr-RNAi* does not lead to neuronal loss (B, C, and D respectively). Similarly arrangement of adherens junction complex was also observed by staining the eye discs with antibody against Armadillo protein. Armadillo arrangement in different *mdr-RNAi* condition (F, G, and H) was similar to wild type control (E). Scale bar= 5μm.