

Disruption of Axonal Transport by Loss of Huntingtin or Expression of Pathogenic PolyQ Proteins in *Drosophila*

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Summary

We tested whether proteins implicated in Huntington's and other polyglutamine (polyQ) expansion diseases can cause axonal transport defects. Reduction of *Drosophila* huntingtin and expression of proteins containing pathogenic polyQ repeats disrupt axonal transport. Pathogenic polyQ proteins accumulate in axonal and nuclear inclusions, titrate soluble motor proteins, and cause neuronal apoptosis and organismal death. Expression of a cytoplasmic polyQ repeat protein causes adult retinal degeneration, axonal blockages in larval neurons, and larval lethality, but not neuronal apoptosis or nuclear inclusions. A nuclear polyQ repeat protein induces neuronal apoptosis and larval lethality but no axonal blockages. We suggest that pathogenic polyQ proteins cause neuronal dysfunction and organismal death by two non-mutually exclusive mechanisms. One mechanism requires nuclear accumulation and induces apoptosis; the other interferes with axonal transport. Thus, disruption of axonal transport by pathogenic polyQ proteins could contribute to early neuropathology in Huntington's and other polyQ expansion diseases.

Introduction

Huntington's disease (HD) is one of nine neurodegenerative diseases that result from the expansion of CAG repeats leading to proteins containing abnormally long polyQ tracts. Although little is known about the mechanism by which polyQ expansion leads to pathogenesis, it has been proposed that misfolding of the mutant protein triggers a cascade of events, ultimately causing disease. The misfolded protein may undergo proteolytic cleavage, interact with other proteins, self-aggregate, and in many cases, translocate into the nucleus. Indeed, a common characteristic of all polyQ diseases is the formation of nuclear or cytoplasmic and axonal or dendritic inclusions of the disease protein (Li et al., 2000, 2001; Piccioni et al., 2002; Paulson et al., 1997; Becher et al., 1998; Ishikawa et al., 1999). In the nucleus, aggregated polyQ proteins have been suggested to recruit

transcription factors, caspases, and molecular chaperones and other proteins, which may stimulate apoptosis.

We previously found that expression in *Drosophila* of proteins that cause Alzheimer's disease in humans disrupts axonal transport and induces neuronal cell death, suggesting that perturbation in axonal transport might play a role in neurodegenerative disease (Gunawardena and Goldstein, 2001). Here, we test if proteins implicated in HD and other polyQ expansion diseases can also impair axonal transport pathways.

Huntingtin is a cytoplasmic protein with unknown functions. In mice, deletion of the huntingtin gene results in early embryonic lethality, whereas later deletion of huntingtin by conditional mutagenesis causes neuronal degeneration (Dragatsis et al., 2000; Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). In rat sciatic nerve axons, huntingtin is transported in both anterograde and retrograde pathways (Block-Galarza et al., 1997). Immunohistochemical studies in human and rat brains reveal cytoplasmic huntingtin within neurons, and biochemical analysis indicates that huntingtin is enriched in compartments containing vesicle-associated proteins (DiFiglia et al., 1995, 1997). Huntingtin interacts with many proteins, including nuclear, transcriptional, and signaling proteins (Cattaneo et al., 2001; Freiman and Tjian, 2002). One protein of particular interest is the huntingtin-associated protein 1 (HAP1; Li et al., 1996). Although its function is unknown, HAP1 is also transported in both anterograde and retrograde pathways (Block-Galarza et al., 1997) and is found associated with vesicle membranes in synaptosomal fractions, indicating that the HAP1 interaction with huntingtin may occur within axons (Engelender et al., 1997). Additionally, HAP1 strongly associates with p150^{Glued}, a critical component of the dynein-based transport system (Engelender et al., 1997; Li et al., 1998). Recent work suggests a role for a *Drosophila* HAP1-like protein in kinesin-dependent transport of mitochondria (Stowers et al., 2002). Together, these findings lead to the still untested suggestion that huntingtin has an important function in the axonal transport machinery itself.

In HD brain, aggregates of mutant huntingtin are observed in nuclear inclusions and in dystrophic neurites (DiFiglia et al., 1997; Becher et al., 1998). In HD transgenic mice, N-terminal huntingtin fragments and their aggregates initially accumulate in striatal neurons, and later these neurons form aggregates in axonal processes and terminals (Li et al., 2000). Neuropil aggregates were observed in the striatum in the lateral globus pallidus (LGP), a region into which medium spiny neurons project. How a function that may normally be associated with cytoplasmic vesicles can contribute to nuclear dysfunction and whether this reflects a normal nuclear signaling role of huntingtin is unknown. A testable possibility is that (1) normal huntingtin has a role in axonal transport and (2) mutant huntingtin, and possibly other pathogenic polyQ proteins, cause neuronal dysfunction by poisoning vesicular transport within neurons, which can ultimately contribute to neurodegenera-

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tion. Here we directly test this hypothesis in vivo in *Drosophila*.

Results

Reduction of *Drosophila* Huntingtin Expression Causes Axonal Transport Defects in Larval Nerves and Neurodegeneration in Adult Eyes

Drosophila larvae with mutations in genes encoding axonal transport proteins show dramatic neuromuscular pathology. Segmental nerves of such mutant animals contain prominently stained accumulations of synaptic vesicle proteins such as synaptotagmin (SYT) and cysteine string protein (CSP) (Hurd and Saxton, 1996; Bowman et al., 1999). Recently, deletion of the *Drosophila* APPL gene, which is proposed to function as a vesicular receptor for kinesin-I, was found to exhibit phenotypes characteristic of axonal transport defects (Gunawardena and Goldstein, 2001). To test if reduction of *Drosophila* huntingtin (htt) causes axonal transport phenotypes, we generated tissue-specific reduction of *Drosophila* htt by using a modified RNAi method (Figure 1A; Piccin et al., 2001). Three independent UAS double-strand htt hairpin RNAi transgenic lines (dhtt#1, #9, and #13) were combined with the neuronal APPL-GAL4 driver. Expression of htt RNA in the dhtt-RNAi lines was observed by RT-PCR to be reduced, in some instances to as little as 10%–30% of normal (see Supplemental Figure S1B at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>). All three lines exhibited defects characteristic of axonal transport problems (organelles accumulate within larval nerves) as well as a small amount of neuronal cell death in the larval brain (Figures 1B and 1C; Supplemental Figure S1A; data not shown). As controls, UAS double-strand RNAi lines of RhoGAP (Bilguat et al., 2001) were combined with APPL-GAL4. These combinations did not cause axonal transport defects, although disruption of RhoGAP in the adult eye using GMR-GAL4 or eyeless-GAL4 caused a rough eye phenotype (data not shown).

Ordinarily, deleting one of two copies of motor genes does not have a significant phenotype, but such a reduction combined with a reduction of a putative cargo binding partner is predicted to enhance an axonal transport phenotype because of the additional reduction in components required for transport. To test this prediction, we generated larvae that had reduced levels of both htt and motor proteins. Axonal blockages in the presence of reduced htt gene function were enhanced by a 50% reduction in kinesin heavy chain (KHC) or kinesin light chain (KLC) gene dosage; a small enhancement of neuronal cell death was also observed. A 50% reduction of dynein heavy chain (DHC) or dynein light chain gene (DLC) dose did not dramatically increase the amount of organelle accumulations or the amount of neuronal cell death (Figures 1B and 1C; Supplemental Figure S1A; data not shown).

Since adults and pupae mutant for DLC (Bowman et al., 1999), dynein intermediate chain (Boylan et al., 2000), and the dominant-negative mutation p150^{Glued} have rough eye phenotypes, we asked if loss or disruption of htt would also exhibit this phenotype. Reduction of htt in the eye using the GMR-GAL4 driver caused a rough

eye phenotype in adults, which is also characteristic of neurodegeneration (Figure 1D). This phenotype was progressive over time (10 days), causing severe problems of morphology and pigmentation, leading to patches of dark areas indicative of death. Sections of aged mutant eyes showed loss of cells beneath the external surface of the eye and disruption in the normal ommatidial morphology, suggesting loss of photoreceptor integrity. These results suggest that htt has a function in the axonal transport machinery and that loss of htt can lead to neurodegeneration.

Expression of Human Huntingtin Exon 1 with Expanded PolyQ Repeats Causes Axonal Transport Defects

The observation that reduction of htt causes an axonal transport phenotype, combined with previous work linking htt to the transport machinery, leads to the hypothesis that excess htt, particularly htt containing pathogenic polyQ repeats, should cause axonal blockages by titrating motor proteins away from other cargo. To test this proposal, we expressed human huntingtin exon 1 with either a “normal” length of polyQ (httex1-20Q) or with a disease-causing length of polyQ (httex1-93Q; Steffan et al., 2001) in larval neurons. These transgenic lines were crossed to two neuron-specific GAL4 driver lines, APPL-GAL4 and 179Y-GAL4, and their nerves were stained for synaptic vesicle markers. While httex1-20Q nerves were similar to wild-type, two different httex1-93Q lines had organelle accumulations characteristic of defects in axonal transport (Figure 2A; Supplemental Figure S1A at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>).

If expression of httex1 with pathogenic polyQ repeats causes axonal transport phenotypes by titrating motor proteins from other cargoes and pathways, then reduction of motor protein gene dosage in larvae overexpressing httex1 is predicted to significantly enhance the axonal transport phenotype by further reducing the available motor protein pool. To test this prediction, we generated larvae that overexpressed httex1-20Q or httex1-93Q and were heterozygous for motor protein gene mutations. A 50% reduction in KHC or DLC with httex1-93Q enhanced the amount of organelle accumulations, while KHC or DLC reductions combined with httex1-20Q were comparable to wild-type (Figure 2A; Supplemental Figure S1A).

We used TUNEL analysis to test whether perturbations caused by httex1 causes neuronal apoptosis. While the transgenic line expressing httex1-20Q was comparable to wild-type, the transgenic lines expressing httex1-93Q showed some neuronal apoptosis (Figure 2B); a strong enhancement of neuronal death was also observed with 50% reduction in KHC and a small increase with 50% reduction in DLC.

Pathogenic PolyQ Proteins Cause Axonal Transport Defects and Neuronal Apoptosis

To address whether axonal transport defects were selective to the pathogenic htt protein, or whether they were a feature of polyQ proteins in general, we examined the effects on axonal transport of various proteins containing polyQ tracts of different lengths and in different contexts. 179Y-GAL4 and APPL-GAL4 were crossed to

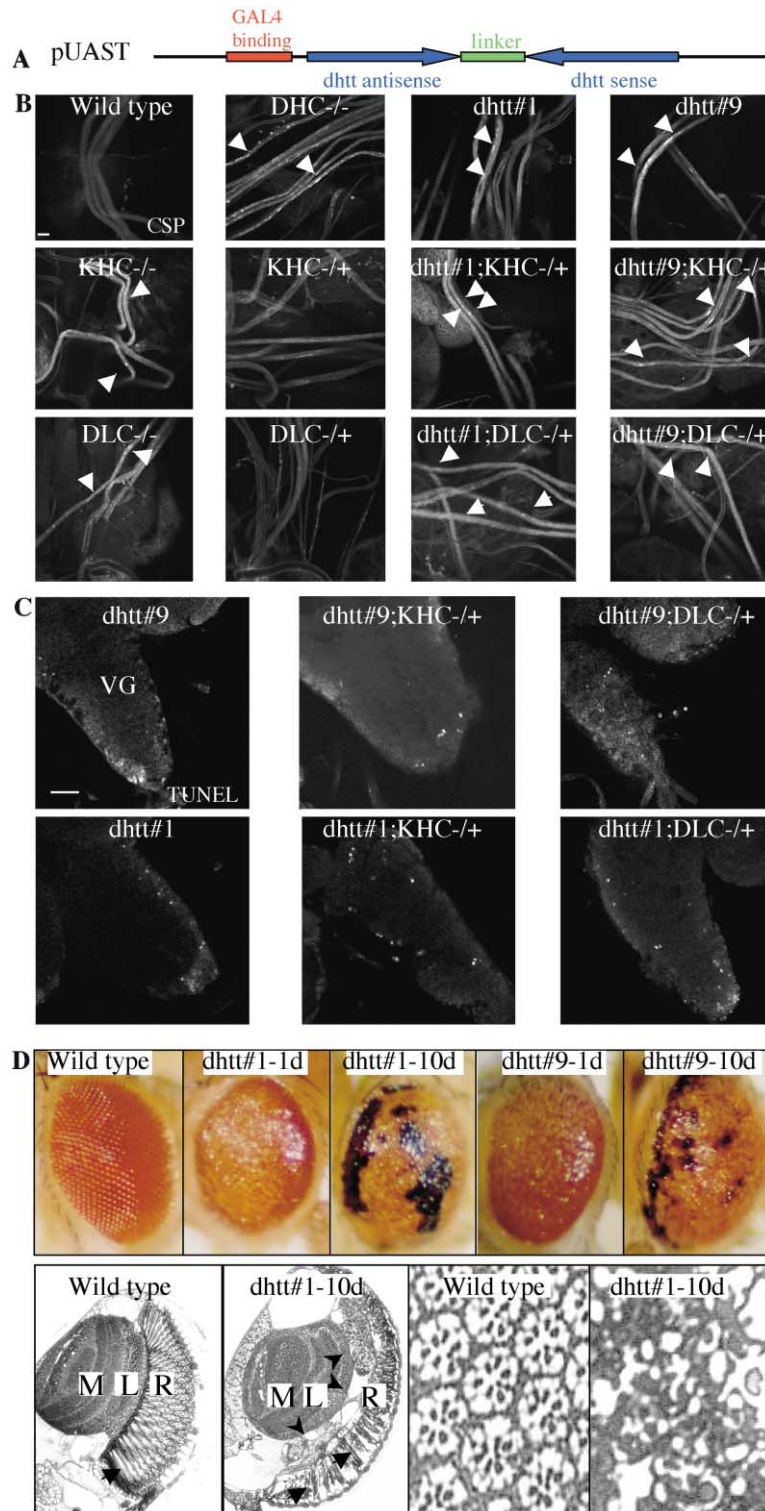


Figure 1. Reduction of *Drosophila* Huntingtin Causes Axonal Transport Defects and Neurodegeneration

(A) The UAS double-strand RNAi construct contains 1 kb each of sense and antisense DNA from the N-terminal region of the *Drosophila* htt gene (blue) separated by 300 bp of linker DNA from GFP (green).

(B) Two RNAi lines, dhdt#1 and dhdt#9, with APPL-GAL4 show organelle accumulations within larval segmental nerves (arrows) similar to DHC^{-/-} larvae when stained with synaptic marker CSP. Segmental nerves from wild-type larvae stain smoothly. 50% reduction of KHC with RNAi lines dhdt#1 and dhdt#9 (dhdt#1;KHC^{-/+} and dhdt#9;KHC^{-/+}) enhance the organelle accumulation phenotype. Larvae that are KHC^{-/-} show extensive accumulations while larvae that are KHC^{-/+} are normal. 50% reduction of DLC with RNAi lines dhdt#1 and dhdt#9 (dhdt#1;DLC^{-/+} and dhdt#9;DLC^{-/+}) does not significantly enhance the organelle accumulation phenotype. Larvae that are DLC^{-/-} show extensive accumulations while larvae that are DLC^{-/+} are normal. Scale bar equals 10 μ m.

(C) Cell death assayed by TUNEL analysis shows some neuronal cell death within the larval brains of dhdt#1 and dhdt#9. Some neuronal death is observed in dhdt#1; KHC^{-/+}, dhdt#9;KHC^{-/+}, dhdt#1;DLC^{-/+}, and dhdt#9;DLC^{-/+}. (VG, ventral ganglion). Scale bar equals 10 μ m.

(D) Disruption of *Drosophila* htt causes degeneration in the adult eye. The rough eye phenotype is observed in dhdt#1 and dhdt#9 with GMR-GAL4 compared to wild-type (GMR-GAL4/Oregon R). The rough eye phenotype becomes progressively worse as the flies age (10 days). Frontal sections of 10 day dhdt#1 and dhdt#9 flies show cell degeneration in the retina (R, arrows) with severely disrupted internal morphology. Brain regions lamina (L) and medulla (M) appear normal. Large holes (arrowheads) are visible in the most lateral part of the lamina. Tangential sections through the eyes of 10 day dhdt#1 and dhdt#9 show loss of cell morphology of the rhabdomere lattice compared to wild-type (GMR-GAL4/Oregon R).

lines encoding proteins with either a “normal” length, nondisease-causing polyQ repeat region or proteins with an expanded, disease-causing polyQ repeat region. These proteins consisted either entirely of polyQ repeats (20Q, 22Q, 108Q, 127Q; Marsh et al., 2000; Kazemi-Esfarjani and Benzer, 2000) or polyQ repeats embedded in the C-terminal region of the MJD protein (MJD-27Q,

MJD-78Q; Warrick et al., 1998). We found that proteins with normal length polyQ regions (22Q, MJD-27Q) were present within axons based upon the smooth staining seen with either an antibody against polyQ or an antibody against the HA tag (Figure 3A) and that these proteins accumulate at neuromuscular junctions (Figure 3B), suggesting that they are normally transported within

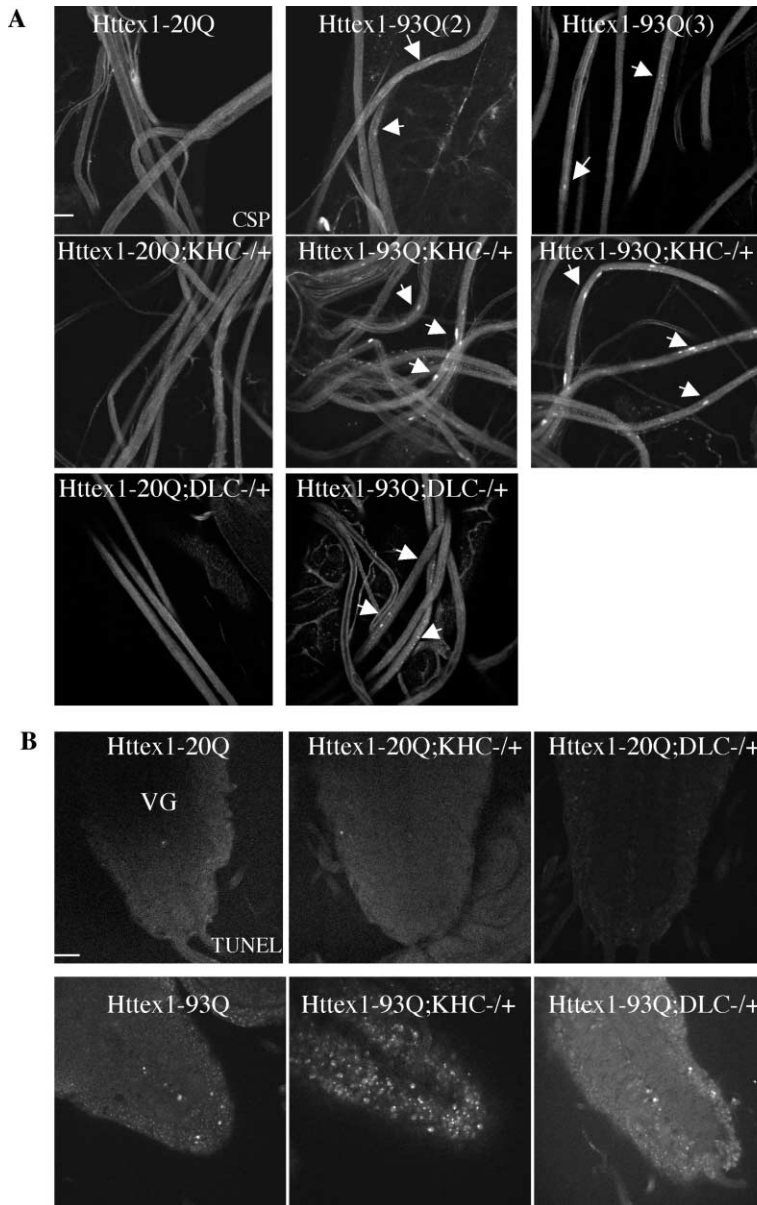


Figure 2. Overexpression of Human Huntingtin Exon 1 with Expanded PolyQ Repeats Results in Axonal Transport Defects and Neuronal Cell Death

(A) Expression of *httex1-20Q* resulted in a normal phenotype indistinguishable from wild-type, while two different lines on the second and third chromosome expressing *httex1-93Q* showed organelle accumulations with CSP (arrows). Accumulations were enhanced with 50% reduction of *KHC* (*httex1-93Q;KHC^{-/+}*). 50% reduction of *DLC* also exhibited accumulations but there was no significant increase in the amount of accumulations (*httex1-93Q;DLC^{-/+}*). Scale bar equals 10 μ m.

(B) Expression of *httex1-93Q* caused neuronal cell death, which was enhanced by 50% reduction of *KHC*. 50% reduction of *DLC* did not dramatically enhance neuronal death. Expression of *httex1-20Q* was comparable to wild-type and no phenotypic change was observed with 50% reduction in *KHC* or *DLC*.

larval axons. In contrast, proteins with expanded polyQ repeats (MJD-78Q, 127Q) exhibited prominent polyQ staining within organelle blockages (Figure 3A), while reduced staining was observed at the neuromuscular junctions (Figure 3B), suggesting impaired transport of pathogenic polyQ proteins.

The extent of axonal accumulations induced by polyQ repeats was length dependent, as we observed a correlation between the number of polyQ repeats and the amount of axonal accumulations (Supplemental Figures S2A and S2B at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>). Larvae expressing 20Q, 22Q, or MJD-27Q were similar to wild-type in that they exhibited no axonal accumulations. Larvae expressing the pathogenic proteins MJD-78Q, 108Q, or 127Q exhibited a severe sluggish larval movement phenotype, with prominent axonal accumulations observed in all instances (Figure 3A; Supplemental Figures S2A and S2B). We also observed that

the expression of MJD-78Q and 127Q at 29°C was very toxic such that larvae expressing these proteins never survived to adulthood and died at second or third instar larval stage. Western blot analysis ruled out a general expression difference between proteins with normal length polyQ repeats and proteins with expanded polyQ repeats since, if anything, more MJD-27Q expression was observed compared to MJD-78Q (Figure 5B). To reduce the level of toxicity, we reduced the amount of MJD-78Q and 127Q made by growing animals at 25°C (GAL4 activity is temperature dependent; Phelps and Brand, 1998). We still observed organelle accumulations although these larvae now survived much longer, dying at early pupal stages (data not shown).

To confirm the results seen by immunofluorescent staining, we conducted EM analysis on larvae expressing 127Q and on severe genotypes in which expression of MJD-78Q or 127Q was combined with a 50% reduc-

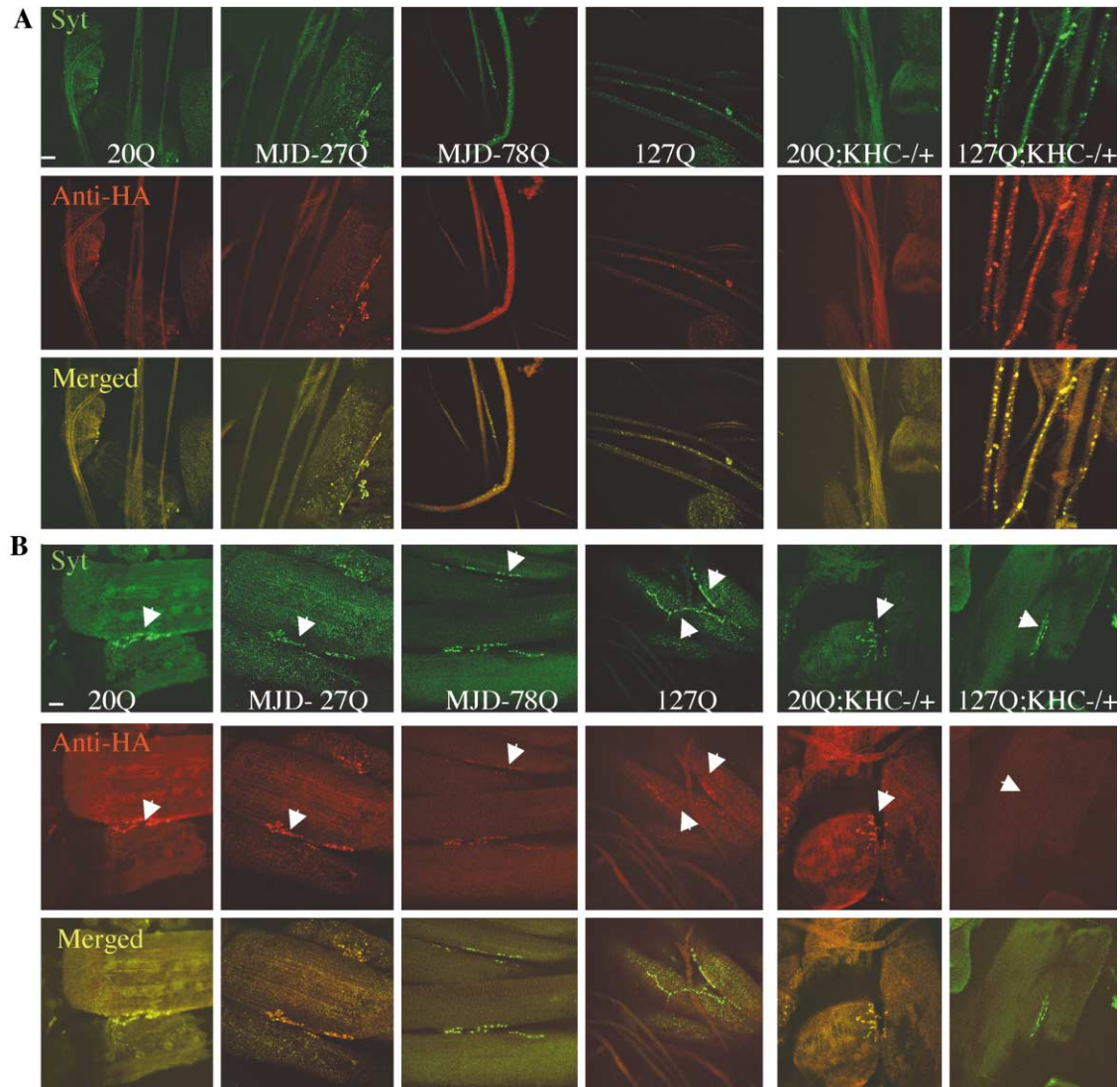


Figure 3. Nonpathogenic PolyQ Proteins Are Transported Normally within Larval Nerves, while Pathogenic PolyQ Proteins Are Not
(A) Nonpathogenic polyQ proteins are present within larval nerves, while pathogenic polyQ proteins (anti-HA, red) are found within organelle accumulations (anti-SYT, green). Excess 127Q with 50% reduction in KHC enhances 127Q containing organelle accumulations. Scale bar equals 10 μ m.
(B) Nonpathogenic polyQ proteins are transported to the neuromuscular junctions while pathogenic polyQ proteins are not (arrows). Complete blockage is observed with 50% reduction of KHC with 127Q.

tion in KHC gene dose (see below). We observed prominent axonal blockages characteristic of those observed in homozygous mutations of motor protein genes (compare Figure 4A to Bowman et al. [1999] Figure 6 [DLC^{-/-}] and Hurd and Saxton [1996] Figure 2B [KHC^{-/-}]). Mutant larval nerves also contained enlarged axons, some almost four or five times the diameter of those observed in wild-type. Sometimes we observed “holes” lacking organelles within the nerve, perhaps indicative of degeneration.

To test directly whether pathogenic polyQ proteins block transport by inducing nonmoving blockages in axonal processes, we performed live analysis of vesicular movement within whole-mount larval axons. We expressed YFP-tagged human amyloid precursor protein (APP-YFP) either in the presence or absence of MJD-

78Q, using the GAL4 driver pGAL4-62B SG26-1, which is expressed in only a small population of motor neurons (R.G.B., unpublished data). Neurons expressing only APP-YFP contained many actively motile vesicles moving at velocities of approximately 1 μ m/s (Figure 4C; Supplemental Figure S3 and Movies S1 and S2 at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>); we never observed any large bright nonmotile accumulations such as those seen in the presence of MJD-78Q (below). Neurons coexpressing MJD-78Q and APP-YFP revealed nonmoving large, bright aggregates of APP-YFP. Thus, polyQ expression can interfere with transport of APP-YFP vesicles.

To test whether proteins with expanded polyQ repeats can induce neuronal apoptosis, we used TUNEL analysis. A large increase in neuronal apoptosis was observed

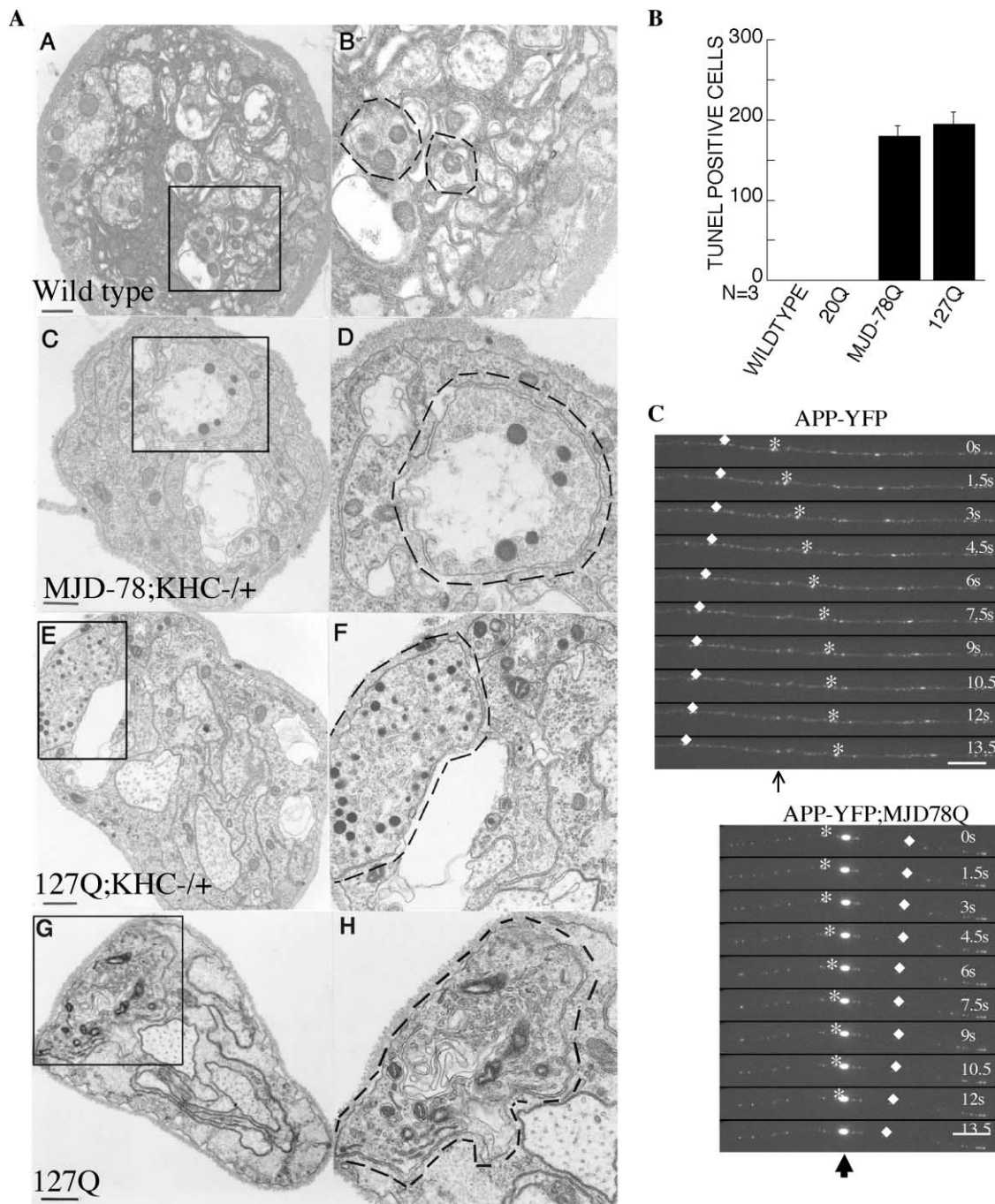


Figure 4. Expression of Pathogenic PolyQ Proteins Causes Organelle Accumulations and Neuronal Cell Death

(A–H) EM analysis shows organelle accumulations within one larval segmental nerve in lines expressing pathogenic polyQ proteins. In wild-type, many axons can be observed (box) and organelles can be observed within them (dashed outline). In 127Q (G and H), MJD-78Q;KHC^{-/+} (C and D), and 127Q;KHC^{-/+} (E and F) nerve, organelle accumulations can be observed within axons (box and dashed outline). Only a few axons can be observed and they are enlarged about four or five times that of wild-type (A and B). Scale bar equals 500 nm.

(B) Quantitative analysis depicts the extent of TUNEL-positive cells caused by expression of pathogenic polyQ proteins within the larval brain. (C) Live analysis of YFP-tagged vesicle movement within whole-mount larvae expressing MJD-78Q compared to normal YFP vesicle movement. Note the nonmoving large, bright APP-YFP aggregate in the neuron coexpressing MJD-78Q and APP-YFP. Asterisk, anterograde movement; diamond, retrograde movement; thin arrow, nonmoving vesicle; broad arrow, nonmoving accumulation. Scale bar equals 10 μ m.

in lines expressing MJD-78Q and 127Q, but not in lines expressing the nonpathogenic control proteins 22Q or MJD-27Q (Figure 4B; Supplemental Figure S2C). Anti-polyQ staining revealed obvious nuclear inclusions

within larval brain cells (Supplemental Figure S2D). Many stained nuclei were obviously enlarged and may be undergoing apoptosis. Expression during embryonic cycle 14 revealed smooth cytoplasmic staining for MJD-78Q

protein, while staining for 127Q revealed obvious punctate cytoplasmic aggregates with some nuclear aggregates (Supplemental Figure S2E). At later cycles, both MJD-78Q and 127Q were observed as punctate cytoplasmic and nuclear aggregates (data not shown). In addition, embryos expressing both MJD-78Q and 127Q died soon after they hatched into larvae, indicating substantial polyQ toxicity on normal development. Taken together, these results confirm that proteins with expanded polyQ repeats cause axonal transport defects, perhaps by blocking axonal processes by polyQ accumulations, neuronal cell death, and neurodegeneration.

Pathogenic PolyQ Proteins May Titrate Motor Proteins from Other Cargoes and Pathways

It is possible that polyQ proteins bind and deplete critical components of the molecular motor machinery, perhaps via a *Drosophila* version of HAP1. This hypothesis makes two predictions: (1) genetic reduction of motor protein dosage should worsen the phenotypes caused by proteins with polyQ expansions by further depleting the motor protein supply; and (2) motor protein depletion should be observable with biochemical methods.

To test this hypothesis, we expressed proteins with expanded polyQ repeats and reduced levels of dynein and kinesin. We found that while a 50% reduction in the dose of KHC has no significant phenotype on its own, when combined with pathogenic polyQ repeats, it dramatically enhanced the axonal organelle accumulation phenotype (Supplemental Figures S2A and S2B at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>). Similarly, while a 50% reduction in the dose of DLC or components of the dynactin complex (p150^{Glued}, Arp1, and dynamitin) also normally have no significant phenotypes on their own, when combined with pathogenic polyQ proteins, these reductions substantially enhanced the organismal phenotype leading to early larval lethality (Supplemental Figures S2A and S2B). This finding is consistent with our observation that the neuronal APPL-GAL4 driver turns on during embryonic stage 15 as observed by the expression pattern of UAS-GFP (data not shown). The enhanced lethality precluded analysis of axonal transport in these genotypes. Interestingly, organelle accumulations now appeared in transgenic lines expressing normally nonpathogenic poly Q repeats (22Q and MJD-27Q) with a 50% reduced dose of dynein (Supplemental Figures S2A and S2B), consistent with the hypothesis that all of these proteins may titrate motor proteins, but to varying extents (see below). To test directly for motor protein depletion mediated by expression of proteins with expanded polyQ regions, we analyzed early embryos expressing httex1-20Q, MJD-27Q, MJD-78Q, httex1-93Q, and 127Q, using the early embryonic GAL4 driver da-GAL4, which turns on at the blastoderm stage based on its UAS-GFP expression pattern (data not shown).

Two considerations led us to evaluate the effects of polyQ proteins on available motor protein pools by assessing soluble levels of motor proteins. First, if motor proteins are titrated from normal cargoes by binding to large aggregates, it may be difficult to distinguish motor proteins bound to sedimentable cargoes from sedimentable aggregates. Second, it is not possible to mea-

sure the amount of each motor protein associated with normal cargoes owing to the lack of information about such cargoes and how such cargoes fractionate relative to polyQ aggregates. Thus, we assessed soluble levels of motor proteins under the hypothesis that aggregated polyQ proteins may bind motor proteins and deplete both soluble and cargo bound pools in parallel.

At 6 hr of development, we observed no significant change in the amount of total motor protein present in these embryos. In contrast to the normal amounts of total motor proteins, we observed an obvious reduction in the amount of soluble motor proteins in embryos expressing MJD-27Q, MJD-78Q, and 127Q compared to wild-type embryos (i.e., da-GAL4 alone and *yw*; Figure 5A). The amount of soluble DHC, DIC, p150^{Glued}, KHC, and KLC were reduced, with no change observed in tubulin, actin, HDAC3, and Rab8. However, for reasons that are not clear, syntaxin was upregulated. Similar observations were evident from 12 and 16 hr embryo collections (data not shown). The effect of httex1-93Q expression on levels of soluble motor proteins was not obvious at 6 hr of development (data not shown). However, at 18 hr of development, there is an obvious reduction in soluble p150^{Glued} and KLC in embryos expressing httex1-93Q but not httex1-20Q or wild-type. Expression of polyQ proteins in embryos was obvious as detected by anti-HA antibody and confirmed by anti-polyQ antibody (Figure 5B). The level of 127Q was difficult to evaluate, perhaps due to the formation of aggregates, and was convincingly observed only after immunoprecipitation with anti-HA antibody (Figure 5C). These observations indicate that expanded polyQ proteins can deplete or sequester available soluble motor proteins, perhaps into polyQ aggregates. The high expression level of MJD-27Q in embryos and the observed depletion of soluble motor proteins in these embryos are consistent with the finding that axonal blockages can be observed in larvae expressing MJD-27Q when motor protein gene dose is reduced by 50% (Supplemental Figure S2A at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>). This finding is also consistent with the proposal (see Discussion) that motor titration and aggregation may act in concert to poison axonal transport.

Enhanced Expression of Chaperones Restores Neuronal Transport and Suppresses Cell Death Caused by Pathogenic PolyQ Proteins

Previous work found that the neurodegenerative adult eye phenotype caused by polyQ expansion proteins in *Drosophila* is suppressed by excess chaperone proteins (Warrick et al., 1999). This suppression has been proposed to occur by modulating soluble properties of pathogenic polyQ proteins, by preventing abnormal interactions with other proteins, or by rescuing chaperone depletion (Bonini, 2002). We tested whether expression of excess HSC70 protein would suppress axonal blockages and neuronal cell death. Expression of UAS-HSC70 using APPL-GAL4 in the presence of MJD-78Q and 127Q restored axonal transport within larval nerves and suppressed neuronal death (Figure 6A; Supplemental Figure S4A at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>). PolyQ accumulations were absent within larval nerves, while cytoplasmic and punctate

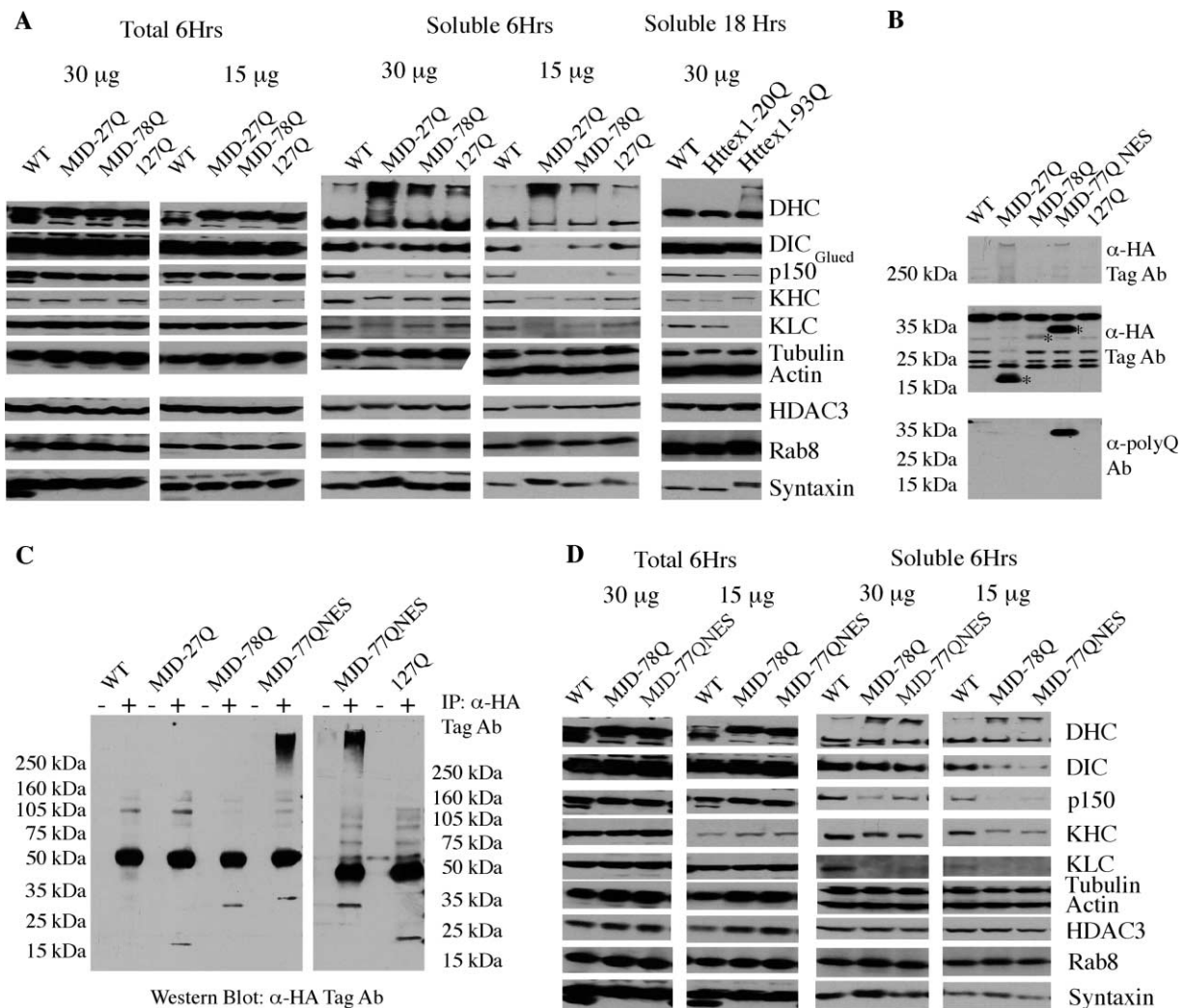


Figure 5. Expression of Pathogenic PolyQ Proteins Depletes the Amount of Soluble Motor Proteins

(A) Dramatic reduction is observed in the amount of soluble motor proteins, DHC, DIC, p150^{Glued}, KHC, and KLC when MJD-27Q, MJD-78Q, and 127Q are expressed; no change is observed in the amount of total motor proteins at 6 hr, using da-GAL4. A reduction in the amount of soluble p150^{Glued} and KLC is also seen when httex1-93Q is expressed. No change is seen in tubulin, actin, HDAC3, and Rab 8. Note that high molecular weight aggregates containing DHC immunoreactivity are consistently observed in MJD-27Q, MJD-78Q, 127Q, and httex1-93Q compared to wild-type.

(B) Expression of MJD-27Q, MJD-78Q, and MJD-77QNES are observed by anti-HA (asterisks), while MJD-77QNES is also observed by anti-polyQ staining. The levels of MJD-27Q and MJD-77QNES are significantly higher than observed in MJD-78Q.

(C) Immunoprecipitation with anti-HA reveals 127Q protein. Note that the high molecular weight MJD-77QNES aggregate can also be precipitated by anti-HA.

(D) Dramatic reduction in the amount of soluble motor proteins is also observed in embryos expressing MJD-77QNES, similar to (A). High molecular weight aggregate containing DHC is also evident with MJD-77QNES.

polyQ staining was present within larval brains (Figure 6B). However, while these larvae were now able to pupate (expression of MJD-78Q or 127Q alone causes death at second or third instar larval stages), they still failed to eclose, suggesting that polyQ toxicity was still sufficient to cause lethality. Expression of HSC70 by itself did not cause axonal blockages or neuronal cell death (data not shown). These results suggest that chaperones could “clear” larval axons of blockages caused by polyQ proteins and suppress cell death within the larval brain, although organismal toxicity was not completely suppressed.

Do Axonal Defects Instigate Neuronal Dysfunction?

The pathogenic polyQ proteins studied in the preceding sections accumulate in both axonal and nuclear inclusions. To dissect the relative contributions of nuclear and axoplasmic inclusions to the phenotype, we used transgenes that expressed proteins that had different subcellular localizations.

One transgene encoded a protein with an expanded polyQ repeat with a nuclear localization sequence (MJD-65QNLS). While expression of MJD-65QNLS within the larval brain caused neuronal apoptosis as observed by TUNEL staining, organelle accumulations within larval

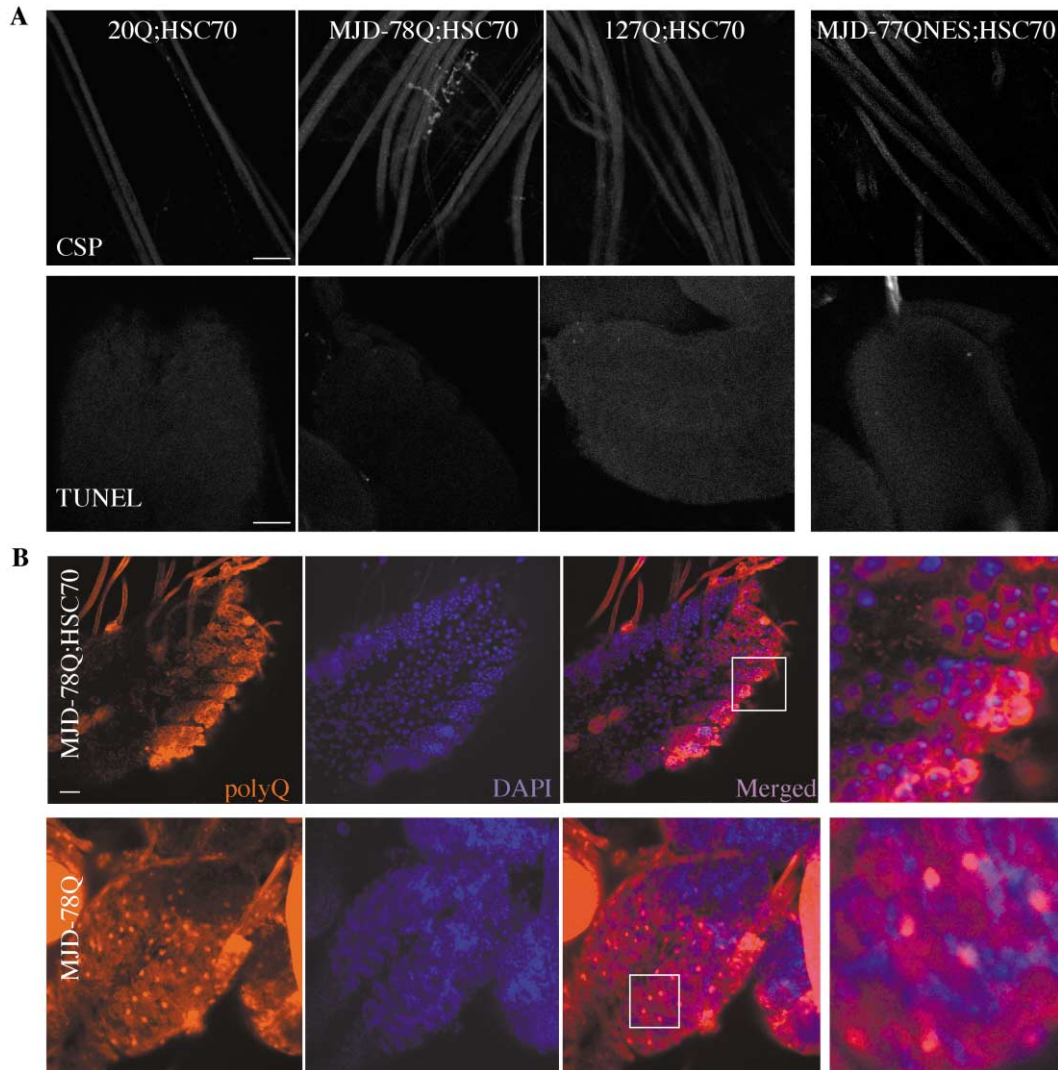


Figure 6. Excess Chaperones Restore Neuronal Transport and Suppresses Cell Death Caused by Pathogenic PolyQ Proteins

(A) Overexpression of pathogenic polyQ proteins with excess HSC70 clears organelle accumulations and suppresses neuronal cell death. Scale bar equals 10 μ m.

(B) Expression of excess HSC70 with pathogenic polyQ proteins leads to cytoplasmic polyQ staining within larval brains compared to nuclear staining observed in brains expressing MJD-78Q alone (box enlarged on right; anti-polyQ, red; DAPI, blue).

axons were absent (Figure 7A). These larvae pupated but failed to eclose. While reduction in dynein dose by 50% with excess MJD-65QNLS had no effect, reduction in kinesin dose by 50% with excess MJD-65QNLS caused a small number of accumulations, perhaps due to continued motor titration by these proteins even when targeted to nuclei (Figure 7A).

To test further if dying neuronal cells induce axonal transport defects, we also analyzed the axonal transport phenotype of the cell death gene reaper, which also induces neuronal apoptosis. Transport following reaper expression in these genotypes appeared to be normal based on immunostaining with synaptic vesicle markers even though high levels of neuronal apoptosis were induced (Supplemental Figure S4B at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>). In addition, a 50% reduction in KHC combined with excess reaper expression

had no effect on axonal transport. These findings emphasize that not all neuronal death is associated with axonal accumulations, and that the axonal transport defects induced by pathogenic polyQ proteins may be specific to cytoplasmic aggregations of expanded polyQ proteins.

To test for cytoplasmic or axoplasmic toxicity, we studied a protein with an expanded polyQ repeat with a nuclear export sequence (MJD-77QNES). Expression of MJD-77QNES within larval neurons caused large numbers of synaptotagmin-containing organelle accumulations within larval nerves (Figure 7B). Consistent with primarily cytoplasmic localization of this protein, polyQ/HA staining was absent from cell nuclei within the larval brain, with bright anterior staining (just distal to the brain) present within larval nerves (Figure 7D; compare to nuclear polyQ expression in MJD-78Q [Fig-

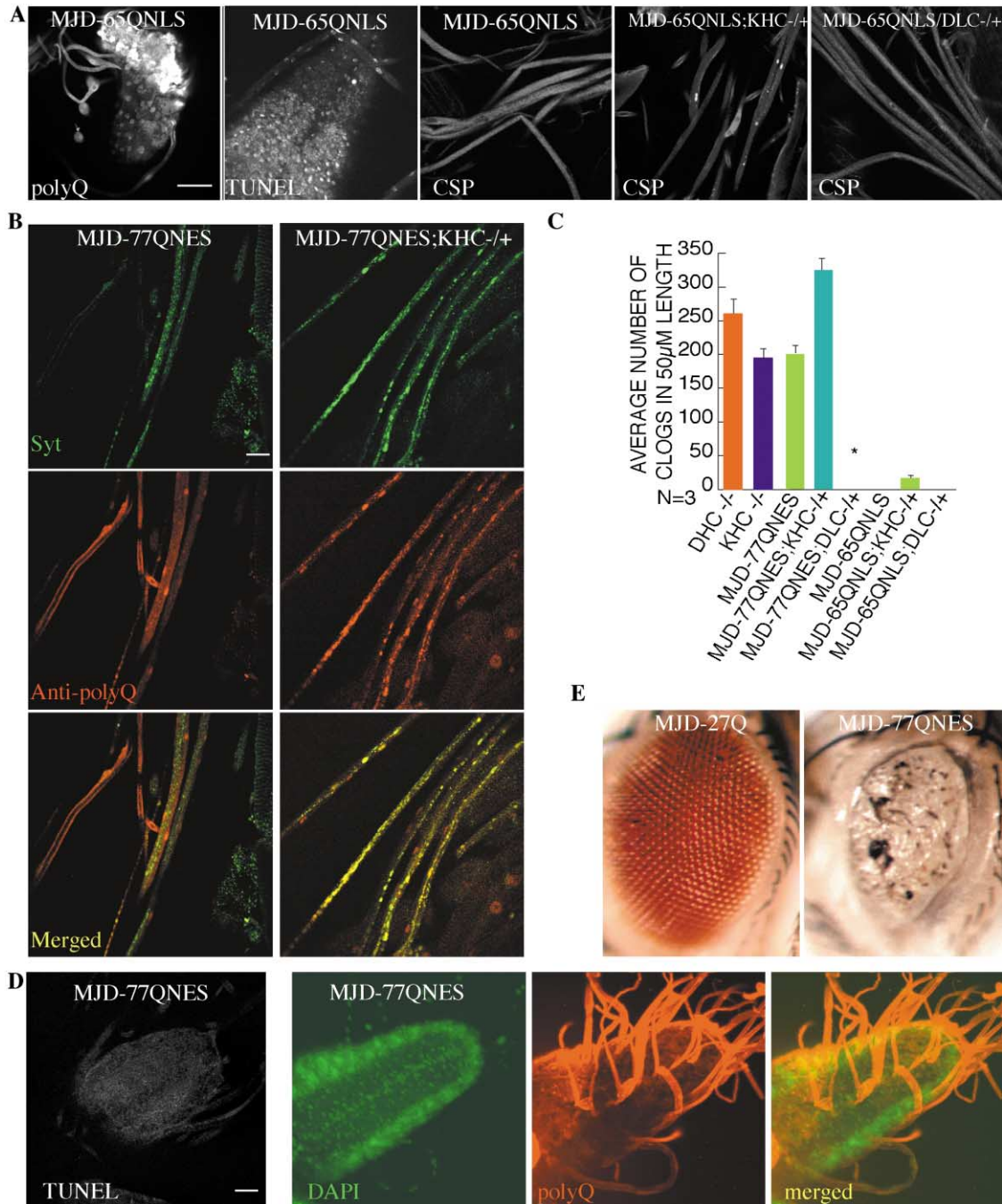


Figure 7. Axonal Transport Defects Caused by Pathogenic PolyQ Proteins Are Specific to Cytoplasmic Aggregations

(A) Expression of MJD-65QNLS does not cause organelle accumulations within larval nerves as observed by CSP, but causes neuronal cell death as observed by TUNEL within the brain. PolyQ staining is observed within brains. 50% reduction in KHC with MJD-65QNLS shows some organelle accumulations (also see C) while 50% reduction in DLC has no effect.

(B) Expression of MJD-77QNES causes organelle accumulations, which are enhanced by 50% reduction in KHC. Anti-SYT, green; anti-polyQ, red. Scale bar equals 10 µm.

(C) Quantitative analysis indicates that the extent of accumulations in MJD-77QNES is comparable to homozygous mutations of motor proteins. 50% reduction in KHC with MJD-77QNES enhances this phenotype, while 50% reduction in DLC causes early larval lethality (asterisk).

(D) Expression of MJD-77QNES does not cause TUNEL-positive nuclei within the brain, and polyQ staining is not observed within neuroblast nuclei as in MJD-78Q. Bright polyQ staining is observed within larval nerves (compare to Figure 6B; DAPI, green; anti-polyQ, red).

(E) Expression of MJD-77QNES within the adult eye using GMR-GAL4 shows degeneration, including complete loss of pigmentation compared to MJD-27Q.

ure 6B] or MJD-65QNLS [Figure 7A]). Neuronal apoptosis as determined by TUNEL staining was completely absent (Figure 7D) and these larvae died at second or third instar, similar to MJD-78Q. Quantitative analysis indicates that the extent of organelle accumulations within MJD-77QNES is comparable to accumulations observed in mutations of motor proteins, suggesting that perhaps the extent of accumulations causes lethality (Figure 7C). Similar to MJD78Q, a 50% reduction in the dose of KHC with MJD-77QNES enhanced organelle blockages (Figures 7B and 7C), while 50% reduction in DLC combined with MJD-77QNES caused early larval lethality. Additionally, expression of MJD-77QNES in the adult eye using GMR-GAL4 caused a severe degenerative eye phenotype, indicating that cytoplasmic polyQ protein can cause degeneration of adult neurons (Figure 7E).

To directly test if excess MJD-77QNES sequestered motor proteins, we compared embryos expressing MJD-27Q, MJD-78Q, and MJD-77QNES with wild-type (da-GAL4). While embryos expressing both MJD-78Q and MJD-77QNES showed normal levels of total motor proteins, they exhibited a striking reduction in the amount of soluble motor proteins (Figure 5D). MJD-77QNES also exhibited high molecular weight aggregates, which could be immunoprecipitated with the HA antibody (Figures 5B and 5C). These high molecular weight aggregates also contained sequestered DHC (Figures 5A and 5D). Although phenotypically normal when expressed in larvae, MJD-27Q appears to titrate more DHC into high molecular weight aggregates than do MJD-78Q, MJD-77QNES, or 127Q. It is conceivable that pathogenic MJD-78Q, MJD-77QNES, and 127Q form high molecular weight aggregates that we are unable to trap or to solubilize in our current protocols. Indeed, dramatic phenotypes are only observed in MJD-78Q, MJD-77QNES, and 127Q (Figures 3A and 7B). Additionally, similar to embryos expressing MJD-78Q or 127Q, embryos expressing MJD-77QNES died soon after they hatch into larvae, indicating significant polyQ toxicity on normal development. It is possible that the "soluble" polyQ aggregates we observe in embryos expressing MJD-77QNES represent a subclass of misfolded proteins, while the class of insoluble aggregates, which we are unable to observe directly on SDS-PAGE, may be responsible for polyQ toxicity.

To distinguish if MJD-78Q blockages and MJD-77QNES blockages are comparable, we tested whether blockages caused by MJD-77QNES expression can be suppressed by excess HSC70. Expression of MJD-77QNES with excess HSC70 completely suppressed axonal accumulations, polyQ aggregates, and rescued larval lethality to pupae, suggesting that axonal blockages caused by either MJD-78Q or MJD-77QNES were comparable (Figure 6A). PolyQ-containing accumulations were also absent in larval nerves. However, excess HSC70 was not sufficient to suppress organismal lethality.

Discussion

Within axons, vital neuronal cargoes must be transported over great distances along microtubule tracks.

This transport is critical to support signaling and other aspects of neuronal viability and function. We previously found that disruption of axonal transport by proteins implicated in Alzheimer's disease can cause neuronal cell death, suggesting that axonal transport perturbation may play a role in neurodegenerative disease. Here we tested whether proteins that cause polyQ expansion diseases could also cause defects in axonal transport pathways. We found that wild-type htt is needed for normal axonal transport. In addition, pathogenic polyQ proteins alone, or in the context of human htt exon 1 or the MJD C terminus, can interfere with the axonal transport machinery and cause neuronal apoptosis and organismal death (Table 1). Thus, our findings demonstrate that pathogenic polyQ proteins can poison the axonal transport system and support the proposal that defects in axonal transport may contribute to neuronal failure in HD and other polyQ expansion diseases.

An important concern in interpreting our data is whether the transport failures we observe are caused by direct poisoning of the transport machinery by pathogenic polyQ proteins, or if it is an indirect consequence in neurons that have become sick or are dying from other causes. There are four strong arguments that support the interpretation that pathogenic polyQ proteins themselves poison the transport machinery leading to neuronal failure. First, two different screens for axonal transport mutants in our lab suggest that this phenotype is relatively rare. In one screen, only 4 out of 12,000 mutagenized chromosomes exhibited a phenotype diagnostic of transport abnormalities, namely abnormal larval motility combined with organelle accumulations in axons (Bowman et al., 1999, 2000). In a recent screen, 446 out of 13,000 mutants exhibited the larval motility phenotype. 114 of these were tested for organelle accumulations in axons, but only 3 were found to exhibit this phenotype (M. Haghnia and L.S.B.G., unpublished data). In this context, among mutations where the gene product is known, only mutations in genes encoding motor protein subunits, or genes encoding proteins where there is strong evidence to support a role in the transport machinery, cause this phenotype. Second, an overexpression screen using EP elements yielded 36 lines that had the larval motility phenotype out of 2300 tested when driven with 179Y-GAL4; only 3 of these had organelle accumulations within their axons (S.G. and L.S.B.G., unpublished data). Third, we previously showed that overexpression of GFP, or of any protein lacking the C terminus of APP relatives (which is thought to interact with the transport machinery), did not cause a transport phenotype (Gunawardena and Goldstein, 2001). In our current analysis of proteins implicated in polyQ expansion diseases (Table 1), we observed similar phenotypic selectivity. Proteins with short polyQ regions do not cause transport failures unless motor protein dosage is reduced. Fourth, induction of neuronal apoptosis by excess expression of the cell death gene reaper failed to cause axonal transport problems. Expression of MJD-65QNLS, which primarily targets to the nucleus and induces apoptosis, also did not cause transport failures. Thus, cell death or sick cells do not generally cause axonal transport defects.

If expression of pathogenic polyQ proteins directly causes transport failures, what might be the mecha-

Table 1. Summary of Constructs, Mutants, and Phenotypes

	Axonal Transport Phenotype APPL-GAL4			Eye Degeneration Phenotype GMR-GAL4
	Normal Kinesin, Normal Dynein	50% Reduction in Kinesin	50% Reduction in Dynein	Normal Kinesin, Normal Dynein
dhhtt RNAi Knockout Lines	JAMS death ^a	↓JAMS death	JAMS death	yes
UAS Transgenic Lines				
20Q	normal	normal	normal	no (Kazemi-Esfarjani and Benzer, 2000)
htt exon1 20Q	normal	normal	normal	no (Steffan et al., 2001)
22Q	normal	normal	JAMS no death	no (Marsh et al., 2000)
MJD-27Q	normal	normal	JAMS no death	no (Warrick et al., 1998)
MJD-65QNL	No JAMS death	JAMS	no effect	ND
MJD-77QNES	JAMS death	↓JAMS no death	larval lethal	yes
MJD-78Q	JAMS death	↓JAMS ↓death	larval lethal	yes (Warrick et al., 1998)
htt exon1 93Q	JAMS death	↓JAMS ↓death	JAMS death	yes (Steffan et al., 2001)
108Q	JAMS death	↓JAMS ↓death	larval lethal	yes (Marsh et al., 2000)
127Q	JAMS death	↓JAMS ↓death	larval lethal	yes (Kazemi-Esfarjani and Benzer, 2000)
reaper	No JAMS death	no effect	ND	yes

^aDeath refers to neuronal cell death.

nism? One possibility is that aggregation of pathogenic polyQ proteins in narrow axons can physically impair transport of large organelles or vesicles. While attractive, this possibility on its own does not easily account for the observation that polyQ proteins with repeat lengths that are not pathogenic can cause transport failures when combined with reductions in motor protein gene dose (e.g., MJD-27Q; Supplemental Figure S2A at <http://www.neuron.org/cgi/content/full/40/1/25/DC1>). In addition, recent work argues that aggregation per se may not be required for neuronal toxicity (Klement et al., 1998; Yoo et al., 2003). A second possibility is that titration of motor proteins from other critical pathways induces vesicle stalling during transport in narrow axons, which can nucleate organelle accumulations that block subsequent transport. While this possibility accounts for the observed ability of pathogenic polyQ proteins to significantly reduce the soluble pool of motor proteins we observe, it does not easily account for the ability of short polyQ repeats to titrate motor proteins without causing transport failure. The third possibility, which we favor, is that motor protein titration and a propensity to aggregate and physically block transport in narrow axons act in concert to poison axonal transport. This mechanism, while more complex than the others, best accounts for a number of important observations including those that are not easily explained by the simplest models. For example, this mechanism accounts for the observed ability of short polyQ repeats to titrate soluble motor proteins but to poison the transport machinery only when motor protein gene dosage is further reduced. It is also consistent with the observation that motor protein gene dosage generally needs to be reduced by more than 50% to cause significant transport phenotypes. This mechanism also accounts for our (e.g., Figure 3A) and others' observations (Piccioni et al., 2002; Li et al., 2000, 2001) that expression of polyQ proteins in neurons causes axonal inclusions that contain the polyQ proteins themselves. EM examination of the ultrastructure of these inclusions in a mouse model of HD revealed a morphology of accumulated vesicles, organelles, and distended axons that is virtually identical to what we observe in axonal blockages formed in the

Drosophila system (compare Figure 4A to Figure 4 in Li et al., 2001). Similarly, axonal abnormalities, perinuclear and nuclear accumulations, with severe dysfunction in mechanosensory neurons were observed in *C. elegans* expressing pathogenic polyQ repeats in the context of htt (Parker et al., 2001). In addition, axonopathies are prominent in a number of polyQ expansion diseases (Li et al., 2000), and this view also accounts for the beneficial effects of chaperone increases upon transport phenotypes. Live analysis also showed the accumulation of YFP-tagged vesicles into nonmotile aggregates in axons of larvae expressing expanded polyQ repeats. This mechanism would also suggest that there is a phenotypic continuum caused by motor protein reductions or physical aggregation where the burden of either one alone, if substantial enough, can cause phenotypes. However, the relative extent to which axonal blockages contribute to transport failure as compared to motor protein depletion needs to be investigated further. We also note that this proposal has the virtue that it provides a plausible explanation for the otherwise puzzling ability of broadly expressed proteins to cause neuron-specific toxicity in human disease. Thus, it is conceivable that defects in axonal pathways may contribute to early disease neuropathology.

The Normal Functions of Huntingtin

We provide direct evidence that htt is required for normal axonal transport. Neuronal depletion of *Drosophila* htt using RNAi caused an axonal blockage phenotype, which is characteristic of mutations not only in cytoskeletal motor proteins that are required for axonal transport, but also in proteins that function as receptors for motors (Bowman et al., 2000; Gunawardena and Goldstein, 2001). These axonal blockages were enhanced by a 50% reduction in kinesin. Loss of htt in the eye caused a distinct degenerative phenotype similar to what has been observed in weak mutations of DLC (Bowman et al., 1999), DIC, and heterozygous dominant mutations of p150^{Glued} (Boylan et al., 2000). Until now, previous work has only hinted at a possible transport function for htt but provided no direct evidence in support of this important proposal. For example, mouse models of HD and

conditional htt knockout mice all exhibited degeneration of axon fibers, compatible with, but not establishing, a function for htt in axonal transport (Dragatsis et al., 2000; DiFiglia et al., 1997; Hodgson et al., 1999; Li et al., 2000). Thus, our data together with htt localization data strongly support a functional role for htt in fast axonal transport. Although how htt associates with the axonal transport machinery is still unclear, we propose that a subclass of vesicles containing htt may associate with motor proteins via HAP1, or a similar protein, which establishes the link between htt and p150^{Glued}, thereby enabling transport. Although a true *Drosophila* homolog of HAP1 is yet to be identified, *Drosophila* Milton is related to HAP1 and has been suggested to be required for kinesin-dependent transport of mitochondria (Stowers et al., 2002). In addition, many coiled-coil linker proteins exist that could facilitate this connection.

Our proposal that htt is required for axonal transport explains why both reduction and gain of function cause similar phenotypes, since both can lead to failures of vesicle transport that might physically and biochemically cause organelle blockages in axons. For example, in mouse models, both loss (Dragatsis et al., 2000) and gain of function of htt causes neurodegeneration (Mangiarini et al., 1996; DiFiglia et al., 1997; Li et al., 2000) and axonal pathology (Sapp et al., 1999; Li et al., 2000, 2001). It is striking that in the *Drosophila* system too, both loss of htt function and polyQ-induced gain of function cause similar axonal blockage phenotypes including neurodegeneration in the adult eye, which may result due to disruption of a specialized neuronal pathway. Both processes could contribute to the observed reduced trafficking of the neurotrophic factor BDNF in mouse HD brains (Zuccato et al., 2001). Indeed, HD is a dominantly inherited disease with both homozygous and heterozygous individuals affected similarly by a gained toxic function.

Nuclear versus Cytoplasmic Mechanisms of PolyQ Diseases

An important point of controversy is whether neuronal toxicity in HD and other polyQ diseases results from nuclear or cytoplasmic events. We found that polyQ-induced disease pathogenesis can occur via two mechanisms—one that induces apoptosis by nuclear accumulation, and the other that induces neuronal dysfunction by disrupting axonal transport, although these two pathways may not be mutually exclusive. PolyQ-induced neuronal death did not result upon expression of a pathogenic polyQ protein restricted to the cytoplasm by the addition of a nuclear export signal (NES), although axonal blockages formed and organismal death resulted. In contrast, expression of a nuclear-targeted polyQ protein (NLS) caused nuclear accumulations, apoptosis, and lethality. These findings suggest that translocation of polyQ protein into the nucleus is required for cell death and that cytoplasmic polyQ proteins can cause axonal blockage. In fact, similar to the situation of polyQ proteins without an NES, a 50% reduction in kinesin-enhanced organelle blockages and a 50% reduction in dynein caused early organismal lethality of polyQ-NES expressing animals. Taken together, our data support two pathways for pathogenesis by polyQ proteins. In

the first, polyQ-induced cytoplasmic perturbations in axonal transport pathways could directly instigate neuronal failure and organismal death. In the second, accumulation of pathogenic polyQ proteins within the nucleus (perhaps enhanced by axonal blockages) and events triggered by the nuclear presence of pathogenic polyQ protein could trigger neuronal death, neuronal degeneration, and finally organismal death. These ideas are consistent with recent findings on the androgen receptor, which, when expanded by a polyQ stretch in the N-terminal A/B domain, causes spinal and bulbar muscular atrophy (SBMA), an X-linked, adult-onset neurodegenerative disorder. While expression of expanded polyQ repeats in the context of the androgen receptor also caused neuropil aggregates and altered the distribution of kinesin (Piccioni et al., 2002), abnormal binding of the ligand, androgen, to polyQ-expanded human androgen receptor caused neurodegeneration due to ligand-dependent structural alteration that promoted nuclear translocation (Takeyama et al., 2002). Thus, it is possible that pathogenic polyQ proteins cause polyQ-induced cytoplasmic accumulations and these accumulations may promote abnormal protein-protein interactions that could trigger a cascade of toxic events, ultimately leading to neurodegeneration and organismal death. Indeed, a recent study suggests that soluble oligomers, which are common to most aggregate forming diseases, may be cytotoxic (Kayed et al., 2003).

Both polyQ toxicity (Warrick et al., 1999; Kazemi-Esfarjani and Benzer, 2000) and α -synuclein toxicity (Auluck et al., 2002) observed in the *Drosophila* adult eye and brain are dramatically modulated by excess chaperones. It is conceivable that polyQ toxicity within axons is also modulated by chaperones. Indeed, we found that axonal blockages and neuronal cell death were completely suppressed by excess HSC70 together in transgenic lines expressing expanded polyQ repeats, although organismal lethality still persisted. The chaperone interaction with misfolded mutant polyQ protein may prevent abnormal interactions with motor proteins and other proteins, thereby preventing organelle blockages within axons and neuronal death. Consistently, cytoplasmic axonal aggregations caused by excess polyQ repeats with NES were also suppressed by chaperone expression, although organismal lethality still occurred. Lethality, however, may result due to the fact that although chaperones are modulating abnormal or misfolded proteins, they may be unable to completely prevent the toxic activity of abnormal aggregations of disease protein in time for normal development to proceed.

Is Axonal Transport a Common Target in Aggregation Disorders?

Protein aggregation appears to be a common manifestation in many neurodegenerative diseases, and increasing evidence suggests that such accumulations can be a major trigger of cellular stress and neuronal death (Wyss-Coray and Mucke, 2002). In Alzheimer's disease, accumulation of the 4 kDa A β fragment in amyloid plaques and aggregation of phosphorylated Tau in neurofibrillary tangles is observed surrounded by degenerating neurites; deposits of aggregated prion proteins with amyloid-like structures are observed in mad cow or

Creutzfeldt-Jacobs disease; in Parkinson's disease, abnormal α -synuclein accumulations known as Lewy bodies are seen; and in HD and other expanded polyQ diseases, abnormal accumulations of mutant protein are observed as nuclear and sometimes axonal inclusions. The widespread occurrence of axonal (or dendritic) inclusions leads us to wonder whether perturbations in transport could be a common pathway in neurodegenerative disease. In support of this idea, recent findings indicate that dynein and dynactin mutations can induce motor neuron degeneration in mice and humans (Hafezparast et al., 2003; Puls et al., 2003).

In this context, the strongest evidence comes from HD, which is characterized by the preferential loss of striatal neurons. Strikingly, htt accumulations are found in axons of striatal projection neurons (Li et al., 2001), and it has been argued that these striatal axonal inclusions are better correlated with striatal neuron loss than the presence of nuclear inclusions. Expression of expanded polyQ repeats in the context of the androgen receptor also forms neuropil aggregates and alters the distribution of kinesin (Piccioni et al., 2002), further supporting the idea that early pathology can occur within axonal processes together with axonal inclusions. It is possible that wild-type htt is required for efficient vesicle trafficking of cortical BDNF, since mutant htt interfered with its anterograde transport, contributing to BDNF depletion in the striatum (Cattaneo et al., 2001). The importance in transport of neurotrophic factors is also evident in Alzheimer's disease, where one of the earliest detectable signs of disease is the loss of synapses and retrograde degeneration of neurons, accompanied by the decay of intracellular traffic (Terry, 2000). In addition, excess of APP proteins containing the toxic A β region perturbed axonal transport pathways and caused neuronal cell death (Gunawardena and Goldstein, 2001). Thus, we propose that perturbation in axonal transport can contribute to early disease pathology owing to disruption in proper transport of essential neuronal components, triggering a cascade of events leading to neuronal failure and death.

Experimental Procedures

Drosophila Stocks

UAS-MJD-27Q and UAS-MJD-78Q have been previously described (Warrick et al., 1998). The NES sequence from the Rev protein (Meyer and Malim, 1994) was added to the 3' end of the MJD-78Q sequence (Warrick et al., 1998) and subcloned into the pUAST vector (B.G.-G. and N.M.B., unpublished data), and the injected construct had 77Q repeats. A polyQ construct with an added NLS (Perez et al., 1998) was subcloned into pUAST, and the injected construct had 65Q repeats (B.G.-G. and N.M.B., unpublished data). UAS-20Q and UAS-127Q were obtained from Dr. Seymour Benzer (Caltech), and UAS-22Q, UAS-108Q httex1-20Q, and httex1-93Q were obtained from Dr. Leslie Thompson (University of California, Irvine). Transgenic lines were expressed using APPL-GAL4 (Gunawardena and Goldstein, 2001), 179Y-GAL4, daughterless-GAL4, and GMR-GAL4 (Bloomington) at 29°C unless otherwise stated. The onset of APPL-GAL4 and daughterless-GAL4 was determined by crossing these to UAS-GFP (Bloomington). Genetic interaction tests were performed as in Gunawardena and Goldstein (2001). Males that were APPL-GAL4/Y;khc⁸/B3, APPL-GAL4/Y;Df(3L)8ex⁹⁴ (KLC)/B3, APPL-GAL4/Y;rob¹/B3, APPL-GAL4/Y;Df(3L)GN24 (DHC)/B3, APPL-GAL4/Y;Df(3R)ry615 (Arp1)/B3, APPL-GAL4; Df(3L)fz-GF3b (p150^{Glued})/B3, or APPL-GAL4/Y;(2)k16109 (Dmn)/B3 were crossed to females from UAS lines and only females

were used. Female tubby siblings were used as controls. Control experiments were done by crossing the above males to Oregon-R females. UAS-RhoGAP RNAi, UAS-HSC70, and UAS-reaper lines were obtained from Bloomington.

Generation of RNAi Knockout of *Drosophila* Huntingtin

The UAS double-strand htt hairpin construct was generated similar to Piccini et al., 2001. The inverted repeat RNAi clone utilized two 1 kb PCR-amplified fragments from the EST clone LD23533 corresponding to the 5' end of the *Drosophila* htt gene (CG9995). A 300 bp GFP linker fragment was PCR amplified from the GFP vector (Clontech). Fragments were cloned into pBS.SK (Stratagene) in a three part ligation. The 2.3 kb EagI/KpnI fragment was excised and cloned into pUAST; identity and orientation were confirmed by digests and sequence analysis. P element-mediated germline transformation was performed using yw as the parental strain. A total of eight lines were obtained and three lines (dhth#1, dhth#9, and dhth#13) were used for tissue-specific RNAi knockout experiments.

RT-PCR and RNA Quantitative Analysis

To determine the extent of RNAi knockout of the *Drosophila* htt gene, total RNA was isolated from single yw, APPL-GAL4;dhth#1, APPL-GAL4;dhth#9, and APPL-GAL4;dhth#13 larval brains using RNeasy (Qiagen). Any remaining DNA was removed using DNA-free system (Ambion). First strand cDNA synthesis was performed using superscript system for RT-PCR (Invitrogen). PCR was done using HotStart Taq (Qiagen) using two sets of HD primers corresponding to the middle and the 3' end of the gene to amplify a 156 bp and a 349 bp fragment of the htt gene. As a control, a 500 bp fragment of the ELAV gene was amplified. Intensity versus exposure time and intensity versus loading were analyzed and determined to be linear using line plot analysis on the Stratagene Eagle Eye II system (data not shown). The best intensity range was selected and used to measure intensity of PCR bands from experimental brains.

Cell Immunohistochemistry and Histology

Larvae were dissected, fixed, immunostained, and analyzed as described in Gunawardena and Goldstein (2001). Synaptic vesicle markers anti-DSYT2 (Hugo Bellen) and anti-CSP (Konrad Zinsmaier) were used. Anti-polyQ (Chemicon) and anti-HA (Roche and Santa Cruz) were used at 1:100. For histology, adult heads were prepared for light microscopy as described in Warrick et al. (1998).

Embryos were generated by crossing da-GAL4 females to UAS-MJD-27Q, UAS-MJD-77QNES, UAS-MJD-78Q, UAS-20Q, UAS-127Q, or yw males at 29°C. 6 hr and 12 hr embryo collections were dechorionated and fixed as described in Gunawardena and Rykowski (1994). Briefly, embryos were washed in 50% bleach in ddH₂O and fixed in heptane, 1× PBS, and 4% formaldehyde (16%, TED PELLA). Embryos were incubated overnight in anti-polyQ. DAPI (0.1 μ g/ml; Sigma)-stained embryos were mounted in Vectashield mounting medium (Vector Labs) and observed under a BIO-RAD MRC1024 confocal microscope. No noticeable developmental delays were observed during embryogenesis (data not shown).

For TUNEL analysis, larval brains were carefully dissected. Detection of apoptotic neuronal cells was performed as previously described (Gunawardena and Goldstein, 2001).

For electron microscopy, larvae were dissected, fixed, stained, and cut as previously described in Bowman et al. (1999).

Live Analysis of Vesicle Transport within Larval Axons

The APP-YFP construct (Carlos Dotti) was excised and cloned into pUAST and injected as described. APP-YFP/Y;GAL4/+ males were crossed to homozygous UAS-MJD-78Q females. Larvae were dissected as described and axonal transport was visualized live at 100× magnification on a Nikon TE2000U microscope. Images were captured using a Cool Snap HQ cooled CCD camera and Metamorph imaging system v.6.0 (Universal Imaging Corporation).

Protein Extraction, Immunoprecipitation, and Western Analysis

6 hr and 12 hr aged embryos were dechorionated and homogenized in Buffer A (250 mM sucrose, 15 mM Tris-HCl [pH 7.9], 60 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.5 mM DTT, and protease

inhibitors). Lysate was centrifuged at $4000 \times g$ for 10 min. Supernatant was centrifuged at $18,000 \times g$ for 10 min and the second supernatant was collected. Samples were loaded on 4%–12% Bis-Tris NuPage gels (Invitrogen) and transferred to Immobilon-P membrane (Millipore). Anti-*Drosophila* p150^{Glued} (Erika Holzbaur), anti-*Drosophila* DHC (Tom Hayes), anti-DIC (Chemicon), anti-*Drosophila* KHC (Bill Saxton), anti-*Drosophila* KLC, anti-HDAC3 (BD Bioscience), anti-Rab8 (BD Bioscience), anti- α -tubulin (Sigma), anti-actin (Boehringer Mannheim), anti-polyQ (Chemicon), and anti-HA (Roche) were used as recommended. Respective secondary antibodies were used and detected using ECL (Amersham Pharmacia). For immunoprecipitation, 500 μ g of lysate was precleared with 50 μ l of protein-G beads (Roche). 1 μ g of antibody was added to supernatant and incubated. 20 μ l of BSA-blocked protein-G beads were added and incubated. The beads were washed in cold Buffer A and 30 μ l of $1 \times$ LDS-sample buffer (Invitrogen) was added to beads and analyzed by Western blot analysis.

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