Characterization of a *Drosophila* Model of Huntington's Disease

by

Wyan-Ching Mimi Lee

A.B. Biology
A.B. Visual Arts
Brown University, 1999

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2006

Signature of Author: _____X
Department of Biology
May 26, 2006

Certified by: _____X
Dr. J. Troy Littleton
Associate Professor of Biology
Thesis Supervisor

Accepted by: _____X
Dr. Stephen P. Bell
Professor of Biology
Chairman of the Graduate Committee

Characterization of a *Drosophila* Model of Huntington's Disease

by

Wyan-Ching Mimi Lee

Submitted to the Department of Biology on May 19th, 2006
in Partial Fulfillment of the Requirements for The Degree of
Doctor of Philosophy in Neurobiology

ABSTRACT

Huntington's disease (HD) is an autosomal dominant neurological disorder caused by a polyglutamine (polyQ) repeat expansion in the huntingtin (Htt) protein. The disease is characterized by neurodegeneration and formation of neuronal intracellular inclusions primarily in the striatum and cortex, leading to personality changes, motor impairment, and dementia. To date, the molecular mechanisms that underlie the neurodegenerative process remain to be defined. Development of transgenic *Drosophila* HD models may facilitate dissection of molecular and cellular pathways that lead to disease pathology and suggest potential strategies for treatment.

To explore mutant Htt-mediated mechanisms of neuronal dysfunction, we generated transgenic *Drosophila* that express the first 548 amino acids of the human Htt gene with either a pathogenic polyglutamine tract of 128 repeats (Htt-Q128) or a nonpathogenic tract of 0 repeats (Htt-Q0). Characterization of these transgenic lines indicates formation of cytoplasmic and neuritic Htt aggregates in our *Drosophila* HD model that sequester other non-nuclear polyQ-containing proteins and block axonal transport.

To further explore axonal transport defects in Huntington's disease, we generated *Drosophila* transgenic strains expressing 588 aa or exon 1 N-terminal fragments of human huntingtin encoding pathogenic (HttQ138) or nonpathogenic (HttQ15) proteins tagged with mRFP and/or eGFP. These transgenic lines enable *in vivo* imaging of Htt aggregation and trafficking in live *Drosophila*, providing a unique resource for tracking Htt in real time. Our findings indicate that expression of mutant Htt may impair axonal transport through both aggregate-dependent and –independent means.

Finally, to assay the therapeutic effect of expression of an intracellular antibody (intrabody) against Htt, we generated double transgenic lines coexpressing pathogenic Htt (mRFP-HttQ138) with the V_L12.3 intrabody. Intrabody expression caused suppression of aggregation in both neuronal and non-neuronal cell types, but failed to rescue mutant Htt-mediated cellular dysfunction.

In summary, our *Drosophila* HD model provides an ideal *in vivo* system for examination of mutant Htt-mediated cellular defects, particularly impairment of axonal transport, and may facilitate rapid development and validation of potential treatments for Huntington's disease.

Thesis Supervisor: J. Troy Littleton
Title: Associate Professor of Biology

Acknowledgments

I thank my parents and my sister and brother for supporting me in my graduate career as in all other aspects of my life. During the long grad school years, it's been a huge comfort to know that I can always return to a warm, loving home for restorative visits as needed. My parents have given me every opportunity to find happiness, and I will always be grateful to them for the influence they have had and continue to have on my life. My sister allows me to live vicariously through her college exploits, and is never too busy or too bogged down by drama to offer a few words of encouragement. As for my brother… well, until a couple months ago, he thought I had spent six years here in pursuit of my *Master's* degree, which goes to show how much interest he takes in my scientific career. But I know that at heart, he, like the rest of my family, wishes for my success and happiness. I'm very thankful for their love and support.

I can't imagine how the past five years would have been without Salil Soman, my fiancé and other (and much better) half, who has provided me with an unending supply of food, comfort, laughter and love during the writing of this thesis and throughout the years of research that preceded it. His love and his belief in me give me strength in everything I do. Also, his ability to turn on the tough-love approach and give me a kick where I need it, when I need it, has gotten me over some of the most difficult hurdles of my graduate career. I am so fortunate to have him in my life.

I also thank my close friends Pia and Steve Owens, who have always welcomed me into their home and made me part of their family, especially during the years when my own family was out of the country. Long conversations with them about everything and nothing have kept me in touch with life outside the scientific bubble. I have also greatly enjoyed their many thoughtful gestures, their delicious home-cooked dinners, and their admiration of my ability to shake the head off a fly.

My graduate years have been a true pleasure, thanks to past and present members of the Littleton Lab. No matter how frustrating things got on the research front, I could always look forward to lab conversations on topics ranging from the taste of cat food to the tenets of Catholic theology to the iron content of plantains. It has been wonderful to work in an environment in which every colleague can truly be called a friend.

Finally, my deepest thanks go to Troy Littleton, my teacher and mentor, who has helped me in every way throughout my graduate career and has supported all my scientific and personal goals. It has made all the difference to work with an advisor who is passionate not only about the science but also about the happiness of the people doing the science. His generosity with his time and effort has been astounding, not least in the editing of this thesis. I couldn't ask for a better advisor or friend.

TABLE OF CONTENTS

CHAPTER 1

Introduction

Wyan-Ching Mimi Lee

Huntington's disease: an overview

Huntington's disease (HD), a devastating autosomal dominant neurodegenerative disease affecting 1 in 10,000 (HDCRG, 1993), is the most common inherited neurodegenerative disorder. The age of onset is highly variable; in most cases, onset occurs between the ages of 35-50, but HD has been reported in patients from the ages of 2 to >80 (Myers, 2004). Symptoms of HD include involuntary choreiform movements and loss of motor coordination, cognitive impairment, and psychiatric disturbances, particularly depression. (Vonsattel et al., 1985). Disease manifestation is progressive, with death occurring 15-20 years after onset of the first symptoms; earlier onset is usually associated with more rapid progression (Myers, 2004). At present, there is no cure.

A substantial research effort has focused on neurodegenerative disorders caused by protein misfolding, including Huntington's disease and other trinucleotide repeat disorders, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and prion diseases. Each disease is characterized by mutation and subsequent misfolding of specific protein(s), leading to the formation of intracellular inclusions and development of neuropathology in selective brain regions (Ross and Poirier, 2004). In the case of polyglutamine (polyQ) repeat disorders, CAG expansions in the open reading frame of specific polyQ-tract containing proteins lead to nine known diseases, including Huntington's disease, spinal and bulbar muscular atrophy (SBMA), dentatorubral and pallidoluysian atrophy (DRPLA), and spinocerebellar ataxias (SCAs) 1, 3, 6, and 17. With the exception of SBMA, all of the polyQ diseases are autosomal dominant and exhibit late-onset progressive neurodegeneration that roughly correlates with the formation of neuronal inclusions in regions of the brain specific to each disease (Ho et al., 2001b). The selective vulnerability of neuronal subtypes is an ongoing question in polyglutamine disease research; although the causative proteins are expressed ubiquitously, characteristic patterns of neurodegeneration are seen in each disorder.

In Huntington's disease, pathology is characterized by atrophy of the caudate and putamen in the basal ganglia, as well as the cerebral cortex, reducing brain weight by up to 25-30% (Aylward et al., 1997; Rosas et al., 2002) (Fig. 1). Most affected are GABAergic type II medium spiny projection neurons, which constitute about 80% of striatal neurons, and large neurons in layers III, IV, and V of the cortex (Hedreen et al.,

1991). Interestingly, striatal interneurons are spared (Zucker et al., 2005). Medium spiny neurons receive glutamatergic signals from the cerebral cortex; defects in the basal ganglia-thalamocortical pathways involved in motor control may contribute to the choreiform disorders seen in HD (Albin et al., 1990).

The Huntington's disease gene, huntingtin (Htt), was mapped to chromosome 4p16.3 in 1983, and the causative mutation was identified in 1993 as an expansion in a CAG repeat at the N-terminus of the protein, coding for a glutamine tract (HDCRG, 1993). In concordance with the other polyQ repeat disorders, the normal, nonpathogenic range for the polyQ tract in Htt is 6-35 repeats, while 36-39 repeats are variably penetrant, and expansion to greater than 40 repeats results in a fully penetrant disease phenotype (Rubinsztein et al., 1996). An inverse correlation exists between polyQ repeat length and age of HD onset, with expansion past 70 glutamines resulting in a severe juvenile form of HD (Duyao et al., 1993; Telenius et al., 1993). Pathogenic repeat lengths are unstable, with a bias towards expansion, especially during paternal transmission (Ranen et al., 1995). This phenomenon, or "anticipation", results in increasing severity of symptoms or earlier age of onset through successive generations (McInnis, 1996; Ross et al., 1993); as a result, most cases of juvenile HD are inherited from the father (Ridley et al., 1988).

Only 70% of the variability in age of onset can be accounted for by CAG repeat length (Ho et al., 2001b), suggesting the existence of genetic and environmental modifiers of HD. Recent studies indicate that the size of the polyQ repeat in the normal Htt allele in HD patients influences the age of disease onset; surprisingly, in one report, larger polyQ repeats in the wild-type protein are correlated with later onset (Djousse et al., 2003). Additionally, variations in the glutamate receptor 6 (GluR6) subunit of the kainate receptor are thought to affect the onset of symptoms. A genome scan has identified other loci that may encode genetic modifiers (Li et al., 2003b). Environmental factors may also play a role in mediating HD onset and progression, as suggested by the fact that monozygotic twins with polyQ repeats of identical length can exhibit different ages of onset and severity of HD symptoms (Anca et al., 2004; Georgiou et al., 1999). Environmental enrichment has been shown to delay the progression of motor symptoms and neuronal loss in mouse models of HD (Hockly et al., 2002; van Dellen et al., 2000), potentially by enhancing adult neurogenesis (Lazic et al., 2006; van Dellen and Hannan, 2004). Dietary restriction and supplementation with essential fatty acids may also delay disease onset in HD mice (Clifford et al., 2002; Duan et al., 2003).

Figure 1

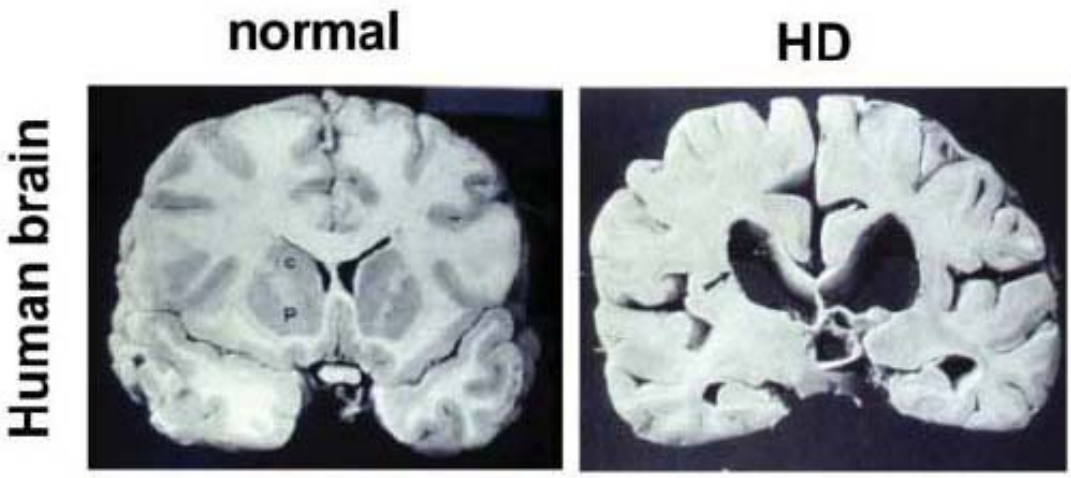

FIGURE 1. **Severe atrophy of the striatum and cortex in Huntington's disease brain.** Particularly evident is loss of cells in the caudate (c) and putamen (p), resulting in enlarged lateral ventricles in HD brain. (Reproduced from Marsh et al., 2003)

The structure and putative function of wild-type huntingtin

Wild-type Htt is expressed ubiquitously in humans and rodents (Ferrante et al., 1997; Fusco et al., 1999), and is particularly enriched in the brain (Cattaneo et al., 2005). The protein is predominantly cytoplasmic, and is localized to various subcellular compartments, including the ER, Golgi, neurites and synapses (DiFiglia et al., 1995; Hilditch-Maguire et al., 2000; Kegel et al., 2002; Li et al., 2003a; Velier et al., 1998), but may also have a role in the nucleus (Kegel et al., 2002). Expression of Htt is essential for embryogenesis (Duyao et al., 1995; Nasir et al., 1995) and remains necessary throughout development (Bhide et al., 1996; Dragatsis et al., 2000; Nasir et al., 1995; Reiner et al., 2001) and in adulthood (O'Kusky et al., 1999), but the function of the Htt protein has yet to be conclusively identified.

The Htt protein is comprised of 3144 amino acids, coding for a 348 kDa, 67 exon protein that shows little homology to other known proteins. A polyQ stretch at the amino-terminus of the protein is followed by a polyproline (polyP) tract and 37 putative HEAT repeats of about 40 aa in 3 clusters (Cattaneo et al., 2005). Htt may also contain both a nuclear localization signal and a nuclear export signal, indicating that it may have a role in transporting molecules between the nucleus and cytoplasm (Xia et al., 2003) (Fig. 2).

The structure of Htt allows it to interact with many binding partners; the polyQ tract, for instance, may bind to polyQ stretches in other proteins. The polyP domain helps to maintain Htt solubility (Steffan et al., 2004) and interacts with Src homology 3 (SH3) or tryptophan domains of other proteins, which are thought to include SH3GL3/endophilin 3 (Sittler et al., 1998), protein kinase C, PACSIN1 (Modregger et al., 2002), p53 (Steffan et al., 2000), and PSD-95 (Sun et al., 2001). The HEAT repeats are found in proteins involved in intracellular transport, microtubule dynamics, and chromosome segregation (Neuwald and Hirano, 2000) and form hydrophobic α-helices that assemble into a superhelix with a groove for interactions with proteins such as HIP1, HAP1, and HIP14 (Andrade et al., 2001; Harjes and Wanker, 2003; Li et al., 2006).

The function of proteins known to interact with Htt may help to define its normal activity. PolyQ- and HEAT repeat-containing proteins often play a role in regulation of transcription, and studies have shown interactions between Htt and various transcription factors such as the cAMP response-element binding protein (CBP) (McCampbell et al., 2000; Steffan et al., 2000), p53 (McCampbell et al., 2000; Steffan et al., 2000), Sp1, TAFII130 (Dunah et al., 2002), N-CoR, and Sin3A (Boutell et al., 1999). Htt may also

play a role in the cytoplasm; it associates with clathrin-coated vesicles and endosomal compartments, as well as with microtubules (Li et al., 2003c), indicating that it may play a role in clathrin-mediated endocytosis. This possibility is underscored by the postulated interaction of Htt with proteins involved in endocytosis, such as HIP1, HIP12, PACSIN1, SH3GL3, and HIP14 (Harjes and Wanker, 2003). Additionally, Htt is associated with both vesicles and proteins involved in vesicle transport, such as HAP1, which binds to the p150glued subunit of dynactin (Engelender et al., 1997; Li et al., 1998). Htt and HAP1 are cotransported in both the anterograde and retrograde direction (Block-Galarza et al., 1997), indicating that Htt may function as an adaptor linking transport cargoes to motor systems. The palmitoylation of Htt by HIP14, a palmitoyl transferase, supports the hypothesis that it is involved in vesicular trafficking; palmitoylated proteins are often involved in the regulation of vesicle transport and function (DiFiglia et al., 1995; Huang et al., 2004). Htt may also function in post-synaptic signaling through interaction with PSD-95, a scaffolding protein that links glutamate receptors and cytoplasmic signaling proteins (Sheng and Kim, 2002), and proteins that mediate dendritic morphogenesis such as CIP4 (Holbert et al., 2003) and FIP-2 (Hattula and Peranen, 2000). Lastly, Htt binding to HIP1, which reduces the ability of HIP1 to induce procaspase-8 cleavage and apoptosis (Gervais et al., 2002), and Htt phosphorylation by the serine/threonine kinase Akt, a component in cellular survival pathways (Humbert et al., 2002), indicate that Htt may play an anti-apoptotic role in neurons.

The discovery of several vertebrate and invertebrate Htt homologues has identified conserved domains that may be important for Htt function. Homologues have been identified in mouse, rat, pufferfish, zebrafish, and *Drosophila*. Htt is highly conserved among vertebrates, with greater than 90% peptide sequence identity to human Htt in rodents (Barnes et al., 1994; Schmitt et al., 1995); however, the polyQ repeat is greatly reduced, with only 7Q in mouse Htt (mHtt) (Barnes et al., 1994) and 4Q in pufferfish and zebrafish Htt (pHtt and zHtt, respectively) (Baxendale et al., 1995; Karlovich et al., 1998). The polyQ and polyP regions are less conserved than the HEAT repeats (Takano and Gusella, 2002b), suggesting that binding of Htt interactors to HEAT superhelices may play a key part in normal Htt function. The *Drosophila* homologue of Htt (dHtt) is the only entirely known invertebrate Htt sequence and is composed of 3583 aa coding for a 394 kDa, 29 exon protein that is expressed throughout development and in the adult fly (Li et al., 1999b). dHtt lacks polyQ and polyP tracts, as well as consensus caspase cleavage sites found in vertebrate Htts, but contains five regions of similarity to

Figure 2

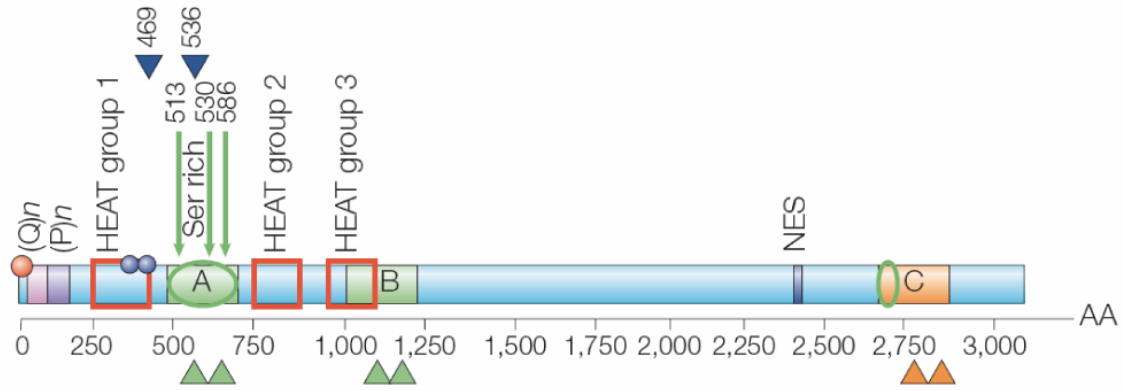
469
536
513
530
586
HEAT group 1
Ser rich
HEAT group 2
HEAT group 3
(Q)n
(P)n
NES
A
B
C
AA
0
250
500
750
1,000
1,250
1,500
1,750
2,000
2,250
2,500
2,750
3,000

FIGURE 2. **Structure of wild-type huntingtin.** The polyglutamine and polyproline repeats are indicated by (Q)n and (P)n, respectively., while the nuclear export signal is identified by NES. The red squares outline the locations of three main clusters of HEAT repeats. Caspase cleavage sites are indicated by the green arrows, while calpain cleavage sites are marked by blue arrowheads. Green and orange arrowheads show sites of protease cleavage selective for areas of the brain; A identifies regions cleaved in both the striatum and cortex, while B indicates regions preferentially cleaved in cortex and C indicates regions preferentially cleaved in striatum. (Adapted from Cattaneo et al., 2005)

vertebrate Htt that may represent functional domains (Li et al., 1999b). dHtt also contains 28 putative consensus HEAT repeats (Takano and Gusella, 2002b), underscoring the importance of these repeats in Htt function. No Htt homologues have been identified in *C. elegans, S. cerevisiae,* or *Arabidopsis thaliana* (Takano and Gusella, 2002b).

The polyQ repeat is conserved throughout vertebrate evolution, but is only highly polymorphic in humans (Cattaneo et al., 2005); concordantly, only humans develop the polyQ expansions in Htt that lead to Huntington's disease. The mechanism and effect of polyQ repeat expansion in the Htt protein are subjects of intense study.

The role of mutant huntingtin in Huntington's disease pathology

The repeat instability of the polyQ tract found in Htt is thought to occur through defective replication, recombination and repair during gametogenesis. Trinucleotide repeat sequences form unusual DNA structures prone to replication slippage when expanded past the disease threshold (Pearson et al., 2005). The mutated protein then takes on an abnormal conformation that promotes formation of globular and protofibrillar intermediates (Fig. 3) (Poirier et al., 2002; Sanchez et al., 2003) that then assemble into SDS-resistant aggregates with a β-sheet conformation (Scherzinger et al., 1997); this type of amyloid structure is found in many late-onset neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and the prion diseases (Ross and Poirier, 2004). The precise β-sheet structure formed by aggregates of polyglutamine-expanded proteins remains unknown; several potential structures are proposed by computer modeling (Fig. 4). Aggregation may exhibit "seeded polymerization" kinetics, with a lag phase followed by a rapid growth phase (Chen et al., 2001; Scherzinger et al., 1999). The "polar zipper" hypothesis of aggregate formation states that polyQ tracts associate through hydrogen bonding between side-chain and main-chain amides, with self-interaction more rapid and energetically favorable for longer polyQ chains (Perutz, 1996; Perutz et al., 1994). There is also some evidence for formation of aggregates through the action of transglutaminases (Cooper et al., 1999; Zainelli et al., 2005), which catalyze formation of cross-linkages between glutamine and lysine residues.

Much evidence suggests that the Htt protein is cleaved *in vivo* into N-terminal fragments, which are then able to form aggregates (Poirier et al., 2002); inclusions in HD

brain tissue are selectively labeled with antibodies to the N-terminus of Htt (Becher et al., 1998; DiFiglia et al., 1997). In most mouse and cellular HD models, N-terminal fragments of mutant Htt are more toxic than the full-length protein (de Almeida et al., 2002). The Htt protein contains three well-characterized caspase cleavage consensus sites (Goldberg et al., 1996; Wellington et al., 1998; Wellington et al., 2000) that are highly conserved in all vertebrate homologues of Htt (Goldberg et al., 1996; Wellington et al., 1998). In addition, calpain cleavage sites are found in human and mouse Htt (Gafni and Ellerby, 2002; Gafni et al., 2004). Mutant Htt is more susceptible to caspase- and calpain-mediated cleavage, producing toxic N-terminal Htt fragments that are found in both the nucleus and the cytoplasm (DiFiglia et al., 1997; Kim et al., 1999; Lunkes et al., 2002). Proteolytic processing appears to facilitate HD pathogenesis, as impairment of caspase and calpain activity reduces Htt toxicity and delays disease progression in cell cuture (Gafni et al., 2004; Ona et al., 1999; Wellington et al., 2000). Phosphorylation at serines 421 and 434, which inhibits cleavage of Htt, is reduced in HD brain (Humbert et al., 2002; Luo et al., 2005; Warby et al., 2005). Interestingly, Htt is cleaved at different sites in the cortex and striatum (Mende-Mueller et al., 2001), providing a potential reason for selective neuronal vulnerability in HD.

An ongoing controversy in the field of HD research is whether HD pathology is caused by gain or loss of Htt function. With the exception of SBMA, all of the polyQ disorders are inherited in an autosomal dominant manner, suggesting a gain of function in the disease protein. However, haploinsufficiency of the normal allele or dominant negative activity of the mutant allele may contribute to disease pathogenesis. Inactivation of mHtt expression in the brains of adult mice results in a progressive neurological phenotype similar to that seen in transgenic mouse models of polyQ diseases (Dragatsis et al., 2000). In addition, one-third of mice with a 50% decrease in full-length wild-type mHtt expression develop behavioral and cognitive abnormalities coupled with neurodegeneration in adulthood (Nasir et al., 1995; O'Kusky et al., 1999), suggesting that reduction in levels of normal Htt can lead to an HD-like pathology in the absence of an expanded polyQ protein. Wild-type Htt has an anti-apoptotic function in neurons (Gervais et al., 2002; Rigamonti et al., 2000; Rigamonti et al., 2001); 50% or greater loss of this function may contribute to neuronal apoptosis see in human HD brain (Portera-Cailliau et al., 1995; Thomas et al., 1995) and in mHtt knockout mice (Dragatsis et al., 2000). Compromising the role of normal Htt in production and transport of the neurotrophic factor BDNF (Zuccato et al., 2001) may also lead to neurodegeneration.

Figure 3

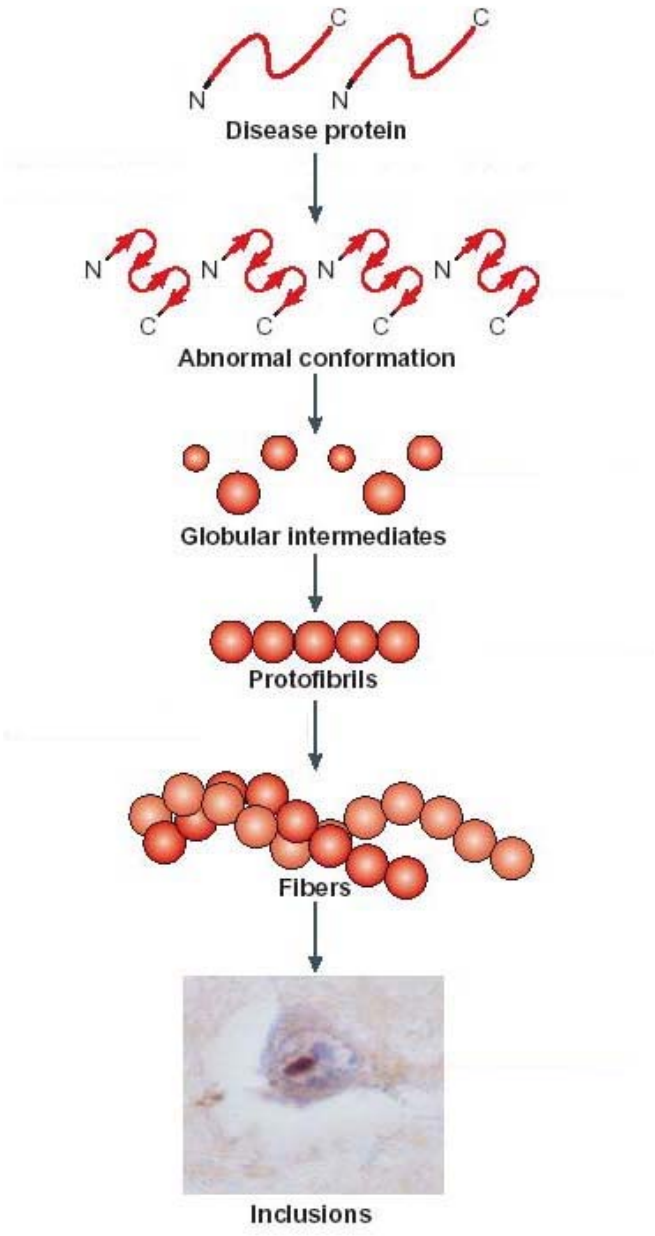
C
C
N
N
Disease protein
N
N
N
N
C
C
C
C
Abnormal conformation
Globular intermediates
Protofibrils
Fibers
Inclusions

FIGURE 3. **Formation of intermediates and aggregates by mutant huntingtin.** Mutation of the protein allows adoption of an abnormal conformation which then promotes sequential formation of globular intermediates, protofibrils, fibers, and SDS-resistant inclusions. (Reproduced from Ross and Poirier, 2004)

Figure 4

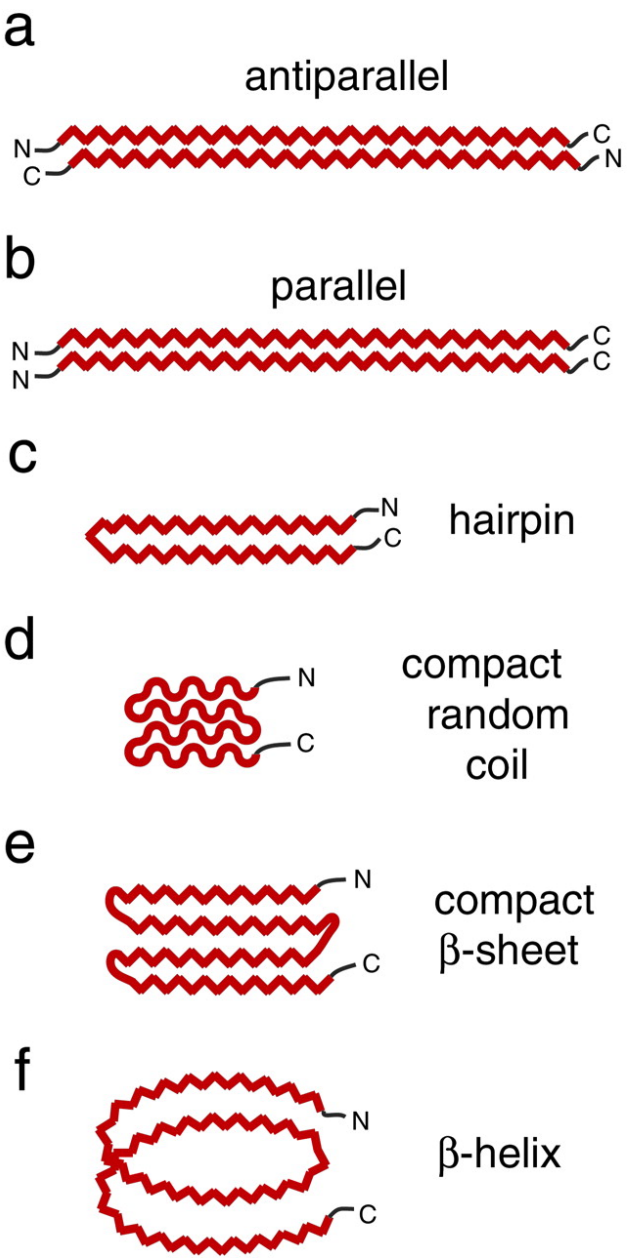
a
antiparallel
N
C
C
N
b
parallel
N
N
C
C
c
N
C
hairpin
d
N
C
compact
random
coil
e
N
C
compact
β-sheet
f
N
C
β-helix

FIGURE 4. **Potential β-sheet models for expanded polyglutamine aggregates.** (A) Anti-parallel β-sheet "polar zipper" structure described by Perutz (Perutz et al., 1994). (B) Parallel β-sheet. (C) Anti-parallel β-hairpin. (D) Compact random coil containing four anti-parallel elements. (E) Compact β-sheet structure consisting of four β-strand elements. (F) Parallel β-helix structure with 20 residues per turn. (Reproduced from Ross et al., 2003).

However, much evidence indicates that HD is not caused by a simple loss of wild-type Htt function. Patients hemizygous for normal Htt do not develop any symptoms of HD (Ambrose et al., 1994). In addition, expression of mutant Htt can rescue lethality in mHtt knockout mice (White et al., 1997), indicating that expansion of the polyQ tract does not cause complete loss of normal Htt function.

More evidence exists for a gain of toxic function through expansion of the polyQ tract in the mutant Htt protein. While expansion of the polyQ repeat in Htt does not abolish its function, insertion of a pathogenic polyQ repeat into HPRT, a protein not related to neurological disease, results in a late-onset neurodegenerative phenotype and premature death (Ordway et al., 1997), providing evidence for toxicity mediated by the expanded polyQ. Transgenic mice expressing either full-length mutant Htt or an N-terminal fragment of mutant Htt in addition to two wild-type alleles of mHtt develop signs of HD pathology (Hodgson et al., 1999; Mangiarini et al., 1996; Reddy et al., 1998; Schilling et al., 1999); knock-in mice in which the polyQ tract of mHtt has been expanded past the pathogenic threshold also exhibit HD-like symptoms (Lin et al., 2001; Shelbourne et al., 1999; White et al., 1997). Additionally, expression of expanded polyQ proteins in *C. elegans*, which has no functional Htt homologue, is enough to produce a neurodegenerative phenotype (Faber et al., 1999). Expansion of the polyQ repeat causes the Htt protein to assume an abnormal conformation, which may allow Htt to undergo abnormal associations with other proteins, including its self-association into aggregates. Toxicity of mutant Htt may be mediated either by a soluble monomeric form, by protofibrillar intermediates, or by fully formed aggregates.

While visible aggregates of mutant Htt are a hallmark of HD pathology, another hotly debated question in the field of HD research concerns whether the aggregates are harmful, neuroprotective, or neither; evidence exists for all sides, and is mostly correlative. Resolution of this issue is important in deciding whether aggregation may be a target for HD therapeutics. Evidence for aggregate-mediated pathology includes the correlation between the polyQ threshold for *in vitro* aggregation and the threshold for disease manifestation (Davies et al., 1997; Scherzinger et al., 1999). Longer polyQ tracts undergo more rapid aggregate formation and require a lower critical concentration for aggregation, correlating with an earlier onset of disease symptoms (Chen et al., 2001; Scherzinger et al., 1999). Compounds that suppress aggregation, including chaperone proteins (Cummings et al., 1998; Warrick et al., 1999), chemical chaperones (Yoshida et al., 2002), small peptides (Kazantsev et al., 2002; Nagai et al., 2000),

intracellular antibodies (Colby et al., 2004a; Khoshnan et al., 2002; Lecerf et al., 2001), and drugs (Heiser et al., 2000; Wang et al., 2005), have also been found to suppress polyQ toxicity. In addition, when the polyQ tract of the SCA1 polyQ disease protein is disrupted by insertion of histidine, aggregation is greatly reduced and the disease does not manifest (Sen et al., 2003). In a conditional mouse model of HD, turning off Htt expression causes disappearance of nuclear aggregates and improvement in motor performance (Yamamoto et al., 2000). Aggregates may cause pathology by sequestering various proteins, including mutant or wild-type Htt, away from their regular sites of function, or may disrupt or physically block cellular processes.

However, other studies argue that aggregates play no role in polyQ-mediated pathology. Evidence indicates that neurons exhibiting aggregates do not correspond entirely with neurons that undergo degeneration in HD; while some aggregates are found in medium spiny projection neurons of the striatum (Vonsattel et al., 1985), which are most vulnerable to neurodegeneration, more aggregates are found in striatal interneurons (Kuemmerle et al., 1999), which are spared. A mouse model of HD expressing full-length mutant Htt exhibits a behavioral phenotype and neuronal cell loss, but little aggregation is observed (Hodgson et al., 1999; Reddy et al., 1998). Some studies suggest that entry into the nucleus, rather than aggregate formation, is the cause of Htt toxicity (Bae et al., 2006; Peters et al., 1999; Saudou et al., 1998), and that aggregation may play a neuroprotective role by reducing the surface area with which aggregates can associate with other proteins. In support of this neuroprotective function, aggregate formation correlates with a decrease in diffuse intracellular mutant Htt and an increase in cellular survival in neuronal culture (Arrasate et al., 2004). In addition, disrupting ubiquitin conjugation suppresses aggregation but enhances toxicity in primary striatal neurons expressing mutant Htt (Saudou et al., 1998). Visualization of neuronal nuclei in SCA1 and SCA3 brains show that nuclei appear healthier in neurons with visible aggregates than those without (Nagaoka et al., 2003; Uchihara et al., 2002), suggesting that aggregate formation may present a general mechanism to decrease toxicity of expanded polyQ disease proteins. One hypothesis explains this neuroprotectivity as a result of stimulation of autophagy through aggregate-mediated sequestration of mTOR, a negative regulator of the autophagic pathway (Ravikumar et al., 2004). Impairment of mTOR function may lead to subsequent degradation of toxic polyQ protein in the cytoplasm.

It remains a possibility that small “microaggregates”, rather than fully formed, visible aggregates, are the cause of HD pathology (Meredith, 2006). Globular and protofibrillar intermediate structures found in HD (Poirier et al., 2002; Sanchez et al., 2003) may be responsible for Htt toxicity, which may be decreased by assembly of the toxic intermediates into aggregates. However, administration of the dye Congo red, which increases the ratio of protofibrils to aggregates, improves the behavioral phenotype and extends lifespan in transgenic HD mice (Sanchez et al., 2003), indicating that fully-formed aggregates of Htt are more toxic than intermediate structures.

While it is likely that gain of toxic Htt function plays a key role in HD pathogenesis, at present it is impossible to rule out loss of wild-type Htt function as a contributing factor. Use of animal models of HD may elucidate the normal function of Htt, the role of aggregates in HD pathology, and the cellular pathways affected in disease pathogenesis.

Animal models of Huntington’s disease

A range of animal models of HD have been developed to determine normal Htt function and to identify the molecular pathways affected in HD. Such models are particularly useful to determine early, causative events in HD, as there is insufficient availability of brain tissue from presymptomatic HD patients to make studies possible. Genetic tools available in different model systems have made it possible to create a variety of knockout, transgenic, and knock-in models of HD.

To date, mouse models of HD have been most commonly used for HD research. These models fall into three general categories: (1) homozygous or heterozygous knock-out mice with inactivation of one or both mHtt alleles, (2) transgenic mice expressing truncated or full-length mutant human Htt, in addition to two wild-type alleles of mHtt, (3) mice with a pathogenic polyQ tract inserted into the existing polyQ tract (normally only 7 Qs) of one allele of mHtt, in the presence of a wild-type mHtt allele.

Studies using knock-out mouse models of HD have determined that Htt expression is essential throughout development and in adulthood. Homozygous deletion of mHtt results in embryonic lethality before day 8.5 of embryogenesis, possibly due to defects in extra-embryonic tissue (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Htt is also necessary post-gastrulation; conditional inactivation of Htt expression

in the brain and testis of adult mice causes neurodegeneration and impaired spermatogenesis (Dragatsis et al., 2000).

Several transgenic mouse models of HD have also been generated, expressing a range of N-terminal fragments or the full-length form of the human Htt protein with varying expansions in the polyQ repeat. In general, toxicity of transgene expression is correlated with expression levels and with expansion size of the CAG repeat, and is inversely correlated with transgene length. The most well-studied transgenic mouse HD model is the R6/2 mouse, which expresses exon 1 of human Htt with around 145 Qs (Mangiarini et al., 1996). The R6/2 mouse exhibits a progressive behavioral and neuropathological phenotype with extensive formation of intranuclear and cytoplasmic aggregates (Davies et al., 1997; Li et al., 1999a), but without overt neuronal loss (Hockly et al., 2003b; Turmaine et al., 2000). R6/1 mice, which harbor a shorter polyQ repeat of 115 Q, exhibit slower disease progression with delayed formation of nuclear aggregates when compared to the R6/2 model (Davies et al., 1997). N-171-82Q mice express both exons 1 and 2 of human Htt with 82 Qs (Schilling et al., 1999). While the behavioral phenotype is more subtle in this model, the neuropathological profile more closely resembles that seen in HD brain, with a greater density of aggregates in cortical than in striatal neurons, but with more evidence of neurodegeneration in the striatum (Yu et al., 2003).

Mice expressing full-length human Htt with 48 or 89 Qs driven by the CMV promoter exhibit both a progressive motor phenotype and striatal neurodegeneration (Reddy et al., 1998). A yeast artificial chromosome HD mouse model (YAC 128) expresses full-length human Htt with 128 Qs along with flanking genomic sequence that might contain Htt regulatory elements, and exhibits both motor defects and cortical and striatal neuronal cell loss correlating with behavioral abnormalities (Slow et al., 2003). Interestingly, very few aggregates are observed in full-length Htt-expressing mouse models, while even very low expression levels of truncated Htt lead to aggregate formation and a disease phenotype (Schilling et al., 1999), possibly highlighting the role of protein cleavage in HD pathogenesis.

Knock-in mouse models of HD may be the most relevant for elucidating the role of the expanded polyQ repeat in the Htt protein, as the mHtt gene with an expanded CAG insertion is expressed under its natural promoter and in the proper genomic context. A CAG repeat insertion of 72-80 in the polyQ tract of mHtt gene causes mice to exhibit aggressive behavior. Neuropil aggregates are present, but no neuronal loss is

evident (Lin et al., 2001; Shelbourne et al., 1999). Knocking in 94 CAG repeats results in progressive behavioral abnormalities accompanied by formation of nuclear microaggregates, but lifespan is normal (Menalled et al., 2002). Insertion of 111 CAG repeats results in progressive formation of nuclear aggregates of N-terminal mHtt fragments in the striatum, with some striatal neurodegeneration observed, but no behavioral phenotype (Duan et al., 2003; Wheeler et al., 2002), while insertion of 140 repeats leads to behavioral symptoms that precede the neuropathological phenotype of nuclear aggregrate formation in the striatum and neuropil aggregate formation in the globus pallidus and cerebral cortex (Menalled et al., 2003). Lastly, insertion of 150 CAG repeats results in motor impairment, striatal gliosis, and the development of striatal intranuclear aggregates (Lin et al., 2001). The knock-in models exhibit alterations in molecular and cellular processes, but do not display overt neuronal loss, suggesting that neuronal dysfunction precedes neurodegeneration in HD.

Invertebrate models of HD have also proven invaluable in disease research. While HD mouse models allow investigation of disease pathogenesis in a mammalian system that harbors more genetic and anatomical similarity to humans, invertebrate organisms such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* provide many experimental advantages that are not available in more complex organisms. These include small size, short generation time, and sophisticated tools for genetic and molecular manipulations. While *Drosophila* and *C. elegans* are phylogenetically more distant from humans, many important cellular pathways are highly conserved (Bargmann, 1998; Yoshihara et al., 2001).

Transgenic *C. elegans models* exist for Alzheimer's disease (Link et al., 2001) and Parkinson's disease (Nass et al., 2001), as well as for HD (Faber et al., 1999) (Parker et al., 2001). Neuronal expression of N-terminal Htt fragments containing 88 or 128 glutamines results in neuronal dysfunction (Parker et al., 2001), while expression of an N-terminal Htt fragment with 150 Qs leads to progressive degeneration (Faber et al., 1999). Both models exhibit formation of cytoplasmic aggregates.

Several transgenic *Drosophila* models of polyQ disease have also been generated (Fernandez-Funez et al., 2000; Gunawardena et al., 2003; Jackson et al., 1998; Kazemi-Esfarjani and Benzer, 2000; Lee et al., 2004; Marsh et al., 2000; Steffan et al., 2001; Takeyama et al., 2002; Warrick et al., 1999), and replicate many key features of the human diseases, including late-onset, progressive cellular pathology as a function of polyQ repeat length. Fly models also exhibit behavioral phenotypes and

premature death. Models commonly express a range of N-terminal mutant Htt fragments; expression of truncated mutant human Htt with 75 or 120 Qs in the fly eye leads to progressive neuronal degeneration in the absence of nuclear inclusions (Jackson et al., 1998), while pan-neuronal expression of either exon 1 or a 548 N-terminal fragment of mutant human Htt results in formation of cytoplasmic and neuritic aggregates that disrupt axonal transport (Gunawardena et al., 2003; Lee et al., 2004).

Invertebrate models of HD may prove invaluable in elaborating cellular pathways affected in HD. Importantly, these models can be utilized to perform non-biased genetic screens to identify *in vivo* suppressors and enhancers that may provide insights into both normal Htt function and mechanisms of mutant Htt toxicity (Kazemi-Esfarjani and Benzer, 2000). These organisms are also well-suited for *in vivo* testing of putative genetic and pharmacological Htt therapies (Kazantsev et al., 2002; Steffan et al., 2001; Zhang et al., 2005), and can be used for high-throughput screening of large compound libraries (Bates and Hockly, 2003). Continued study of invertebrate HD models will contribute greatly to the elucidation of HD pathogenesis and treatment.

Theories of Huntington's disease pathogenesis

The molecular pathways leading to HD pathogenesis have yet to be defined. Many cellular processes are proposed to play a key role in disease pathology; however, it is difficult to distinguish between early, causative events and secondary changes resulting from massive neuronal dysfunction. In addition, it is likely that multiple pathogenic mechanisms, rather than a single initiating mechanism, contribute to HD onset. Mutant Htt is found in both the nucleus and the cytoplasm of HD brain (Benn et al., 2005; Gutekunst et al., 1999); it is unknown whether toxic Htt activity occurs in the nucleus, in the cytoplasm, or in both. Nuclear theories of HD pathogenesis focus mainly on transcriptional dysregulation, while toxicity of Htt in the cytoplasm may lead to ubiquitin/proteasome dysfunction, aberrant caspase activity, synaptic pathology, excitotoxicity, mitochondrial dysfunction, and/or impaired axonal transport.

Transcriptional dysregulation

DNA transcription is a highly regulated cellular process that is impaired in HD, resulting in altered levels of expression for a number of genes. Both wild-type and mutant Htt have been shown to interact with a range of transcription factors, giving rise to the hypothesis that abnormal interactions between mutant Htt and proteins involved in transcription lead to transcriptional dysregulation, which may be an early event in HD pathogenesis (Sugars and Rubinsztein, 2003).

PolyQ repeats commonly occur in transcription factor proteins, suggesting that wild-type Htt may play a role in transcription via interaction with these proteins. While Htt localizes mainly to the cytoplasm (DiFiglia et al., 1995), it has also been observed in the nucleus in human fibroblasts; mutant Htt in the nucleus has been shown to repress transcription (Kegel et al., 2002). Mutation of Htt may result in dysregulation of transcription either through loss of normal Htt activity or abnormal binding of transcription factors by the mutant protein, leading to widespread alterations in gene expression. Analysis of HD brain reveals changes in gene expression profiles that are most pronounced in the caudate nucleus and the motor cortex (Hodges et al., 2006). In a conditional PC12 cell model expressing exon 1 of mutant human Htt, transcriptional changes occur within hours of turning on mutant Htt expression (Kita et al., 2002); these include decreased expression of genes involved in glucose and lipid metabolism and altered expression of genes involved in oxidative stress response. Alterations in transcription are also seen in R6/2 mice, which exhibit downregulation of genes involved in transcriptional regulation, synaptic function, and calcium and retinoid signaling pathways, and upregulation of genes associated with cellular stress and inflammation (Luthi-Carter et al., 2000). Less changes in expression levels are detected in mice expressing longer or full-length mutant Htt transgenes (Chan et al., 2002), suggesting that cleavage of Htt may be an important step in mediation of transcriptional dysregulation.

Mutant Htt is observed to undergo abnormal associations with transcriptional regulators that include the cAMP response element binding protein binding protein (CBP) (Kazantsev et al., 1999; McCampbell et al., 2000; Nucifora et al., 2001; Steffan et al., 2000), TBP-associated factor $_{II}$130 (TAFII130) (Shimohata et al., 2000), and specificity protein 1 (Sp1) (Shimohata et al., 2000); these proteins localize with Htt aggregates in HD brain, and disruption of transcriptional pathways mediated by these proteins is proposed to contribute to HD pathology. Alterations in CRE- and Sp1-

mediated transcription have received special attention, due to their roles in neuronal survival and neural gene expression, respectively.

Many studies have focused on mutant Htt disruption of the function of the transcriptional coactivator CBP, a protein that functions in neuroprotective pathways. CBP associates with the cAMP response element binding protein (CREB) to activate transcription of cAMP-responsive genes, which mediate cellular stress responses and promote neuronal survival. CBP contains a polyQ tract of 18 glutamines that allows for its interaction with mutant Htt through both its polyQ domain (Nucifora et al., 2001) and its acetyltransferase domain (Steffan et al., 2001), indicating that mutant Htt may inhibit both its transcriptional and acetyltransferase activity. CBP has been detected in mutant Htt aggregates, and levels of soluble CBP are decreased in HD brain (Nucifora et al., 2001), corresponding to downregulation of cAMP-responsive genes (Cha et al., 1999; Glass et al., 2000; Luthi-Carter et al., 2000; Timmers et al., 1996). Downregulation of cAMP-responsive genes may contribute to a neurodegenerative phenotype; indeed, loss of CREB results in HD-like hippocampal and striatal pathology in mice (Mantamadiotis et al., 2002), and suppression of either CREB or CBP expression enhances polyQ toxicity in C. elegans (Bates et al., 2006), while CBP overexpression rescues polyQ toxicity in neuronal cell culture (McCampbell et al., 2000).

The Sp1-mediated transcriptional pathway is also implicated in HD pathology; in HD brain, HD striatal neurons, and transgenic HD mouse brain, soluble mutant Htt is shown to have an enhanced interaction with Sp1, weakening its interaction with TAFII130 and with promoters for Sp1-mediated gene transcription (Chen-Plotkin et al., 2006; Dunah et al., 2002). This results in downregulation of genes that include the dopamine D2 receptor and nerve growth factor receptor genes (Dunah et al., 2002; Li et al., 2002). Coexpression of Sp1 and TAFII130 in cultured striatal neurons from transgenic mice reverses alterations in transcription and protects neurons from polyQ-mediated toxicity (Dunah et al., 2002).

Recent studies indicate that wild-type Htt may regulate the nuclear transport of neuron-restrictive silencer element (NRSE) transcription factors, thus playing a role in regulation of genes that contain NRSE sequences. NRSEs are found in genes responsible for neuronal development and function; suppression of NRSE-containing gene expression is mediated by the repressor element-1 transcription factor (REST) (Schoenherr and Anderson, 1995). Wild-type Htt binds to REST in the cytoplasm and prevents it from entering the nucleus, allowing the expression of NRSE-containing

genes, including brain-derived neurotrophic factor (BDNF) (Zuccato et al., 2003). However, mutant Htt has an attenuated interaction with REST, leading to increased REST entry into the nucleus, where it inhibits expression of BDNF and other NRSE-containing genes (Zuccato et al., 2003). BDNF expression is reduced in the caudate and putamen of HD patients (Ferrer et al., 2000) and in the cortex and striatum of HD mouse models (Luthi-Carter et al., 2000; Luthi-Carter et al., 2002b; Zuccato et al., 2001), providing evidence for disruption of the interaction between Htt and REST. Disruption of the important role of BDNF in neuronal survival and corticostriatal synaptic function (Ivkovic and Ehrlich, 1999; Jovanovic et al., 2000; Nakao et al., 1995; Widmer and Hefti, 1994) may lead to neuronal dysfunction and striatal neurodegeneration.

Mutant Htt may also cause transcriptional dysregulation by inhibiting the action of histone acetylases such as CBP, p300, and P/CAF through binding of the expanded polyQ tract to acetyltransferase domains (Steffan et al., 2001). Acetylation of histones through histone acetyltransferase activity facilitates unwinding of chromatin, rendering it transcriptionally active; conversely, inhibition of histone acetylase activity results in repression of gene transcription. Administration of histone deacetylase inhibitors rescues neurodegeneration in cellular, fly, and mouse models of HD (Ferrante et al., 2003; Hockly et al., 2003b; Steffan et al., 2001), underscoring a role for mutant Htt-mediated inhibition of histone acetylases in HD.

Apoptosis/caspase activity

Apopotosis is involved in the pathology of several neurodegenerative diseases, including Alzhemier's disease and ALS, and has been suggested to play a causative role in Huntington's disease. Mutation of Htt may lead to HD through activation of caspases, which initiate and execute the apopototic program of cell death. Signs of cell death, such as DNA fragmentation, have been observed in HD brain (Dragunow et al., 1995; Portera-Cailliau et al., 1995; Thomas et al., 1995), with the degree of fragmentation positively correlated with polyQ expansion length (Butterworth et al., 1998). Additionally, expression of mutant Htt induces apoptosis in cell culture (Kim et al., 1999; Saudou et al., 1998). Activation of caspases is observed in HD striatum (Ona et al., 1999; Sanchez et al., 1999) and in mutant-Htt expressing lymphoblasts (Maglione et al., 2006). An increase in caspase-1 activity is also observed in presymptomatic and early symptomatic transgenic HD mice, while inhibition of caspase-1 activity slows disease

pathology (Ona et al., 1999), indicating that caspase-mediated cell death may play a key role in initiation and progression of HD pathogenesis.

Normal and mutant Htt are both cleaved into N-terminal fragments by caspase-1 and caspase-3 (Goldberg et al., 1996; Wellington et al., 2002; Wellington et al., 2000); in a positive feedback loop, increased nuclear entry of N-terminal mutant Htt fragments then upregulates caspase-1 expression, leading to more Htt cleavage (Li et al., 2000b). Caspase-1 may then activate downstream effector caspases such as caspase-3, which can execute the apoptotic program (Li et al., 2000b). Additionally, initiator caspases-8 and -10 are auto-activated through sequestration into mutant Htt aggregates (Sanchez et al., 1999; U et al., 2001), while cytochrome c release from dysfunctional mitochondria found in HD activates caspase-9, (Kiechle et al., 2002), also triggering cascades leading to apoptosis.

Mutation of Htt may also lead to apoptosis by diminishing the anti-apoptotic function of wild-type Htt. Abnormally high levels of apoptosis are seen in Htt knockout mouse embryos, while overexpression of an N-terminal fragment of wild-type Htt provides protection against a range of apoptotic stimuli in neuronal culture (Rigamonti et al., 2000), including the pathogenic effect of mutant Htt exon 1 expression (Ho et al., 2001a). This neuroprotective effect may be due to wild-type Htt-mediated inhibition of pro-caspase-9 processing into active caspase-9 (Rigamonti et al., 2001). Wild-type Htt also associates with the pro-apoptotic factor HIP-1, repressing its activation of caspase-8-dependent cell death (Gervais et al., 2002; Hackam et al., 2000); mutation of Htt decreases this interaction and frees HIP-1 to induce apoptosis (Gervais et al., 2002). Lastly, Htt may be a component in a pro-survival pathway through phosphorylation by the serine/threonine kinase Akt (Humbert et al., 2002; Rangone et al., 2004). Thus, if apoptosis plays a causal role in HD pathogenesis, both gain of mutant Htt function of loss of wild-type Htt function are likely to be involved.

Chaperone and ubiquitin-proteasome dysfunction

Normal cellular function requires the constant synthesis and degradation of proteins. The correct folding of newly synthesized proteins into soluble conformations is mediated by chaperones; misfolded proteins are either refolded or tagged with ubiquitin for proteasomal degradation (Voges et al., 1999). Impairment of chaperone or proteasome systems can lead to accumulation of misfolded or damaged proteins and

aggregate formation, inducing a cellular stress response that leads to cell death. Chaperone proteins such as heat shock proteins 70 (Hsp70) and 40 (Hsp40) interact with polyQ proteins and are recruited into Htt aggregates (Chai et al., 1999; Cummings et al., 1998; Wyttenbach et al., 2000), impairing their ability to regulate protein structure and contributing to cellular dysfunction and death. Both polyQ aggregate formation and toxicity can be rescued by overexpression of chaperone proteins in neuronal culture (Chai et al., 1999; Kobayashi et al., 2000; Wyttenbach et al., 2000), *Drosophila* (Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000; Warrick et al., 1999) and mice (Cummings et al., 2001), indicating that mutant Htt-mediated loss of chaperone function may play a role in disease pathogenesis. Evidence for proteasomal dysfunction in HD include inhibition of the ubiquitin-proteasome system in both early and late stage HD brain (Seo et al., 2004), as well as studies in which transient transfection of mutant Htt fragments severely impairs proteasomal degradation (Bence et al., 2001; Holmberg et al., 2004). In addition, expression of expanded polyQ proteins renders neuroblastoma cells more vulnerable to additional cellular stress (Ding et al., 2002), and striatal neurons from R6/2 mice display increased pathology when subjected to oxidative stimuli (Petersen et al., 2001), suggesting mishandling of damaged proteins. The extent of proteasomal dysfunction is correlated with polyQ expansion length (Jana et al., 2001), suggesting that a polyQ-related gain of mutant Htt function, such as aggregate formation, may be responsible for proteasomal impairment. Indeed, Htt aggregates are ubiquitinated and associated with proteasome components (Ciechanover and Brundin, 2003; Wyttenbach et al., 2000), indicating failed cellular attempts to degrade the misfolded Htt protein. Because the ubiquitin-proteasome system plays a key role in regulation of cellular processes such as cell division and apopotosis, disruption of proteasome activity by mutant Htt may be a pathway to neuronal dysregulation and death.

Synaptic dysfunction

A specialized function of neurons involves transmission and reception of signals across the synaptic cleft. Evidence of both pre- and postsynaptic dysfunction is observed in HD, and could underlie cognitive and motor symptoms of the disease. Decreased synaptic vesicle density and neurotransmitter release are seen in a transgenic HD mouse model (Li et al., 2003a), and may result from high levels of mutant

Htt in presynaptic terminals (DiFiglia et al., 1995). Neurotransmitter release may be affected by abnormal associations between mutant Htt and presynaptic proteins such as complexin II, rabphilin 3A, and proteins involved in membrane endocytosis (Smith et al., 2005). After synaptic vesicle fusion to the plasma membrane and neurotransmitter release, endocytosis is necessary to recycle vesicle membranes and may also capture retrograde signals from the postsynaptic cell. Expansion of the polyQ tract in Htt leads to its aberrant interaction with binding partners involved in endocytosis, binding more strongly to HAP1, PACSIN1, endophilin B1b, and SH3G13, and more weakly to HIP1 and HIP14 (Smith et al., 2005). Defects in endocytosis may result in both depletion of synaptic vesicles and loss of neurotrophic support from retrograde signals.

Postsynaptic defects in HD may arise from impaired interaction between mutant Htt and the postsynaptic density protein PSD-95, a protein that plays a key role in regulation of synaptic plasticity and synaptogenesis (Che et al., 2000). Wild-type Htt associates with the SH3 domains of PSD-95, a scaffolding protein that links NMDA and kainate receptors to the postsynaptic density (Sheng and Kim, 2002). Mutation of Htt decreases its interaction with PSD-95, leading to NMDA receptor oversensitivity and excitotoxic cell death (Sun et al., 2001). Other receptors are also affected by expression of mutant Htt; mGluR2 and 3 receptors and dopamine receptors are downregulated, and alpha-amino-3-hydroxy-5-methyl-4-proprionate (AMPA), kainate, and dopamine D1 and D2 receptors all exhibit decreased ligand binding in R6/2 mice (Cha et al., 1998).

Electrophysiological evidence for synaptic pathology in HD include LTP defects in hippocampal slices from R6/2 HD mice (Murphy et al., 2000), YAC HD mice (Hodgson et al., 1999) and knock-in mice (Usdin et al., 1999), as well as LTD defects in R6/1 HD mice (Milnerwood et al., 2006). Striatal neurons from R6/2 mice also exhibit more depolarized resting potentials (Levine et al., 1999), which may indicate removal of the voltage-dependent magnesium block of NMDA channels and vulnerability to excitotoxic neurodegeneration.

Excitotoxicity

Excessive glutamatergic input to the striatum from corticostriatal pathways may lead to glutamate excitotoxicity in HD. Although cortical pyramidal neurons express higher levels of Htt than striatal cells (Fusco et al., 1999), overactivity of glutamate neurotransmission from cortical to striatal neurons may explain the selective vulnerability

of the striatum. In addition, NMDA receptors are selectively depleted in the striatum of HD patients and asymptomatic carriers, suggesting that neurons responsive to glutamate neurotransmission are susceptible to neurodegeneration in HD (Albin et al., 1990; Dure et al., 1991), and that this may be an early event in HD pathology.

In excitotoxic cell death, binding of glutamate to NMDA receptors allows high levels of sodium and calcium to enter the neuron, activating calcium-dependent enzymes. Some, such as nitric oxide synthase (NOS), increase free radical production (Nicotera et al., 1997), which can damage the cell and induce apoptotic or necrotic cell death. An increase in intracellular calcium levels can also induce opening of the mitochondrial transition pore, which can release cytochrome C and apoptosis inducing factor (AIF) to activate cell death pathways (Susin et al., 1999). Excitoxicity can be caused by either overtransmission of glutamate from presynaptic neurons, oversensitivity of receptors on postsynaptic neurons, or inefficient removal of glutamate from the synaptic cleft.

Mutant Htt expression may affect cortical cells by decreasing expression of the metabotropic glutamate receptor mGluR2, which downregulates glutamate release at corticostriatal synapses; loss of mGluR2 receptors leads to overactive glutamate neurotransmission (Cha et al., 1998). Excitotoxicity may also result from changes in postsynaptic striatal function. In mouse HD models, increased sensitivity to NMDA receptor agonists is observed in neurons (Laforet et al., 2001; Levine et al., 1999), and administration of the NMDA agonist quinolinic acid to rats produces HD-like striatal lesions (Beal et al., 1991). In addition, reduced mutant Htt binding to PSD-95 may also cause sensitization of NMDA receptors (Sun et al., 2001). Lastly, decreased levels of the glial glutamate transporter GLT-1 are observed in transgenic HD mice (Behrens et al., 2002), suggesting that impaired glutamate handling by glial cells at the corticostriatal synapse may contribute to Htt-mediated excitotoxicity. Blockage of glutamatergic corticostriatal inputs through decortication, glutamate release inhibitors, and NMDA receptor antagonists prevent the ability of mitochondrial toxins to produce HD-like striatal lesions (Schulz et al., 1996), further indicating a potential role for glutamate excitotoxicity in the selective striatal neurodegeneration seen in HD.

Mitochondrial dysfunction and oxidative damage

Many studies have indicated that mitochondrial dysfunction may contribute to many neurodegenerative diseases (Beal, 2000), including HD. Mitochondria are responsible for the synthesis of ATP, which is essential in neurons to fuel ionic pumps, antiporters, and ATP-dependent enzymes (Grunewald and Beal, 1999). Mitochondria also buffer intracellular calcium levels and sequester apoptotic factors, playing a vital role in neuronal function and survival (Hollenbeck and Saxton, 2005). Dysfunction of mitochondria can lead to metabolic insufficiency, oxidative damage, excitotoxicity, and neurodegeneration (Hollenbeck, 1996).

Mitochondrial and metabolic defects are seen in several polyQ repeat disorders, including HD (Beal, 2000) and SCAs 1 and 3 (Mastrogiacomo et al., 1996; Matsuishi et al., 1996). About half of Htt mutation carriers exhibit metabolic defects long before the onset of clinical symptoms (Antonini et al., 1996; Feigin et al., 2001), including severe chorea-independent weight loss (Djousse et al., 2002). Positron emission topography (PET) scans of presymptomatic and symptomatic HD patients show a decrease in rates of glucose metabolism in the caudate nuclei and putamen, as well as in frontal, parietal, and striatal regions (Alavi et al., 1986; Goto et al., 1993; Hayden et al., 1986). Additionally, lactate levels are elevated in the basal ganglia and cerebral cortex of HD patients (Jenkins et al., 1998), suggesting an upregulation of glycolysis to compensate for defects in the oxidative phosphorylation pathway of ATP synthesis. Lastly, the ratio of phosphocreatine to inorganic phosphate in HD resting muscle is inversely correlated with expansion of the polyQ repeat in Htt (Saft et al., 2005). These metabolic defects point to a role for mitochondrial dysfunction in HD; defects observed in presymptomatic HD carriers indicate that mitochondrial impairment may be an early event in disease pathology.

Ultrastructural studies indicate the presence of abnormal mitochondria in HD brain (Li et al., 2001) and in mutant Htt-expressing lymphoblasts (Squitieri et al., 2006), while appearance of degenerated mitochondria in the striatum of late-stage R6/2 mice corresponds with the onset of clinical symptoms (Yu et al., 2003). Some evidence suggests that mutant Htt binds directly to the surface of mitochondria in R6/2 and YAC-72Q HD mouse models (Panov et al., 2002; Yu et al., 2003), which may lead to a reduction in mitochondrial calcium uptake ability (Panov et al., 2002). In addition, mitochondrial respiration and ATP production are greatly reduced in striatal cells from mutant Htt knock-in mice (Milakovic and Johnson, 2005), and biochemical studies show defects in complex II-III and complex IV activity in HD basal ganglia (Browne et al., 1997;

Gu et al., 1996). Further evidence for a role for dysfunctional mitochondria in HD pathology comes from the observation that systemic administration of mitochondrial toxins such as 3-nitropropionic acid (3-NP) and malonate, which selectively inhibit succinate dehydrogenase and mitochondrial complex II, induce striatal lesions and choreiform movement disorders in humans, rodents, and primates that strikingly resemble those seen in HD (Browne and Beal, 2002). The striatal selectivity of many of these findings suggests that mitochondrial dysfunction may play a part in the neuropathological specificity of HD.

Defects in mitochondrial membrane depolarization are observed in HD lymphoblasts, and lead to apoptotic cell death (Sawa et al., 1999); this may be due to an abnormal interaction between mutant Htt and p53. Upregulation of p53 levels is seen in many neurodegenerative disorders (LaFerla et al., 1996; Martin, 2000), and has been observed in affected areas of HD brain and in neurons of transgenic HD mice. Mutation of Htt strengthens its interaction with p53, resulting in elevated nuclear levels of p53 as well as increased p53 transcriptional activity and leading to mitochondrial membrane depolarization in HD neurons and lymphoblasts (Bae et al., 2005). Loss of mitochondrial membrane potential impairs ATP synthesis and calcium handling in the neuron, leading to neuronal cell death (Ruan et al., 2004).

Oxidative damage from mitochondrial dysfunction may also contribute to the disease state. HD patients exhibit increased signs of oxidative damage in the striatum and cortex, including DNA strand breaks and elevated immunohistochemical staining of oxidative damage products (Browne et al., 1997; Polidori et al., 1999). Reactive oxygen species are produced by oxidation-reduction reactions such as those that occur in the mitochondrial electron transport chain; free radicals formed by damaged mitochondria may attack lipid layers, proteins, and DNA, leading to cellular dysfunction and death (Calabrese et al., 2001). 3-NP-induced lesions and oxidative damage are reduced by expressing the free radical scavenger Cu/Zn superoxide dismutase (SOD1) (Browne and Beal, 2002), free radical traps, or nitric oxide synthase (NOS) inhibitors (Schulz et al., 1995) indicating a role for damage by free radicals in the formation of HD-like lesions and potentially in HD neuropathology. Oxidative damage has also been found to downregulate genes involved in synaptic plasticity, vesicular transport, and mitochondrial function (Lu et al., 2004), leading to a widespread neurodegenerative phenotype.

Many arguments can be made for mitochondrial dysfunction as an initial mechanism of HD pathogenesis, leading to impairment of other affected pathways.

Oxidative damage has been shown to enhance formation of mutant Htt aggregates (Goswami et al., 2006), potentially activating toxic pathways downstream of aggregation. Reduced ATP synthesis may trigger excitotoxicity by limiting the activity of ATP-dependent ion pumps; loss of ionic and voltage gradients across the neuronal membrane may then relieve the voltage-gated magnesium block of NMDA channels, allowing ambient levels of glutamate to activate NMDA receptors and cause toxic influx of calcium (Grunewald and Beal, 1999). ATP is also necessary as a precursor for cAMP synthesis; reduction in cAMP levels may decrease CRE-mediated transcription of genes involved in neuronal survival. Lack of ATP and increased damage and misfolding of proteins due to ROS generation may also lead to insufficient activity of the ubiquitin-proteasome system and impairment of its normal cellular function (Beal, 2005). Mutant Htt may impair mitochondrial activity by interacting directly with mitochondria or by causing defects in mitochondrial transport.

Axonal Transport

Recently, many studies have highlighted a potential role for defective axonal transport in neurodegenerative disease. Neurons rely on fast axonal transport to transport vesicles, organelles, nucleic acids, and signaling molecules between the cell body and the synapse via microtubule tracks (Schliwa and Woehlke, 2003). The kinesin family of motor proteins mediates anterograde axonal transport to microtubule plus ends, while the dynein family mediates retrograde movement to microtubule minus ends with the help of the dynactin protein complex. Impairment of axonal trafficking systems can have a devastating effect on neuronal function and survival.

Axonal damage and defects in axonal transport have been implicated in a range of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, motor neuron disease, and Huntington's disease (Raff et al., 2002). Indeed, mutations in motor proteins such as kinesin, dynein, and dynactin can themselves lead to neuropathological phenotypes (Hafezparast et al., 2003; LaMonte et al., 2002; Reid et al., 2002; Zhao et al., 2001). Several lines of evidence indicate that wild-type Htt may play a vital role in axonal trafficking; Htt is associated with synaptosomal vesicle membranes (DiFiglia et al., 1995; Gutekunst et al., 1995), is transported in both anterograde and retrograde directions (Block-Galarza et al., 1997), and interacts with microtubules (DiFiglia et al., 1995) and the $p150^{glued}$ component of dynactin (via HAP1)

(Engelender et al., 1997; Li et al., 1998). Mutation of Htt could disrupt axonal transport through abnormal protein associations or aggregate-dependent physical blockage of axons mediated by the mutant protein, or through loss of wild-type transport function.

Many studies indicate that Htt aggregates in the axon may be involved in early HD pathogenesis. Neuritic degeneration precedes cell body degeneration in cultured striatal neurons (Li et al., 2001), and is observed in presymptomatic HD patients (Albin et al., 1990). In addition, formation of neuropil aggregates is more highly correlated with neuronal dysfunction than formation of nuclear aggregates (Li et al., 1999a). In HD brain, mass spectrometry has shown that Htt aggregates sequester transport components such as α-tubulin, βIII-tubulin, kinesin, and dynactin p150glued; the degree of this sequestration is correlated with disease severity and inversely correlated with neuronal survival (Trushina et al., 2004). In addition, a subset of mutant Htt aggregates are observed to occupy almost the entire cross-sectional diameter of the axon (Li et al., 2003a). Correspondingly, aggregates have been shown to physically block the transport of exogenous markers (Li et al., 2001), synaptic vesicles (Lee et al., 2004), and organelles (Chang et al., 2006). Bidirectional blockage of transport by aggregates may result in synaptic dysfunction, axonal degeneration, and cell death.

Reduced transport velocity of axonal transport cargoes is observed in mice (Trushina et al., 2004), flies (Gunawardena et al., 2003), squid giant axon (Szebenyi et al., 2003), and neuronal cells (Chang et al., 2006; Gauthier et al., 2004; Trushina et al., 2004) expressing mutant Htt, indicating a role for the mutant protein in impairment of transport. In some studies (Szebenyi et al., 2003), decreased rate of transport is seen in the absence of neuritic aggregates, suggesting that the mutant protein may be able to cause aggregate-independent defects in transport. Mutant Htt may cause transport defects by binding more tightly to HAP1 and p150glued, decreasing their interaction with microtubules and slowing movement of cargo that requires HAP1 for transport, such as the neurotrophic support molecule BDNF (Gauthier et al., 2004).

While expression of mutant Htt leads to defects in axonal transport, inhibition of normal Htt function also impairs axonal trafficking in several models of HD. Adult mice expressing less than 50% of the normal dosage of wild-type Htt exhibit defective trafficking of endocytic vesicles and mitochondria in both anterograde and retrograde directions, prior to onset of symptoms (Trushina et al., 2004). In *Drosophila* (Gunawardena et al., 2003) and mammalian neurons (Gauthier et al., 2004; Trushina et

al., 2004), reduction of wild-type Htt levels leads to defective axonal transport of various cargoes, including vesicles, organelles, and BDNF.

BDNF transport, as well as BDNF transcription, may be mediated by the wild-type Htt protein (Gauthier et al., 2004). BDNF colocalizes with Htt in cortical neurons, where it is transported to nerve terminals and released upon striatal neurons, triggering key signaling events (Mizuno et al., 1994; Ventimiglia et al., 1995). Expansion of the polyQ tract in Htt reduces its ability to mediate BDNF transport; in addition, mutant Htt may disrupt the interaction between wild-type Htt and BDNF transport machinery (Gauthier et al., 2004; Saudou et al., 1998). In HD patients (Ferrer et al., 2000) and R6/1 mice (Spires et al., 2004), BDNF is reduced in the striatum but not the cortex, suggesting a disruption in corticostriatal transport pathways that may result in excitotoxic death of striatal neurons.

Disruption of wild-type Htt-mediated transport may also affect other cellular pathways. Recent evidence indicates that impaired axonal transport of mitochondria may be particularly relevant to HD pathology. Mitochondria normally undergo saltatory, bidirectional axonal transport, with individual mitochondria undergoing long stationary periods at target sites in the axon or at the synapse (Hollenbeck, 1996). Proper distribution of mitochondria in the axon is essential to neuronal health; mitochondria with the high membrane potential necessary for ATP production move towards the synapse, while old or damaged mitochondria are transported back to the cell body for repair or autophagy (Miller and Sheetz, 2004). Defects in mitochondrial transport may have a twofold effect: first, lack of healthy mitochondria at sites of high energy demand or tight calcium regulation, such as the axon and the synapse, may result in insufficient response to metabolic and calcium buffering needs, leading to neuronal dysfunction and death. Second, failure to recycle aged or damaged mitochondria may lead to mitochondrial production of reactive oxygen species and release of cytochrome C, resulting in oxidative damage and apoptosis (Lee and Wei, 2000).

Recent studies indicate that Htt may play a role in axonal trafficking of mitochondria. A 50% decrease in wild-type Htt results in a decrease in the motility of mitochondria (Trushina et al., 2004). Additionally, in flies lacking Milton, the *Drosophila* homologue of HAP1, mitochondria are prevented from entering the axon and remain in the cell body (Stowers et al., 2002), further suggesting a link between Htt and mitochondrial transport in the axon. Expansion of the polyQ repeat may disrupt the normal role of Htt in trafficking of mitochondria, and may also lead to mutant Htt-

mediated titration of normal Htt or other mitochondrial trafficking proteins. Analysis of transport in HD cortical neurons (Chang et al., 2006) and in striatal neurons from transgenic HD mice (Trushina et al., 2004) reveals a significant decrease in mitochondrial movement, while degenerated mitochondria are seen in axons (Li et al., 2001) and axon terminals (Li et al., 2003c) of transgenic HD mice, providing evidence for impaired transport of mitochondria in HD models. Defects in mitochondrial transport, and resulting mitochondrial insufficiency and dysfunction, may lead to the excitotoxicity, oxidative damage, and neuronal cell death found in Huntington's disease.

Conclusion

The wealth of evidence implicating a role for Htt mutation in numerous cellular pathways suggests that HD is a multifaceted disorder. Pathology is likely to occur in both the nucleus and in the cytoplasm, and may be mediated by loss of wild-type function in concert with toxic activity of soluble and/or aggregated forms of the mutant Htt protein. Further studies of the normal role of Htt and the cellular processes affected by mutant Htt may help to differentiate early pathological events from secondary ones; identification of causative pathways will facilitate the development of therapies to treat the disorder. Many recent studies have focused on the role of wild-type Htt in axonal transport and in the role of mutant Htt in impairment of axonal transport. In particular, defects in mitochondrial trafficking may play a key role in initiating many of the pathological phenotypes seen in Huntington's disease. Recent evidence suggests that expression of mutant Htt may disrupt axonal transport of mitochondria through loss and sequestration of the wild-type Htt protein and through physical blockage of transport by aggregates (Chang et al., 2006; Trushina et al., 2004); subsequent mitochondrial deficiency and dysfunction can lead to nearly all of the cellular pathology observed in HD, including transcriptional dysregulation, proteasome insufficiency, synaptic pathology, metabolic defects, oxidative damage, excitotoxicity, and apoptotic cell death. Here, we describe the characterization of a transgenic *Drosophila* model of HD and study of cellular processes altered by mutant Htt expression, with special emphasis on axonal transport. Finally, we discuss the use of our *Drosophila* HD model for testing of potential therapies. Findings from invertebrate models of human disease may lead to

greater understanding of basic disease mechanisms and suggest avenues for therapeutic development.

References

Alavi, A., Dann, R., Chawluk, J., Alavi, J., Kushner, M. and Reivich, M. (1986) Positron emission tomography imaging of regional cerebral glucose metabolism. *Semin Nucl Med*, **16**, 2-34.

Albin, R.L., Reiner, A., Anderson, K.D., Penney, J.B. and Young, A.B. (1990) Striatal and nigral neuron subpopulations in rigid Huntington's disease: implications for the functional anatomy of chorea and rigidity-akinesia. *Ann Neurol*, **27**, 357-365.

Ambrose, C.M., Duyao, M.P., Barnes, G., Bates, G.P., Lin, C.S., Srinidhi, J., Baxendale, S., Hummerich, H., Lehrach, H., Altherr, M. and et al. (1994) Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. *Somat Cell Mol Genet*, **20**, 27-38.

Anca, M.H., Gazit, E., Loewenthal, R., Ostrovsky, O., Frydman, M. and Giladi, N. (2004) Different phenotypic expression in monozygotic twins with Huntington disease. *Am J Med Genet A*, **124**, 89-91.

Andrade, M.A., Petosa, C., O'Donoghue, S.I., Muller, C.W. and Bork, P. (2001) Comparison of ARM and HEAT protein repeats. *J Mol Biol*, **309**, 1-18.

Antonini, A., Leenders, K.L., Spiegel, R., Meier, D., Vontobel, P., Weigell-Weber, M., Sanchez-Pernaute, R., de Yebenez, J.G., Boesiger, P., Weindl, A. and Maguire, R.P. (1996) Striatal glucose metabolism and dopamine D2 receptor binding in asymptomatic gene carriers and patients with Huntington's disease. *Brain*, **119 (Pt 6)**, 2085-2095.

Arrasate, M., Mitra, S., Schweitzer, E.S., Segal, M.R. and Finkbeiner, S. (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. *Nature*, **431**, 805-810.

Aylward, E.H., Li, Q., Stine, O.C., Ranen, N., Sherr, M., Barta, P.E., Bylsma, F.W., Pearlson, G.D. and Ross, C.A. (1997) Longitudinal change in basal ganglia volume in patients with Huntington's disease. *Neurology*, **48**, 394-399.

Bae, B.I., Hara, M.R., Cascio, M.B., Wellington, C.L., Hayden, M.R., Ross, C.A., Ha, H.C., Li, X.J., Snyder, S.H. and Sawa, A. (2006) Mutant huntingtin: nuclear translocation and cytotoxicity mediated by GAPDH. *Proc Natl Acad Sci U S A*, **103**, 3405-3409.

Bae, B.I., Xu, H., Igarashi, S., Fujimuro, M., Agrawal, N., Taya, Y., Hayward, S.D., Moran, T.H., Montell, C., Ross, C.A., Snyder, S.H. and Sawa, A. (2005) p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. *Neuron*, **47**, 29-41.

Bargmann, C.I. (1998) Neurobiology of the Caenorhabditis elegans genome. *Science*, **282**, 2028-2033.

Barnes, G.T., Duyao, M.P., Ambrose, C.M., McNeil, S., Persichetti, F., Srinidhi, J., Gusella, J.F. and MacDonald, M.E. (1994) Mouse Huntington's disease gene homolog (Hdh). *Somat Cell Mol Genet*, **20**, 87-97.

Bates, E.A., Victor, M., Jones, A.K., Shi, Y. and Hart, A.C. (2006) Differential contributions of Caenorhabditis elegans histone deacetylases to huntingtin polyglutamine toxicity. *J Neurosci*, **26**, 2830-2838.

Bates, G.P. and Hockly, E. (2003) Experimental therapeutics in Huntington's disease: are models useful for therapeutic trials? *Curr Opin Neurol*, **16**, 465-470.

Baxendale, S., Abdulla, S., Elgar, G., Buck, D., Berks, M., Micklem, G., Durbin, R., Bates, G., Brenner, S. and Beck, S. (1995) Comparative sequence analysis of the human and pufferfish Huntington's disease genes. *Nat Genet*, **10**, 67-76.

Beal, M.F. (2000) Energetics in the pathogenesis of neurodegenerative diseases. *Trends Neurosci*, **23**, 298-304.

Beal, M.F. (2005) Mitochondria take center stage in aging and neurodegeneration. *Ann Neurol*, **58**, 495-505.

Beal, M.F., Ferrante, R.J., Swartz, K.J. and Kowall, N.W. (1991) Chronic quinolinic acid lesions in rats closely resemble Huntington's disease. *J Neurosci*, **11**, 1649-1659.

Becher, M.W., Kotzuk, J.A., Sharp, A.H., Davies, S.W., Bates, G.P., Price, D.L. and Ross, C.A. (1998) Intranuclear neuronal inclusions in Huntington's disease and dentatorubral and pallidoluysian atrophy: correlation between the density of inclusions and IT15 CAG triplet repeat length. *Neurobiol Dis*, **4**, 387-397.

Behrens, P.F., Franz, P., Woodman, B., Lindenberg, K.S. and Landwehrmeyer, G.B. (2002) Impaired glutamate transport and glutamate-glutamine cycling: downstream effects of the Huntington mutation. *Brain*, **125**, 1908-1922.

Bence, N.F., Sampat, R.M. and Kopito, R.R. (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. *Science*, **292**, 1552-1555.

Benn, C.L., Landles, C., Li, H., Strand, A.D., Woodman, B., Sathasivam, K., Li, S.H., Ghazi-Noori, S., Hockly, E., Faruque, S.M., Cha, J.H., Sharpe, P.T., Olson, J.M., Li, X.J. and Bates, G.P. (2005) Contribution of nuclear and extranuclear polyQ to neurological phenotypes in mouse models of Huntington's disease. *Hum Mol Genet*, **14**, 3065-3078.

Bhide, P.G., Day, M., Sapp, E., Schwarz, C., Sheth, A., Kim, J., Young, A.B., Penney, J., Golden, J., Aronin, N. and DiFiglia, M. (1996) Expression of normal and mutant huntingtin in the developing brain. *J Neurosci*, **16**, 5523-5535.

Block-Galarza, J., Chase, K.O., Sapp, E., Vaughn, K.T., Vallee, R.B., DiFiglia, M. and Aronin, N. (1997) Fast transport and retrograde movement of huntingtin and HAP 1 in axons. *Neuroreport*, **8**, 2247-2251.

Boutell, J.M., Thomas, P., Neal, J.W., Weston, V.J., Duce, J., Harper, P.S. and Jones, A.L. (1999) Aberrant interactions of transcriptional repressor proteins with the Huntington's disease gene product, huntingtin. *Hum Mol Genet*, **8**, 1647-1655.

Browne, S.E. and Beal, M.F. (2002) Toxin-induced mitochondrial dysfunction. *Int Rev Neurobiol*, **53**, 243-279.

Browne, S.E., Bowling, A.C., MacGarvey, U., Baik, M.J., Berger, S.C., Muqit, M.M., Bird, E.D. and Beal, M.F. (1997) Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. *Ann Neurol*, **41**, 646-653.

Butterworth, N.J., Williams, L., Bullock, J.Y., Love, D.R., Faull, R.L. and Dragunow, M. (1998) Trinucleotide (CAG) repeat length is positively correlated with the degree of DNA fragmentation in Huntington's disease striatum. *Neuroscience*, **87**, 49-53.

Calabrese, V., Scapagnini, G., Giuffrida Stella, A.M., Bates, T.E. and Clark, J.B. (2001) Mitochondrial involvement in brain function and dysfunction: relevance to aging, neurodegenerative disorders and longevity. *Neurochem Res*, **26**, 739-764.

Cattaneo, E., Zuccato, C. and Tartari, M. (2005) Normal huntingtin function: an alternative approach to Huntington's disease. *Nat Rev Neurosci*, **6**, 919-930.

Cha, J.H., Frey, A.S., Alsdorf, S.A., Kerner, J.A., Kosinski, C.M., Mangiarini, L., Penney, J.B., Jr., Davies, S.W., Bates, G.P. and Young, A.B. (1999) Altered neurotransmitter receptor expression in transgenic mouse models of Huntington's disease. *Philos Trans R Soc Lond B Biol Sci*, **354**, 981-989.

Cha, J.H., Kosinski, C.M., Kerner, J.A., Alsdorf, S.A., Mangiarini, L., Davies, S.W., Penney, J.B., Bates, G.P. and Young, A.B. (1998) Altered brain neurotransmitter receptors in transgenic mice expressing a portion of an abnormal human huntington disease gene. *Proc Natl Acad Sci U S A*, **95**, 6480-6485.

Chai, Y., Koppenhafer, S.L., Bonini, N.M. and Paulson, H.L. (1999) Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease. *J Neurosci*, **19**, 10338-10347.

Chan, E.Y., Luthi-Carter, R., Strand, A., Solano, S.M., Hanson, S.A., DeJohn, M.M., Kooperberg, C., Chase, K.O., DiFiglia, M., Young, A.B., Leavitt, B.R., Cha, J.H., Aronin, N., Hayden, M.R. and Olson, J.M. (2002) Increased huntingtin protein length reduces the number of polyglutamine-induced gene expression changes in mouse models of Huntington's disease. *Hum Mol Genet*, **11**, 1939-1951.

Chang, D.T., Rintoul, G.L., Pandipati, S. and Reynolds, I.J. (2006) Mutant huntingtin aggregates impair mitochondrial movement and trafficking in cortical neurons. *Neurobiol Dis*, **22**, 388-400.

Che, Y.H., Tamatani, M. and Tohyama, M. (2000) Changes in mRNA for post-synaptic density-95 (PSD-95) and carboxy-terminal PDZ ligand of neuronal nitric oxide synthase following facial nerve transection. *Brain Res Mol Brain Res*, **76**, 325-335.

Chen, S., Berthelier, V., Yang, W. and Wetzel, R. (2001) Polyglutamine aggregation behavior in vitro supports a recruitment mechanism of cytotoxicity. *J Mol Biol*, **311**, 173-182.

Chen-Plotkin, A.S., Sadri-Vakili, G., Yohrling, G.J., Braveman, M.W., Benn, C.L., Glajch, K.E., Dirocco, D.P., Farrell, L.A., Krainc, D., Gines, S., Macdonald, M.E. and Cha, J.H. (2006) Decreased association of the transcription factor Sp1 with genes downregulated in Huntington's disease. *Neurobiol Dis*.

Ciechanover, A. and Brundin, P. (2003) The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. *Neuron*, **40**, 427-446.

Clifford, J.J., Drago, J., Natoli, A.L., Wong, J.Y., Kinsella, A., Waddington, J.L. and Vaddadi, K.S. (2002) Essential fatty acids given from conception prevent topographies of motor deficit in a transgenic model of Huntington's disease. *Neuroscience*, **109**, 81-88.

Colby, D.W., Chu, Y., Cassady, J.P., Duennwald, M., Zazulak, H., Webster, J.M., Messer, A., Lindquist, S., Ingram, V.M. and Wittrup, K.D. (2004) Potent inhibition of huntingtin aggregation and cytotoxicity by a disulfide bond-free single-domain intracellular antibody. *Proc Natl Acad Sci U S A*, **101**, 17616-17621.

Cooper, A.J., Sheu, K.F., Burke, J.R., Strittmatter, W.J., Gentile, V., Peluso, G. and Blass, J.P. (1999) Pathogenesis of inclusion bodies in (CAG)n/Qn-expansion diseases with special reference to the role of tissue transglutaminase and to selective vulnerability. *J Neurochem*, **72**, 889-899.

Cummings, C.J., Mancini, M.A., Antalffy, B., DeFranco, D.B., Orr, H.T. and Zoghbi, H.Y. (1998) Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat Genet*, **19**, 148-154.

Cummings, C.J., Sun, Y., Opal, P., Antalffy, B., Mestril, R., Orr, H.T., Dillmann, W.H. and Zoghbi, H.Y. (2001) Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum Mol Genet*, **10**, 1511-1518.

Davies, S.W., Turmaine, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L. and Bates, G.P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell*, **90**, 537-548.

de Almeida, L.P., Ross, C.A., Zala, D., Aebischer, P. and Deglon, N. (2002) Lentiviral-mediated delivery of mutant huntingtin in the striatum of rats induces a selective neuropathology modulated by polyglutamine repeat size, huntingtin expression levels, and protein length. *J Neurosci*, **22**, 3473-3483.

DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonsattel, J.P., Carraway, R., Reeves, S.A. and et al. (1995) Huntingtin is a

cytoplasmic protein associated with vesicles in human and rat brain neurons. *Neuron*, **14**, 1075-1081.

DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P. and Aronin, N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science*, **277**, 1990-1993.

Ding, Q., Lewis, J.J., Strum, K.M., Dimayuga, E., Bruce-Keller, A.J., Dunn, J.C. and Keller, J.N. (2002) Polyglutamine expansion, protein aggregation, proteasome activity, and neural survival. *J Biol Chem*, **277**, 13935-13942.

Djousse, L., Knowlton, B., Cupples, L.A., Marder, K., Shoulson, I. and Myers, R.H. (2002) Weight loss in early stage of Huntington's disease. *Neurology*, **59**, 1325-1330.

Djousse, L., Knowlton, B., Hayden, M., Almqvist, E.W., Brinkman, R., Ross, C., Margolis, R., Rosenblatt, A., Durr, A., Dode, C., Morrison, P.J., Novelletto, A., Frontali, M., Trent, R.J., McCusker, E., Gomez-Tortosa, E., Mayo, D., Jones, R., Zanko, A., Nance, M., Abramson, R., Suchowersky, O., Paulsen, J., Harrison, M., Yang, Q., Cupples, L.A., Gusella, J.F., MacDonald, M.E. and Myers, R.H. (2003) Interaction of normal and expanded CAG repeat sizes influences age at onset of Huntington disease. *Am J Med Genet A*, **119**, 279-282.

Dragatsis, I., Levine, M.S. and Zeitlin, S. (2000) Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. *Nat Genet*, **26**, 300-306.

Dragunow, M., Faull, R.L., Lawlor, P., Beilharz, E.J., Singleton, K., Walker, E.B. and Mee, E. (1995) In situ evidence for DNA fragmentation in Huntington's disease striatum and Alzheimer's disease temporal lobes. *Neuroreport*, **6**, 1053-1057.

Duan, W., Guo, Z., Jiang, H., Ware, M., Li, X.J. and Mattson, M.P. (2003) Dietary restriction normalizes glucose metabolism and BDNF levels, slows disease progression, and increases survival in huntingtin mutant mice. *Proc Natl Acad Sci U S A*, **100**, 2911-2916.

Dunah, A.W., Jeong, H., Griffin, A., Kim, Y.M., Standaert, D.G., Hersch, S.M., Mouradian, M.M., Young, A.B., Tanese, N. and Krainc, D. (2002) Sp1 and TAFII130 transcriptional activity disrupted in early Huntington's disease. *Science*, **296**, 2238-2243.

Dure, L.S.t., Young, A.B. and Penney, J.B. (1991) Excitatory amino acid binding sites in the caudate nucleus and frontal cortex of Huntington's disease. *Ann Neurol*, **30**, 785-793.

Duyao, M., Ambrose, C., Myers, R., Novelletto, A., Persichetti, F., Frontali, M., Folstein, S., Ross, C., Franz, M., Abbott, M. and et al. (1993) Trinucleotide repeat length instability and age of onset in Huntington's disease. *Nat Genet*, **4**, 387-392.

Duyao, M.P., Auerbach, A.B., Ryan, A., Persichetti, F., Barnes, G.T., McNeil, S.M., Ge, P., Vonsattel, J.P., Gusella, J.F., Joyner, A.L. and et al. (1995) Inactivation of the mouse Huntington's disease gene homolog Hdh. *Science*, **269**, 407-410.

Engelender, S., Sharp, A.H., Colomer, V., Tokito, M.K., Lanahan, A., Worley, P., Holzbaur, E.L. and Ross, C.A. (1997) Huntingtin-associated protein 1 (HAP1) interacts with the p150Glued subunit of dynactin. *Hum Mol Genet*, **6**, 2205-2212.

Faber, P.W., Alter, J.R., MacDonald, M.E. and Hart, A.C. (1999) Polyglutamine-mediated dysfunction and apoptotic death of a Caenorhabditis elegans sensory neuron. *Proc Natl Acad Sci U S A*, **96**, 179-184.

Feigin, A., Leenders, K.L., Moeller, J.R., Missimer, J., Kuenig, G., Spetsieris, P., Antonini, A. and Eidelberg, D. (2001) Metabolic network abnormalities in early Huntington's disease: an [(18)F]FDG PET study. *J Nucl Med*, **42**, 1591-1595.

Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J., McCall, A., Canal, I., Orr, H.T., Zoghbi, H.Y. and Botas, J. (2000) Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature*, **408**, 101-106.

Ferrante, R.J., Gutekunst, C.A., Persichetti, F., McNeil, S.M., Kowall, N.W., Gusella, J.F., MacDonald, M.E., Beal, M.F. and Hersch, S.M. (1997) Heterogeneous topographic and cellular distribution of huntingtin expression in the normal human neostriatum. *J Neurosci*, **17**, 3052-3063.

Ferrante, R.J., Kubilus, J.K., Lee, J., Ryu, H., Beesen, A., Zucker, B., Smith, K., Kowall, N.W., Ratan, R.R., Luthi-Carter, R. and Hersch, S.M. (2003) Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. *J Neurosci*, **23**, 9418-9427.

Ferrer, I., Goutan, E., Marin, C., Rey, M.J. and Ribalta, T. (2000) Brain-derived neurotrophic factor in Huntington disease. *Brain Res*, **866**, 257-261.

Fusco, F.R., Chen, Q., Lamoreaux, W.J., Figueredo-Cardenas, G., Jiao, Y., Coffman, J.A., Surmeier, D.J., Honig, M.G., Carlock, L.R. and Reiner, A. (1999) Cellular localization of huntingtin in striatal and cortical neurons in rats: lack of correlation with neuronal vulnerability in Huntington's disease. *J Neurosci*, **19**, 1189-1202.

Gafni, J. and Ellerby, L.M. (2002) Calpain activation in Huntington's disease. *J Neurosci*, **22**, 4842-4849.

Gafni, J., Hermel, E., Young, J.E., Wellington, C.L., Hayden, M.R. and Ellerby, L.M. (2004) Inhibition of calpain cleavage of huntingtin reduces toxicity: accumulation of calpain/caspase fragments in the nucleus. *J Biol Chem*, **279**, 20211-20220.

Gauthier, L.R., Charrin, B.C., Borrell-Pages, M., Dompierre, J.P., Rangone, H., Cordelieres, F.P., De Mey, J., MacDonald, M.E., Lessmann, V., Humbert, S. and Saudou, F. (2004) Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell*, **118**, 127-138.

Georgiou, N., Bradshaw, J.L., Chiu, E., Tudor, A., O'Gorman, L. and Phillips, J.G. (1999) Differential clinical and motor control function in a pair of monozygotic twins with Huntington's disease. *Mov Disord*, **14**, 320-325.

Gervais, F.G., Singaraja, R., Xanthoudakis, S., Gutekunst, C.A., Leavitt, B.R., Metzler, M., Hackam, A.S., Tam, J., Vaillancourt, J.P., Houtzager, V., Rasper, D.M., Roy, S., Hayden, M.R. and Nicholson, D.W. (2002) Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. *Nat Cell Biol*, **4**, 95-105.

Glass, M., Dragunow, M. and Faull, R.L. (2000) The pattern of neurodegeneration in Huntington's disease: a comparative study of cannabinoid, dopamine, adenosine and GABA(A) receptor alterations in the human basal ganglia in Huntington's disease. *Neuroscience*, **97**, 505-519.

Goldberg, Y.P., Nicholson, D.W., Rasper, D.M., Kalchman, M.A., Koide, H.B., Graham, R.K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N.A., Vaillancourt, J.P. and Hayden, M.R. (1996) Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat Genet*, **13**, 442-449.

Goswami, A., Dikshit, P., Mishra, A., Mulherkar, S., Nukina, N. and Jana, N.R. (2006) Oxidative stress promotes mutant huntingtin aggregation and mutant huntingtin-dependent cell death by mimicking proteasomal malfunction. *Biochem Biophys Res Commun*, **342**, 184-190.

Goto, I., Taniwaki, T., Hosokawa, S., Otsuka, M., Ichiya, Y. and Ichimiya, A. (1993) Positron emission tomographic (PET) studies in dementia. *J Neurol Sci*, **114**, 1-6.

Grunewald, T. and Beal, M.F. (1999) Bioenergetics in Huntington's disease. *Ann N Y Acad Sci*, **893**, 203-213.

Gu, M., Gash, M.T., Mann, V.M., Javoy-Agid, F., Cooper, J.M. and Schapira, A.H. (1996) Mitochondrial defect in Huntington's disease caudate nucleus. *Ann Neurol*, **39**, 385-389.

Gunawardena, S., Her, L.S., Brusch, R.G., Laymon, R.A., Niesman, I.R., Gordesky-Gold, B., Sintasath, L., Bonini, N.M. and Goldstein, L.S. (2003) Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in *Drosophila*. *Neuron*, **40**, 25-40.

Gutekunst, C.A., Levey, A.I., Heilman, C.J., Whaley, W.L., Yi, H., Nash, N.R., Rees, H.D., Madden, J.J. and Hersch, S.M. (1995) Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. *Proc Natl Acad Sci U S A*, **92**, 8710-8714.

Gutekunst, C.A., Li, S.H., Yi, H., Mulroy, J.S., Kuemmerle, S., Jones, R., Rye, D., Ferrante, R.J., Hersch, S.M. and Li, X.J. (1999) Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. *J Neurosci*, **19**, 2522-2534.

Hackam, A.S., Yassa, A.S., Singaraja, R., Metzler, M., Gutekunst, C.A., Gan, L., Warby, S., Wellington, C.L., Vaillancourt, J., Chen, N., Gervais, F.G., Raymond, L., Nicholson, D.W. and Hayden, M.R. (2000) Huntingtin interacting protein 1 induces apoptosis via a novel caspase-dependent death effector domain. *J Biol Chem*, **275**, 41299-41308.

Hafezparast, M., Klocke, R., Ruhrberg, C., Marquardt, A., Ahmad-Annuar, A., Bowen, S., Lalli, G., Witherden, A.S., Hummerich, H., Nicholson, S., Morgan, P.J., Oozageer, R., Priestley, J.V., Averill, S., King, V.R., Ball, S., Peters, J., Toda, T., Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, D., Wattler, S., Wabnitz, P., Dickneite, C., Lampel, S., Boehme, F., Peraus, G., Popp, A., Rudelius, M., Schlegel, J., Fuchs, H., Hrabe de Angelis, M., Schiavo, G., Shima, D.T., Russ, A.P., Stumm, G., Martin, J.E. and Fisher, E.M. (2003) Mutations in dynein link motor neuron degeneration to defects in retrograde transport. *Science*, **300**, 808-812.

Harjes, P. and Wanker, E.E. (2003) The hunt for huntingtin function: interaction partners tell many different stories. *Trends Biochem Sci*, **28**, 425-433.

Hattula, K. and Peranen, J. (2000) FIP-2, a coiled-coil protein, links Huntingtin to Rab8 and modulates cellular morphogenesis. *Curr Biol*, **10**, 1603-1606.

Hayden, M.R., Martin, W.R., Stoessl, A.J., Clark, C., Hollenberg, S., Adam, M.J., Ammann, W., Harrop, R., Rogers, J., Ruth, T. and et al. (1986) Positron emission tomography in the early diagnosis of Huntington's disease. *Neurology*, **36**, 888-894.

HDCRG. (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell*, **72**, 971-983.

Hedreen, J.C., Peyser, C.E., Folstein, S.E. and Ross, C.A. (1991) Neuronal loss in layers V and VI of cerebral cortex in Huntington's disease. *Neurosci Lett*, **133**, 257-261.

Heiser, V., Scherzinger, E., Boeddrich, A., Nordhoff, E., Lurz, R., Schugardt, N., Lehrach, H. and Wanker, E.E. (2000) Inhibition of huntingtin fibrillogenesis by specific antibodies and small molecules: implications for Huntington's disease therapy. *Proc Natl Acad Sci U S A*, **97**, 6739-6744.

Hilditch-Maguire, P., Trettel, F., Passani, L.A., Auerbach, A., Persichetti, F. and MacDonald, M.E. (2000) Huntingtin: an iron-regulated protein essential for normal nuclear and perinuclear organelles. *Hum Mol Genet*, **9**, 2789-2797.

Ho, L.W., Brown, R., Maxwell, M., Wyttenbach, A. and Rubinsztein, D.C. (2001a) Wild type Huntingtin reduces the cellular toxicity of mutant Huntingtin in mammalian cell models of Huntington's disease. *J Med Genet*, **38**, 450-452.

Ho, L.W., Carmichael, J., Swartz, J., Wyttenbach, A., Rankin, J. and Rubinsztein, D.C. (2001b) The molecular biology of Huntington's disease. *Psychol Med*, **31**, 3-14.

Hockly, E., Cordery, P.M., Woodman, B., Mahal, A., van Dellen, A., Blakemore, C., Lewis, C.M., Hannan, A.J. and Bates, G.P. (2002) Environmental enrichment slows disease progression in R6/2 Huntington's disease mice. *Ann Neurol*, **51**, 235-242.

Hockly, E., Woodman, B., Mahal, A., Lewis, C.M. and Bates, G. (2003) Standardization and statistical approaches to therapeutic trials in the R6/2 mouse. *Brain Res Bull*, **61**, 469-479.

Hodges, A., Strand, A.D., Aragaki, A.K., Kuhn, A., Sengstag, T., Hughes, G., Elliston, L.A., Hartog, C., Goldstein, D.R., Thu, D., Hollingsworth, Z.R., Collin, F., Synek, B., Holmans, P.A., Young, A.B., Wexler, N.S., Delorenzi, M., Kooperberg, C., Augood, S.J., Faull, R.L., Olson, J.M., Jones, L. and Luthi-Carter, R. (2006) Regional and cellular gene expression changes in human Huntington's disease brain. *Hum Mol Genet*, **15**, 965-977.

Hodgson, J.G., Agopyan, N., Gutekunst, C.A., Leavitt, B.R., LePiane, F., Singaraja, R., Smith, D.J., Bissada, N., McCutcheon, K., Nasir, J., Jamot, L., Li, X.J., Stevens, M.E., Rosemond, E., Roder, J.C., Phillips, A.G., Rubin, E.M., Hersch, S.M. and Hayden, M.R. (1999) A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron*, **23**, 181-192.

Holbert, S., Dedeoglu, A., Humbert, S., Saudou, F., Ferrante, R.J. and Neri, C. (2003) Cdc42-interacting protein 4 binds to huntingtin: neuropathologic and biological evidence for a role in Huntington's disease. *Proc Natl Acad Sci U S A*, **100**, 2712-2717.

Hollenbeck, P.J. (1996) The pattern and mechanism of mitochondrial transport in axons. *Front Biosci*, **1**, d91-102.

Hollenbeck, P.J. and Saxton, W.M. (2005) The axonal transport of mitochondria. *J Cell Sci*, **118**, 5411-5419.

Holmberg, C.I., Staniszewski, K.E., Mensah, K.N., Matouschek, A. and Morimoto, R.I. (2004) Inefficient degradation of truncated polyglutamine proteins by the proteasome. *Embo J*, **23**, 4307-4318.

Huang, K., Yanai, A., Kang, R., Arstikaitis, P., Singaraja, R.R., Metzler, M., Mullard, A., Haigh, B., Gauthier-Campbell, C., Gutekunst, C.A., Hayden, M.R. and El-Husseini, A. (2004) Huntingtin-interacting protein HIP14 is a palmitoyl transferase involved in palmitoylation and trafficking of multiple neuronal proteins. *Neuron*, **44**, 977-986.

Humbert, S., Bryson, E.A., Cordelieres, F.P., Connors, N.C., Datta, S.R., Finkbeiner, S., Greenberg, M.E. and Saudou, F. (2002) The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. *Dev Cell*, **2**, 831-837.

Ivkovic, S. and Ehrlich, M.E. (1999) Expression of the striatal DARPP-32/ARPP-21 phenotype in GABAergic neurons requires neurotrophins *in vivo* and in vitro. *J Neurosci*, **19**, 5409-5419.

Jackson, G.R., Salecker, I., Dong, X., Yao, X., Arnheim, N., Faber, P.W., MacDonald, M.E. and Zipursky, S.L. (1998) Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron*, **21**, 633-642.

Jana, N.R., Zemskov, E.A., Wang, G. and Nukina, N. (2001) Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. *Hum Mol Genet*, **10**, 1049-1059.

Jenkins, B.G., Rosas, H.D., Chen, Y.C., Makabe, T., Myers, R., MacDonald, M., Rosen, B.R., Beal, M.F. and Koroshetz, W.J. (1998) 1H NMR spectroscopy studies of Huntington's disease: correlations with CAG repeat numbers. *Neurology*, **50**, 1357-1365.

Jovanovic, J.N., Czernik, A.J., Fienberg, A.A., Greengard, P. and Sihra, T.S. (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. *Nat Neurosci*, **3**, 323-329.

Karlovich, C.A., John, R.M., Ramirez, L., Stainier, D.Y. and Myers, R.M. (1998) Characterization of the Huntington's disease (HD) gene homologue in the zebrafish Danio rerio. *Gene*, **217**, 117-125.

Kazantsev, A., Preisinger, E., Dranovsky, A., Goldgaber, D. and Housman, D. (1999) Insoluble detergent-resistant aggregates form between pathological and nonpathological lengths of polyglutamine in mammalian cells. *Proc Natl Acad Sci U S A*, **96**, 11404-11409.

Kazantsev, A., Walker, H.A., Slepko, N., Bear, J.E., Preisinger, E., Steffan, J.S., Zhu, Y.Z., Gertler, F.B., Housman, D.E., Marsh, J.L. and Thompson, L.M. (2002) A bivalent Huntingtin binding peptide suppresses polyglutamine aggregation and pathogenesis in *Drosophila*. *Nat Genet*, **30**, 367-376.

Kazemi-Esfarjani, P. and Benzer, S. (2000) Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science*, **287**, 1837-1840.

Kegel, K.B., Meloni, A.R., Yi, Y., Kim, Y.J., Doyle, E., Cuiffo, B.G., Sapp, E., Wang, Y., Qin, Z.H., Chen, J.D., Nevins, J.R., Aronin, N. and DiFiglia, M. (2002) Huntingtin is present in the nucleus, interacts with the transcriptional corepressor C-terminal binding protein, and represses transcription. *J Biol Chem*, **277**, 7466-7476.

Khoshnan, A., Ko, J. and Patterson, P.H. (2002) Effects of intracellular expression of anti-huntingtin antibodies of various specificities on mutant huntingtin aggregation and toxicity. *Proc Natl Acad Sci U S A*, **99**, 1002-1007.

Kiechle, T., Dedeoglu, A., Kubilus, J., Kowall, N.W., Beal, M.F., Friedlander, R.M., Hersch, S.M. and Ferrante, R.J. (2002) Cytochrome C and caspase-9 expression in Huntington's disease. *Neuromolecular Med*, **1**, 183-195.

Kim, M., Lee, H.S., LaForet, G., McIntyre, C., Martin, E.J., Chang, P., Kim, T.W., Williams, M., Reddy, P.H., Tagle, D., Boyce, F.M., Won, L., Heller, A., Aronin, N. and DiFiglia, M. (1999) Mutant huntingtin expression in clonal striatal cells: dissociation of inclusion formation and neuronal survival by caspase inhibition. *J Neurosci*, **19**, 964-973.

Kita, H., Carmichael, J., Swartz, J., Muro, S., Wyttenbach, A., Matsubara, K., Rubinsztein, D.C. and Kato, K. (2002) Modulation of polyglutamine-induced cell death by genes identified by expression profiling. *Hum Mol Genet*, **11**, 2279-2287.

Kobayashi, Y., Kume, A., Li, M., Doyu, M., Hata, M., Ohtsuka, K. and Sobue, G. (2000) Chaperones Hsp70 and Hsp40 suppress aggregate formation and apoptosis in cultured neuronal cells expressing truncated androgen receptor protein with expanded polyglutamine tract. *J Biol Chem*, **275**, 8772-8778.

Kuemmerle, S., Gutekunst, C.A., Klein, A.M., Li, X.J., Li, S.H., Beal, M.F., Hersch, S.M. and Ferrante, R.J. (1999) Huntington aggregates may not predict neuronal death in Huntington's disease. *Ann Neurol*, **46**, 842-849.

LaFerla, F.M., Hall, C.K., Ngo, L. and Jay, G. (1996) Extracellular deposition of beta-amyloid upon p53-dependent neuronal cell death in transgenic mice. *J Clin Invest*, **98**, 1626-1632.

Laforet, G.A., Sapp, E., Chase, K., McIntyre, C., Boyce, F.M., Campbell, M., Cadigan, B.A., Warzecki, L., Tagle, D.A., Reddy, P.H., Cepeda, C., Calvert, C.R., Jokel, E.S., Klapstein, G.J., Ariano, M.A., Levine, M.S., DiFiglia, M. and Aronin, N. (2001) Changes in cortical and striatal neurons predict behavioral and electrophysiological abnormalities in a transgenic murine model of Huntington's disease. *J Neurosci*, **21**, 9112-9123.

LaMonte, B.H., Wallace, K.E., Holloway, B.A., Shelly, S.S., Ascano, J., Tokito, M., Van Winkle, T., Howland, D.S. and Holzbaur, E.L. (2002) Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. *Neuron*, **34**, 715-727.

Lazic, S.E., Grote, H.E., Blakemore, C., Hannan, A.J., van Dellen, A., Phillips, W. and Barker, R.A. (2006) Neurogenesis in the R6/1 transgenic mouse model of Huntington's disease: effects of environmental enrichment. *Eur J Neurosci*, **23**, 1829-1838.

Lecerf, J.M., Shirley, T.L., Zhu, Q., Kazantsev, A., Amersdorfer, P., Housman, D.E., Messer, A. and Huston, J.S. (2001) Human single-chain Fv intrabodies counteract in situ huntingtin aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci U S A*, **98**, 4764-4769.

Lee, H.C. and Wei, Y.H. (2000) Mitochondrial role in life and death of the cell. *J Biomed Sci*, **7**, 2-15.

Lee, W.C., Yoshihara, M. and Littleton, J.T. (2004) Cytoplasmic aggregates trap polyglutamine-containing proteins and block axonal transport in a *Drosophila* model of Huntington's disease. *Proc Natl Acad Sci U S A*, **101**, 3224-3229.

Levine, M.S., Klapstein, G.J., Koppel, A., Gruen, E., Cepeda, C., Vargas, M.E., Jokel, E.S., Carpenter, E.M., Zanjani, H., Hurst, R.S., Efstratiadis, A., Zeitlin, S. and Chesselet, M.F. (1999) Enhanced sensitivity to N-methyl-D-aspartate receptor activation in transgenic and knockin mouse models of Huntington's disease. *J Neurosci Res*, **58**, 515-532.

Li, H., Li, S.H., Cheng, A.L., Mangiarini, L., Bates, G.P. and Li, X.J. (1999a) Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. *Hum Mol Genet*, **8**, 1227-1236.

Li, H., Li, S.H., Yu, Z.X., Shelbourne, P. and Li, X.J. (2001) Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. *J Neurosci*, **21**, 8473-8481.

Li, H., Wyman, T., Yu, Z.X., Li, S.H. and Li, X.J. (2003a) Abnormal association of mutant huntingtin with synaptic vesicles inhibits glutamate release. *Hum Mol Genet*, **12**, 2021-2030.

Li, J.L., Hayden, M.R., Almqvist, E.W., Brinkman, R.R., Durr, A., Dode, C., Morrison, P.J., Suchowersky, O., Ross, C.A., Margolis, R.L., Rosenblatt, A., Gomez-Tortosa, E., Cabrero, D.M., Novelletto, A., Frontali, M., Nance, M., Trent, R.J., McCusker, E., Jones, R., Paulsen, J.S., Harrison, M., Zanko, A., Abramson, R.K., Russ, A.L., Knowlton, B., Djousse, L., Mysore, J.S., Tariot, S., Gusella, M.F., Wheeler, V.C., Atwood, L.D., Cupples, L.A., Saint-Hilaire, M., Cha, J.H., Hersch, S.M., Koroshetz, W.J., Gusella, J.F., MacDonald, M.E. and Myers, R.H. (2003b) A genome scan for modifiers of age at onset in Huntington disease: The HD MAPS study. *Am J Hum Genet*, **73**, 682-687.

Li, J.Y., Plomann, M. and Brundin, P. (2003c) Huntington's disease: a synaptopathy? *Trends Mol Med*, **9**, 414-420.

Li, S.H., Cheng, A.L., Zhou, H., Lam, S., Rao, M., Li, H. and Li, X.J. (2002) Interaction of Huntington disease protein with transcriptional activator Sp1. *Mol Cell Biol*, **22**, 1277-1287.

Li, S.H., Gutekunst, C.A., Hersch, S.M. and Li, X.J. (1998) Interaction of huntingtin-associated protein with dynactin P150Glued. *J Neurosci*, **18**, 1261-1269.

Li, S.H., Lam, S., Cheng, A.L. and Li, X.J. (2000) Intranuclear huntingtin increases the expression of caspase-1 and induces apoptosis. *Hum Mol Genet*, **9**, 2859-2867.

Li, W., Serpell, L.C., Carter, W.J., Rubinsztein, D.C. and Huntington, J.A. (2006) Expression and characterization of full-length human huntingtin-an elongated heat repeat protein. *J Biol Chem*.

Li, Z., Karlovich, C.A., Fish, M.P., Scott, M.P. and Myers, R.M. (1999b) A putative *Drosophila* homolog of the Huntington's disease gene. *Hum Mol Genet*, **8**, 1807-1815.

Lin, C.H., Tallaksen-Greene, S., Chien, W.M., Cearley, J.A., Jackson, W.S., Crouse, A.B., Ren, S., Li, X.J., Albin, R.L. and Detloff, P.J. (2001) Neurological abnormalities in a knock-in mouse model of Huntington's disease. *Hum Mol Genet*, **10**, 137-144.

Link, C.D., Johnson, C.J., Fonte, V., Paupard, M., Hall, D.H., Styren, S., Mathis, C.A. and Klunk, W.E. (2001) Visualization of fibrillar amyloid deposits in living, transgenic Caenorhabditis elegans animals using the sensitive amyloid dye, X-34. *Neurobiol Aging*, **22**, 217-226.

Lu, T., Pan, Y., Kao, S.Y., Li, C., Kohane, I., Chan, J. and Yankner, B.A. (2004) Gene regulation and DNA damage in the ageing human brain. *Nature*, **429**, 883-891.

Lunkes, A., Lindenberg, K.S., Ben-Haiem, L., Weber, C., Devys, D., Landwehrmeyer, G.B., Mandel, J.L. and Trottier, Y. (2002) Proteases acting on mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. *Mol Cell*, **10**, 259-269.

Luo, S., Vacher, C., Davies, J.E. and Rubinsztein, D.C. (2005) Cdk5 phosphorylation of huntingtin reduces its cleavage by caspases: implications for mutant huntingtin toxicity. *J Cell Biol*, **169**, 647-656.

Luthi-Carter, R., Strand, A., Peters, N.L., Solano, S.M., Hollingsworth, Z.R., Menon, A.S., Frey, A.S., Spektor, B.S., Penney, E.B., Schilling, G., Ross, C.A., Borchelt, D.R., Tapscott, S.J., Young, A.B., Cha, J.H. and Olson, J.M. (2000) Decreased expression of striatal signaling genes in a mouse model of Huntington's disease. *Hum Mol Genet*, **9**, 1259-1271.

Luthi-Carter, R., Strand, A.D., Hanson, S.A., Kooperberg, C., Schilling, G., La Spada, A.R., Merry, D.E., Young, A.B., Ross, C.A., Borchelt, D.R. and Olson, J.M. (2002) Polyglutamine and transcription: gene expression changes shared by DRPLA and Huntington's disease mouse models reveal context-independent effects. *Hum Mol Genet*, **11**, 1927-1937.

Maglione, V., Cannella, M., Gradini, R., Cislaghi, G. and Squitieri, F. (2006) Huntingtin fragmentation and increased caspase 3, 8 and 9 activities in lymphoblasts with heterozygous and homozygous Huntington's disease mutation. *Mech Ageing Dev*, **127**, 213-216.

Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W. and Bates, G.P. (1996) Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell*, **87**, 493-506.

Mantamadiotis, T., Lemberger, T., Bleckmann, S.C., Kern, H., Kretz, O., Martin Villalba, A., Tronche, F., Kellendonk, C., Gau, D., Kapfhammer, J., Otto, C., Schmid, W.

and Schutz, G. (2002) Disruption of CREB function in brain leads to neurodegeneration. *Nat Genet*, **31**, 47-54.

Marsh, J.L., Pallos, J. and Thompson, L.M. (2003) Fly models of Huntington's disease. *Hum Mol Genet*, **12 Spec No 2**, R187-193.

Marsh, J.L., Walker, H., Theisen, H., Zhu, Y.Z., Fielder, T., Purcell, J. and Thompson, L.M. (2000) Expanded polyglutamine peptides alone are intrinsically cytotoxic and cause neurodegeneration in *Drosophila*. *Hum Mol Genet*, **9**, 13-25.

Martin, L.J. (2000) p53 is abnormally elevated and active in the CNS of patients with amyotrophic lateral sclerosis. *Neurobiol Dis*, **7**, 613-622.

Mastrogiacomo, F., LaMarche, J., Dozic, S., Lindsay, G., Bettendorff, L., Robitaille, Y., Schut, L. and Kish, S.J. (1996) Immunoreactive levels of alpha-ketoglutarate dehydrogenase subunits in Friedreich's ataxia and spinocerebellar ataxia type 1. *Neurodegeneration*, **5**, 27-33.

Matsuishi, T., Sakai, T., Naito, E., Nagamitsu, S., Kuroda, Y., Iwashita, H. and Kato, H. (1996) Elevated cerebrospinal fluid lactate/pyruvate ratio in Machado-Joseph disease. *Acta Neurol Scand*, **93**, 72-75.

McCampbell, A., Taylor, J.P., Taye, A.A., Robitschek, J., Li, M., Walcott, J., Merry, D., Chai, Y., Paulson, H., Sobue, G. and Fischbeck, K.H. (2000) CREB-binding protein sequestration by expanded polyglutamine. *Hum Mol Genet*, **9**, 2197-2202.

McInnis, M.G. (1996) Anticipation: an old idea in new genes. *Am J Hum Genet*, **59**, 973-979.

Menalled, L.B., Sison, J.D., Dragatsis, I., Zeitlin, S. and Chesselet, M.F. (2003) Time course of early motor and neuropathological anomalies in a knock-in mouse model of Huntington's disease with 140 CAG repeats. *J Comp Neurol*, **465**, 11-26.

Menalled, L.B., Sison, J.D., Wu, Y., Olivieri, M., Li, X.J., Li, H., Zeitlin, S. and Chesselet, M.F. (2002) Early motor dysfunction and striosomal distribution of huntingtin microaggregates in Huntington's disease knock-in mice. *J Neurosci*, **22**, 8266-8276.

Mende-Mueller, L.M., Toneff, T., Hwang, S.R., Chesselet, M.F. and Hook, V.Y. (2001) Tissue-specific proteolysis of Huntingtin (htt) in human brain: evidence of enhanced levels of N- and C-terminal htt fragments in Huntington's disease striatum. *J Neurosci*, **21**, 1830-1837.

Meredith, S.C. (2006) Protein denaturation and aggregation: cellular responses to denatured and aggregated proteins. *Ann N Y Acad Sci*, **1066**, 181-221.

Milakovic, T. and Johnson, G.V. (2005) Mitochondrial respiration and ATP production are significantly impaired in striatal cells expressing mutant huntingtin. *J Biol Chem*, **280**, 30773-30782.

Miller, K.E. and Sheetz, M.P. (2004) Axonal mitochondrial transport and potential are correlated. *J Cell Sci*, **117**, 2791-2804.

Milnerwood, A.J., Cummings, D.M., Dallerac, G.M., Brown, J.Y., Vatsavayai, S.C., Hirst, M.C., Rezaie, P. and Murphy, K.P. (2006) Early Development of Aberrant Synaptic Plasticity in a Mouse Model of Huntington's Disease. *Hum Mol Genet*.

Mizuno, K., Carnahan, J. and Nawa, H. (1994) Brain-derived neurotrophic factor promotes differentiation of striatal GABAergic neurons. *Dev Biol*, **165**, 243-256.

Modregger, J., DiProspero, N.A., Charles, V., Tagle, D.A. and Plomann, M. (2002) PACSIN 1 interacts with huntingtin and is absent from synaptic varicosities in presymptomatic Huntington's disease brains. *Hum Mol Genet*, **11**, 2547-2558.

Murphy, K.P., Carter, R.J., Lione, L.A., Mangiarini, L., Mahal, A., Bates, G.P., Dunnett, S.B. and Morton, A.J. (2000) Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. *J Neurosci*, **20**, 5115-5123.

Myers, R.H. (2004) Huntington's disease genetics. *NeuroRx*, **1**, 255-262.

Nagai, Y., Tucker, T., Ren, H., Kenan, D.J., Henderson, B.S., Keene, J.D., Strittmatter, W.J. and Burke, J.R. (2000) Inhibition of polyglutamine protein aggregation and cell death by novel peptides identified by phage display screening. *J Biol Chem*, **275**, 10437-10442.

Nagaoka, U., Uchihara, T., Iwabuchi, K., Konno, H., Tobita, M., Funata, N., Yagishita, S. and Kato, T. (2003) Attenuated nuclear shrinkage in neurones with nuclear inclusions of SCA1 brains. *J Neurol Neurosurg Psychiatry*, **74**, 597-601.

Nakao, N., Brundin, P., Funa, K., Lindvall, O. and Odin, P. (1995) Trophic and protective actions of brain-derived neurotrophic factor on striatal DARPP-32-containing neurons in vitro. *Brain Res Dev Brain Res*, **90**, 92-101.

Nasir, J., Floresco, S.B., O'Kusky, J.R., Diewert, V.M., Richman, J.M., Zeisler, J., Borowski, A., Marth, J.D., Phillips, A.G. and Hayden, M.R. (1995) Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell*, **81**, 811-823.

Nass, R., Miller, D.M. and Blakely, R.D. (2001) C. elegans: a novel pharmacogenetic model to study Parkinson's disease. *Parkinsonism Relat Disord*, **7**, 185-191.

Neuwald, A.F. and Hirano, T. (2000) HEAT repeats associated with condensins, cohesins, and other complexes involved in chromosome-related functions. *Genome Res*, **10**, 1445-1452.

Nicotera, P., Ankarcrona, M., Bonfoco, E., Orrenius, S. and Lipton, S.A. (1997) Neuronal necrosis and apoptosis: two distinct events induced by exposure to glutamate or oxidative stress. *Adv Neurol*, **72**, 95-101.

Nucifora, F.C., Jr., Sasaki, M., Peters, M.F., Huang, H., Cooper, J.K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V.L., Dawson, T.M. and Ross, C.A. (2001) Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. *Science*, **291**, 2423-2428.

O'Kusky, J.R., Nasir, J., Cicchetti, F., Parent, A. and Hayden, M.R. (1999) Neuronal degeneration in the basal ganglia and loss of pallido-subthalamic synapses in mice with targeted disruption of the Huntington's disease gene. *Brain Res*, **818**, 468-479.

Ona, V.O., Li, M., Vonsattel, J.P., Andrews, L.J., Khan, S.Q., Chung, W.M., Frey, A.S., Menon, A.S., Li, X.J., Stieg, P.E., Yuan, J., Penney, J.B., Young, A.B., Cha, J.H. and Friedlander, R.M. (1999) Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. *Nature*, **399**, 263-267.

Ordway, J.M., Tallaksen-Greene, S., Gutekunst, C.A., Bernstein, E.M., Cearley, J.A., Wiener, H.W., Dure, L.S.t., Lindsey, R., Hersch, S.M., Jope, R.S., Albin, R.L. and Detloff, P.J. (1997) Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse. *Cell*, **91**, 753-763.

Panov, A.V., Gutekunst, C.A., Leavitt, B.R., Hayden, M.R., Burke, J.R., Strittmatter, W.J. and Greenamyre, J.T. (2002) Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nat Neurosci*, **5**, 731-736.

Parker, J.A., Connolly, J.B., Wellington, C., Hayden, M., Dausset, J. and Neri, C. (2001) Expanded polyglutamines in Caenorhabditis elegans cause axonal abnormalities and severe dysfunction of PLM mechanosensory neurons without cell death. *Proc Natl Acad Sci U S A*, **98**, 13318-13323.

Pearson, C.E., Edamura, K.N. and Cleary, J.D. (2005) Repeat instability: mechanisms of dynamic mutations. *Nat Rev Genet*, **6**, 729-742.

Perutz, M.F. (1996) Glutamine repeats and inherited neurodegenerative diseases: molecular aspects. *Curr Opin Struct Biol*, **6**, 848-858.

Perutz, M.F., Johnson, T., Suzuki, M. and Finch, J.T. (1994) Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. *Proc Natl Acad Sci U S A*, **91**, 5355-5358.

Peters, M.F., Nucifora, F.C., Jr., Kushi, J., Seaman, H.C., Cooper, J.K., Herring, W.J., Dawson, V.L., Dawson, T.M. and Ross, C.A. (1999) Nuclear targeting of mutant Huntingtin increases toxicity. *Mol Cell Neurosci*, **14**, 121-128.

Petersen, A., Hansson, O., Puschban, Z., Sapp, E., Romero, N., Castilho, R.F., Sulzer, D., Rice, M., DiFiglia, M., Przedborski, S. and Brundin, P. (2001) Mice transgenic for exon 1 of the Huntington's disease gene display reduced striatal sensitivity to neurotoxicity induced by dopamine and 6-hydroxydopamine. *Eur J Neurosci*, **14**, 1425-1435.

Poirier, M.A., Li, H., Macosko, J., Cai, S., Amzel, M. and Ross, C.A. (2002) Huntingtin spheroids and protofibrils as precursors in polyglutamine fibrilization. *J Biol Chem*, **277**, 41032-41037.

Polidori, M.C., Mecocci, P., Browne, S.E., Senin, U. and Beal, M.F. (1999) Oxidative damage to mitochondrial DNA in Huntington's disease parietal cortex. *Neurosci Lett*, **272**, 53-56.

Portera-Cailliau, C., Hedreen, J.C., Price, D.L. and Koliatsos, V.E. (1995) Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. *J Neurosci*, **15**, 3775-3787.

Raff, M.C., Whitmore, A.V. and Finn, J.T. (2002) Axonal self-destruction and neurodegeneration. *Science*, **296**, 868-871.

Ranen, N.G., Stine, O.C., Abbott, M.H., Sherr, M., Codori, A.M., Franz, M.L., Chao, N.I., Chung, A.S., Pleasant, N., Callahan, C. and et al. (1995) Anticipation and instability of IT-15 (CAG)n repeats in parent-offspring pairs with Huntington disease. *Am J Hum Genet*, **57**, 593-602.

Rangone, H., Poizat, G., Troncoso, J., Ross, C.A., MacDonald, M.E., Saudou, F. and Humbert, S. (2004) The serum- and glucocorticoid-induced kinase SGK inhibits mutant huntingtin-induced toxicity by phosphorylating serine 421 of huntingtin. *Eur J Neurosci*, **19**, 273-279.

Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J. and Rubinsztein, D.C. (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet*, **36**, 585-595.

Reddy, P.H., Williams, M., Charles, V., Garrett, L., Pike-Buchanan, L., Whetsell, W.O., Jr., Miller, G. and Tagle, D.A. (1998) Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. *Nat Genet*, **20**, 198-202.

Reid, E., Kloos, M., Ashley-Koch, A., Hughes, L., Bevan, S., Svenson, I.K., Graham, F.L., Gaskell, P.C., Dearlove, A., Pericak-Vance, M.A., Rubinsztein, D.C. and Marchuk, D.A. (2002) A kinesin heavy chain (KIF5A) mutation in hereditary spastic paraplegia (SPG10). *Am J Hum Genet*, **71**, 1189-1194.

Reiner, A., Del Mar, N., Meade, C.A., Yang, H., Dragatsis, I., Zeitlin, S. and Goldowitz, D. (2001) Neurons lacking huntingtin differentially colonize brain and survive in chimeric mice. *J Neurosci*, **21**, 7608-7619.

Ridley, R.M., Frith, C.D., Crow, T.J. and Conneally, P.M. (1988) Anticipation in Huntington's disease is inherited through the male line but may originate in the female. *J Med Genet*, **25**, 589-595.

Rigamonti, D., Bauer, J.H., De-Fraja, C., Conti, L., Sipione, S., Sciorati, C., Clementi, E., Hackam, A., Hayden, M.R., Li, Y., Cooper, J.K., Ross, C.A., Govoni, S., Vincenz,

C. and Cattaneo, E. (2000) Wild-type huntingtin protects from apoptosis upstream of caspase-3. *J Neurosci*, **20**, 3705-3713.

Rigamonti, D., Sipione, S., Goffredo, D., Zuccato, C., Fossale, E. and Cattaneo, E. (2001) Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. *J Biol Chem*, **276**, 14545-14548.

Rosas, H.D., Liu, A.K., Hersch, S., Glessner, M., Ferrante, R.J., Salat, D.H., van der Kouwe, A., Jenkins, B.G., Dale, A.M. and Fischl, B. (2002) Regional and progressive thinning of the cortical ribbon in Huntington's disease. *Neurology*, **58**, 695-701.

Ross, C.A., McInnis, M.G., Margolis, R.L. and Li, S.H. (1993) Genes with triplet repeats: candidate mediators of neuropsychiatric disorders. *Trends Neurosci*, **16**, 254-260.

Ross, C.A. and Poirier, M.A. (2004) Protein aggregation and neurodegenerative disease. *Nat Med*, **10 Suppl**, S10-17.

Ross, C.A., Poirier, M.A., Wanker, E.E. and Amzel, M. (2003) Polyglutamine fibrillogenesis: the pathway unfolds. *Proc Natl Acad Sci U S A*, **100**, 1-3.

Ruan, Q., Lesort, M., MacDonald, M.E. and Johnson, G.V. (2004) Striatal cells from mutant huntingtin knock-in mice are selectively vulnerable to mitochondrial complex II inhibitor-induced cell death through a non-apoptotic pathway. *Hum Mol Genet*, **13**, 669-681.

Rubinsztein, D.C., Leggo, J., Coles, R., Almqvist, E., Biancalana, V., Cassiman, J.J., Chotai, K., Connarty, M., Crauford, D., Curtis, A., Curtis, D., Davidson, M.J., Differ, A.M., Dode, C., Dodge, A., Frontali, M., Ranen, N.G., Stine, O.C., Sherr, M., Abbott, M.H., Franz, M.L., Graham, C.A., Harper, P.S., Hedreen, J.C., Hayden, M.R. and et al. (1996) Phenotypic characterization of individuals with 30-40 CAG repeats in the Huntington disease (HD) gene reveals HD cases with 36 repeats and apparently normal elderly individuals with 36-39 repeats. *Am J Hum Genet*, **59**, 16-22.

Saft, C., Zange, J., Andrich, J., Muller, K., Lindenberg, K., Landwehrmeyer, B., Vorgerd, M., Kraus, P.H., Przuntek, H. and Schols, L. (2005) Mitochondrial impairment in patients and asymptomatic mutation carriers of Huntington's disease. *Mov Disord*, **20**, 674-679.

Sanchez, I., Mahlke, C. and Yuan, J. (2003) Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. *Nature*, **421**, 373-379.

Sanchez, I., Xu, C.J., Juo, P., Kakizaka, A., Blenis, J. and Yuan, J. (1999) Caspase-8 is required for cell death induced by expanded polyglutamine repeats. *Neuron*, **22**, 623-633.

Saudou, F., Finkbeiner, S., Devys, D. and Greenberg, M.E. (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell*, **95**, 55-66.

Sawa, A., Wiegand, G.W., Cooper, J., Margolis, R.L., Sharp, A.H., Lawler, J.F., Jr., Greenamyre, J.T., Snyder, S.H. and Ross, C.A. (1999) Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. *Nat Med*, **5**, 1194-1198.

Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G.P., Davies, S.W., Lehrach, H. and Wanker, E.E. (1997) Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and *in vivo*. *Cell*, **90**, 549-558.

Scherzinger, E., Sittler, A., Schweiger, K., Heiser, V., Lurz, R., Hasenbank, R., Bates, G.P., Lehrach, H. and Wanker, E.E. (1999) Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington's disease pathology. *Proc Natl Acad Sci U S A*, **96**, 4604-4609.

Schilling, G., Becher, M.W., Sharp, A.H., Jinnah, H.A., Duan, K., Kotzuk, J.A., Slunt, H.H., Ratovitski, T., Cooper, J.K., Jenkins, N.A., Copeland, N.G., Price, D.L., Ross, C.A. and Borchelt, D.R. (1999) Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. *Hum Mol Genet*, **8**, 397-407.

Schliwa, M. and Woehlke, G. (2003) Molecular motors. *Nature*, **422**, 759-765.

Schmitt, I., Bachner, D., Megow, D., Henklein, P., Hameister, H., Epplen, J.T. and Riess, O. (1995) Expression of the Huntington disease gene in rodents: cloning the rat homologue and evidence for downregulation in non-neuronal tissues during development. *Hum Mol Genet*, **4**, 1173-1182.

Schoenherr, C.J. and Anderson, D.J. (1995) Silencing is golden: negative regulation in the control of neuronal gene transcription. *Curr Opin Neurobiol*, **5**, 566-571.

Schulz, J.B., Matthews, R.T., Henshaw, D.R. and Beal, M.F. (1996) Neuroprotective strategies for treatment of lesions produced by mitochondrial toxins: implications for neurodegenerative diseases. *Neuroscience*, **71**, 1043-1048.

Schulz, J.B., Matthews, R.T., Jenkins, B.G., Ferrante, R.J., Siwek, D., Henshaw, D.R., Cipolloni, P.B., Mecocci, P., Kowall, N.W., Rosen, B.R. and et al. (1995) Blockade of neuronal nitric oxide synthase protects against excitotoxicity *in vivo*. *J Neurosci*, **15**, 8419-8429.

Sen, S., Dash, D., Pasha, S. and Brahmachari, S.K. (2003) Role of histidine interruption in mitigating the pathological effects of long polyglutamine stretches in SCA1: A molecular approach. *Protein Sci*, **12**, 953-962.

Seo, H., Sonntag, K.C. and Isacson, O. (2004) Generalized brain and skin proteasome inhibition in Huntington's disease. *Ann Neurol*, **56**, 319-328.

Shelbourne, P.F., Killeen, N., Hevner, R.F., Johnston, H.M., Tecott, L., Lewandoski, M., Ennis, M., Ramirez, L., Li, Z., Iannicola, C., Littman, D.R. and Myers, R.M. (1999) A Huntington's disease CAG expansion at the murine Hdh locus is unstable and associated with behavioural abnormalities in mice. *Hum Mol Genet*, **8**, 763-774.

Sheng, M. and Kim, M.J. (2002) Postsynaptic signaling and plasticity mechanisms. *Science*, **298**, 776-780.

Shimohata, T., Nakajima, T., Yamada, M., Uchida, C., Onodera, O., Naruse, S., Kimura, T., Koide, R., Nozaki, K., Sano, Y., Ishiguro, H., Sakoe, K., Ooshima, T., Sato, A., Ikeuchi, T., Oyake, M., Sato, T., Aoyagi, Y., Hozumi, I., Nagatsu, T., Takiyama, Y., Nishizawa, M., Goto, J., Kanazawa, I., Davidson, I., Tanese, N., Takahashi, H. and Tsuji, S. (2000) Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. *Nat Genet*, **26**, 29-36.

Sittler, A., Walter, S., Wedemeyer, N., Hasenbank, R., Scherzinger, E., Eickhoff, H., Bates, G.P., Lehrach, H. and Wanker, E.E. (1998) SH3GL3 associates with the Huntingtin exon 1 protein and promotes the formation of polygln-containing protein aggregates. *Mol Cell*, **2**, 427-436.

Slow, E.J., van Raamsdonk, J., Rogers, D., Coleman, S.H., Graham, R.K., Deng, Y., Oh, R., Bissada, N., Hossain, S.M., Yang, Y.Z., Li, X.J., Simpson, E.M., Gutekunst, C.A., Leavitt, B.R. and Hayden, M.R. (2003) Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. *Hum Mol Genet*, **12**, 1555-1567.

Smith, R., Brundin, P. and Li, J.Y. (2005) Synaptic dysfunction in Huntington's disease: a new perspective. *Cell Mol Life Sci*, **62**, 1901-1912.

Spires, T.L., Grote, H.E., Varshney, N.K., Cordery, P.M., van Dellen, A., Blakemore, C. and Hannan, A.J. (2004) Environmental enrichment rescues protein deficits in a mouse model of Huntington's disease, indicating a possible disease mechanism. *J Neurosci*, **24**, 2270-2276.

Squitieri, F., Cannella, M., Sgarbi, G., Maglione, V., Falleni, A., Lenzi, P., Baracca, A., Cislaghi, G., Saft, C., Ragona, G., Russo, M.A., Thompson, L.M., Solaini, G. and Fornai, F. (2006) Severe ultrastructural mitochondrial changes in lymphoblasts homozygous for Huntington disease mutation. *Mech Ageing Dev*, **127**, 217-220.

Steffan, J.S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L.C., Slepko, N., Illes, K., Lukacsovich, T., Zhu, Y.Z., Cattaneo, E., Pandolfi, P.P., Thompson, L.M. and Marsh, J.L. (2004) SUMO modification of Huntingtin and Huntington's disease pathology. *Science*, **304**, 100-104.

Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M., Kurokawa, R., Housman, D.E., Jackson, G.R., Marsh, J.L. and Thompson, L.M. (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature*, **413**, 739-743.

Steffan, J.S., Kazantsev, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y.Z., Gohler, H., Wanker, E.E., Bates, G.P., Housman, D.E. and Thompson, L.M. (2000) The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. *Proc Natl Acad Sci U S A*, **97**, 6763-6768.

Stowers, R.S., Megeath, L.J., Gorska-Andrzejak, J., Meinertzhagen, I.A. and Schwarz, T.L. (2002) Axonal transport of mitochondria to synapses depends on milton, a novel *Drosophila* protein. *Neuron*, **36**, 1063-1077.

Sugars, K.L. and Rubinsztein, D.C. (2003) Transcriptional abnormalities in Huntington disease. *Trends Genet*, **19**, 233-238.

Sun, Y., Savanenin, A., Reddy, P.H. and Liu, Y.F. (2001) Polyglutamine-expanded huntingtin promotes sensitization of N-methyl-D-aspartate receptors via post-synaptic density 95. *J Biol Chem*, **276**, 24713-24718.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*, **397**, 441-446.

Szebenyi, G., Morfini, G.A., Babcock, A., Gould, M., Selkoe, K., Stenoien, D.L., Young, M., Faber, P.W., MacDonald, M.E., McPhaul, M.J. and Brady, S.T. (2003) Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport. *Neuron*, **40**, 41-52.

Takano, H. and Gusella, J.F. (2002) The predominantly HEAT-like motif structure of huntingtin and its association and coincident nuclear entry with dorsal, an NF-kB/Rel/dorsal family transcription factor. *BMC Neurosci*, **3**, 15.

Takeyama, K., Ito, S., Yamamoto, A., Tanimoto, H., Furutani, T., Kanuka, H., Miura, M., Tabata, T. and Kato, S. (2002) Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in *Drosophila*. *Neuron*, **35**, 855-864.

Telenius, H., Kremer, H.P., Theilmann, J., Andrew, S.E., Almqvist, E., Anvret, M., Greenberg, C., Greenberg, J., Lucotte, G., Squitieri, F. and et al. (1993) Molecular analysis of juvenile Huntington disease: the major influence on (CAG)n repeat length is the sex of the affected parent. *Hum Mol Genet*, **2**, 1535-1540.

Thomas, L.B., Gates, D.J., Richfield, E.K., O'Brien, T.F., Schweitzer, J.B. and Steindler, D.A. (1995) DNA end labeling (TUNEL) in Huntington's disease and other neuropathological conditions. *Exp Neurol*, **133**, 265-272.

Timmers, H.J., Swaab, D.F., van de Nes, J.A. and Kremer, H.P. (1996) Somatostatin 1-12 immunoreactivity is decreased in the hypothalamic lateral tuberal nucleus of Huntington's disease patients. *Brain Res*, **728**, 141-148.

Trushina, E., Dyer, R.B., Badger, J.D., 2nd, Ure, D., Eide, L., Tran, D.D., Vrieze, B.T., Legendre-Guillemin, V., McPherson, P.S., Mandavilli, B.S., Van Houten, B., Zeitlin, S., McNiven, M., Aebersold, R., Hayden, M., Parisi, J.E., Seeberg, E., Dragatsis, I., Doyle, K., Bender, A., Chacko, C. and McMurray, C.T. (2004) Mutant huntingtin impairs axonal trafficking in mammalian neurons *in vivo* and in vitro. *Mol Cell Biol*, **24**, 8195-8209.

Turmaine, M., Raza, A., Mahal, A., Mangiarini, L., Bates, G.P. and Davies, S.W. (2000) Nonapoptotic neurodegeneration in a transgenic mouse model of Huntington's disease. *Proc Natl Acad Sci U S A*, **97**, 8093-8097.

U, M., Miyashita, T., Ohtsuka, Y., Okamura-Oho, Y., Shikama, Y. and Yamada, M. (2001) Extended polyglutamine selectively interacts with caspase-8 and -10 in nuclear aggregates. *Cell Death Differ*, **8**, 377-386.

Uchihara, T., Iwabuchi, K., Funata, N. and Yagishita, S. (2002) Attenuated nuclear shrinkage in neurons with nuclear aggregates--a morphometric study on pontine neurons of Machado-Joseph disease brains. *Exp Neurol*, **178**, 124-128.

Usdin, M.T., Shelbourne, P.F., Myers, R.M. and Madison, D.V. (1999) Impaired synaptic plasticity in mice carrying the Huntington's disease mutation. *Hum Mol Genet*, **8**, 839-846.

van Dellen, A., Blakemore, C., Deacon, R., York, D. and Hannan, A.J. (2000) Delaying the onset of Huntington's in mice. *Nature*, **404**, 721-722.

van Dellen, A. and Hannan, A.J. (2004) Genetic and environmental factors in the pathogenesis of Huntington's disease. *Neurogenetics*, **5**, 9-17.

Velier, J., Kim, M., Schwarz, C., Kim, T.W., Sapp, E., Chase, K., Aronin, N. and DiFiglia, M. (1998) Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways. *Exp Neurol*, **152**, 34-40.

Ventimiglia, R., Mather, P.E., Jones, B.E. and Lindsay, R.M. (1995) The neurotrophins BDNF, NT-3 and NT-4/5 promote survival and morphological and biochemical differentiation of striatal neurons in vitro. *Eur J Neurosci*, **7**, 213-222.

Voges, D., Zwickl, P. and Baumeister, W. (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem*, **68**, 1015-1068.

Vonsattel, J.P. and DiFiglia, M. (1998) Huntington disease. *J Neuropathol Exp Neurol*, **57**, 369-384.

Vonsattel, J.P., Myers, R.H., Stevens, T.J., Ferrante, R.J., Bird, E.D. and Richardson, E.P., Jr. (1985) Neuropathological classification of Huntington's disease. *J Neuropathol Exp Neurol*, **44**, 559-577.

Wang, J., Gines, S., MacDonald, M.E. and Gusella, J.F. (2005) Reversal of a full-length mutant huntingtin neuronal cell phenotype by chemical inhibitors of polyglutamine-mediated aggregation. *BMC Neurosci*, **6**, 1.

Warby, S.C., Chan, E.Y., Metzler, M., Gan, L., Singaraja, R.R., Crocker, S.F., Robertson, H.A. and Hayden, M.R. (2005) Huntingtin phosphorylation on serine 421 is significantly reduced in the striatum and by polyglutamine expansion *in vivo*. *Hum Mol Genet*, **14**, 1569-1577.

Warrick, J.M., Chan, H.Y., Gray-Board, G.L., Chai, Y., Paulson, H.L. and Bonini, N.M. (1999) Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat Genet*, **23**, 425-428.

Wellington, C.L., Ellerby, L.M., Gutekunst, C.A., Rogers, D., Warby, S., Graham, R.K., Loubser, O., van Raamsdonk, J., Singaraja, R., Yang, Y.Z., Gafni, J., Bredesen, D., Hersch, S.M., Leavitt, B.R., Roy, S., Nicholson, D.W. and Hayden, M.R. (2002) Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J Neurosci*, **22**, 7862-7872.

Wellington, C.L., Ellerby, L.M., Hackam, A.S., Margolis, R.L., Trifiro, M.A., Singaraja, R., McCutcheon, K., Salvesen, G.S., Propp, S.S., Bromm, M., Rowland, K.J., Zhang, T., Rasper, D., Roy, S., Thornberry, N., Pinsky, L., Kakizuka, A., Ross, C.A., Nicholson, D.W., Bredesen, D.E. and Hayden, M.R. (1998) Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J Biol Chem*, **273**, 9158-9167.

Wellington, C.L., Leavitt, B.R. and Hayden, M.R. (2000) Huntington disease: new insights on the role of huntingtin cleavage. *J Neural Transm Suppl*, 1-17.

Wheeler, V.C., Gutekunst, C.A., Vrbanac, V., Lebel, L.A., Schilling, G., Hersch, S., Friedlander, R.M., Gusella, J.F., Vonsattel, J.P., Borchelt, D.R. and MacDonald, M.E. (2002) Early phenotypes that presage late-onset neurodegenerative disease allow testing of modifiers in Hdh CAG knock-in mice. *Hum Mol Genet*, **11**, 633-640.

White, J.K., Auerbach, W., Duyao, M.P., Vonsattel, J.P., Gusella, J.F., Joyner, A.L. and MacDonald, M.E. (1997) Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. *Nat Genet*, **17**, 404-410.

Widmer, H.R. and Hefti, F. (1994) Neurotrophin-4/5 promotes survival and differentiation of rat striatal neurons developing in culture. *Eur J Neurosci*, **6**, 1669-1679.

Wyttenbach, A., Carmichael, J., Swartz, J., Furlong, R.A., Narain, Y., Rankin, J. and Rubinsztein, D.C. (2000) Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci U S A*, **97**, 2898-2903.

Xia, J., Lee, D.H., Taylor, J., Vandelft, M. and Truant, R. (2003) Huntingtin contains a highly conserved nuclear export signal. *Hum Mol Genet*, **12**, 1393-1403.

Yamamoto, A., Lucas, J.J. and Hen, R. (2000) Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. *Cell*, **101**, 57-66.

Yoshida, H., Yoshizawa, T., Shibasaki, F., Shoji, S. and Kanazawa, I. (2002) Chemical chaperones reduce aggregate formation and cell death caused by the truncated Machado-Joseph disease gene product with an expanded polyglutamine stretch. *Neurobiol Dis*, **10**, 88-99.

Yoshihara, M., Ensminger, A.W. and Littleton, J.T. (2001) Neurobiology and the *Drosophila* genome. *Funct Integr Genomics*, **1**, 235-240.

Yu, Z.X., Li, S.H., Evans, J., Pillarisetti, A., Li, H. and Li, X.J. (2003) Mutant huntingtin causes context-dependent neurodegeneration in mice with Huntington's disease. *J Neurosci*, **23**, 2193-2202.

Zainelli, G.M., Dudek, N.L., Ross, C.A., Kim, S.Y. and Muma, N.A. (2005) Mutant huntingtin protein: a substrate for transglutaminase 1, 2, and 3. *J Neuropathol Exp Neurol*, **64**, 58-65.

Zeitlin, S., Liu, J.P., Chapman, D.L., Papaioannou, V.E. and Efstratiadis, A. (1995) Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. *Nat Genet*, **11**, 155-163.

Zhang, X., Smith, D.L., Meriin, A.B., Engemann, S., Russel, D.E., Roark, M., Washington, S.L., Maxwell, M.M., Marsh, J.L., Thompson, L.M., Wanker, E.E., Young, A.B., Housman, D.E., Bates, G.P., Sherman, M.Y. and Kazantsev, A.G. (2005) A potent small molecule inhibits polyglutamine aggregation in Huntington's disease neurons and suppresses neurodegeneration *in vivo*. *Proc Natl Acad Sci U S A*, **102**, 892-897.

Zhao, C., Takita, J., Tanaka, Y., Setou, M., Nakagawa, T., Takeda, S., Yang, H.W., Terada, S., Nakata, T., Takei, Y., Saito, M., Tsuji, S., Hayashi, Y. and Hirokawa, N. (2001) Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta. *Cell*, **105**, 587-597.

Zuccato, C., Ciammola, A., Rigamonti, D., Leavitt, B.R., Goffredo, D., Conti, L., MacDonald, M.E., Friedlander, R.M., Silani, V., Hayden, M.R., Timmusk, T., Sipione, S. and Cattaneo, E. (2001) Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science*, **293**, 493-498.

Zuccato, C., Tartari, M., Crotti, A., Goffredo, D., Valenza, M., Conti, L., Cataudella, T., Leavitt, B.R., Hayden, M.R., Timmusk, T., Rigamonti, D. and Cattaneo, E. (2003) Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat Genet*, **35**, 76-83.

Zucker, B., Luthi-Carter, R., Kama, J.A., Dunah, A.W., Stern, E.A., Fox, J.H., Standaert, D.G., Young, A.B. and Augood, S.J. (2005) Transcriptional dysregulation in striatal projection- and interneurons in a mouse model of Huntington's disease: neuronal selectivity and potential neuroprotective role of HAP1. *Hum Mol Genet*, **14**, 179-189.

CHAPTER 2

Cytoplasmic aggregates trap polyglutamine-containing proteins and block axonal transport in a *Drosophila* model of Huntington's disease

Wyan-Ching Mimi Lee, Motojiro Yoshihara, and J. Troy Littleton

Picower Center for Learning and Memory, Departments of Biology and Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139

This work was initiated by J. Troy Littleton in the laboratory of Barry Ganetzky. J. Troy Littleton generated the transgenic *Drosophila* lines used in these studies, performed the eye neurodegeneration analysis and electroretinogram and DLM flight muscle electrophysiological recordings, and helped with the immunohistochemical analyses. Motojiro Yoshihara performed the pseudopupil analysis and embryonic immunohistochemistry. All other experiments were performed by Wyan-Ching Mimi Lee. Parts of this work have been published in *Proc Natl Acad Sci U S A*, **101**, 3224-3229 (2004).

Abstract

Huntington's disease is an autosomal dominant neurodegenerative disorder caused by expansion of a polyglutamine tract in the huntingtin protein that results in intracellular aggregate formation and neurodegeneration. Pathways leading from polyglutamine tract expansion to disease pathogenesis remain obscure. To elucidate the means by which polyglutamine expansion causes neuronal dysfunction, we generated *Drosophila* transgenic strains expressing 548 aa N-terminal fragments of human huntingtin encoding pathogenic (Htt-Q128) or nonpathogenic (Htt-Q0) proteins. While expression of Htt-Q0 has no discernible effect on behavior, lifespan, transcriptional regulation or neuronal morphology, pan-neuronal expression of Htt-Q128 led to progressive loss of motor coordination, decreased lifespan, altered gene expression and time-dependent formation of huntingtin aggregates specifically in the cytoplasm and neurites. Huntingtin aggregates sequester other expanded polyglutamine proteins in the cytoplasm and lead to synaptic aggregate accumulation and disruption of axonal transport. In contrast, *Drosophila* expressing an expanded polyglutamine tract alone, or an expanded polyglutamine tract in the context of the spinocerebellar ataxia type 3 protein, display only nuclear aggregates and do not disrupt axonal trafficking. Our findings indicate that non-nuclear events induced by cytoplasmic huntingtin aggregation may play a central role in the progressive neurodegeneration observed in Huntington's disease.

Introduction

Huntington's disease (HD) is characterized by neurodegeneration and formation of neuronal intracellular inclusions secondary to abnormal expansion of a CAG tract (encoding a polyglutamine repeat) in exon 1 of the huntingtin gene (Htt) (HDCRG, 1993). Expansion of the CAG tract past the pathological threshold of ~35-40 repeats ensures disease manifestation (Rubinsztein et al., 1996; Xuereb et al., 1996). As with other glutamine-repeat disorders, abnormal protein conformation(s) promoted by polyglutamine (polyQ) expansion appear to be central to pathogenesis (Persichetti et al., 1999; Scherzinger et al., 1997). Although intracellular aggregates are a prominent hallmark of polyQ disease, their role in disease pathogenesis is debated (Kopito, 2000). In the case of HD, Htt-immunopositive aggregates have been observed in both the nucleus and the cytoplasm of affected neurons, although their primary subcellular localization remains controversial (DiFiglia et al., 1997). One difficulty in characterizing the subcellular localization of Htt aggregates has been the widespread use of exon 1 models, which encode only the first 81 amino acids of the Htt protein with the polyQ tract. These models widely demonstrate nuclear localization of Htt-immunopositive aggregates. Whether expression of exon 1 alone faithfully mimics the localization of the full-length endogenous protein (3144 amino acids) or its putative cleavage products is unclear. However, if aggregate formation plays an essential role in disease progression, it is pertinent to determine the specific subcellular compartments in which aggregates produce detrimental effects.

Although some evidence links intranuclear aggregates and disease manifestation (Becher et al., 1998; DiFiglia et al., 1997), recent findings suggest that the presence of aggregates in the nucleus may not be central to neuropathology (Gutekunst et al., 1999; Saudou et al., 1998). HD disease symptoms have been reported to arise from neuronal cell loss before nuclear aggregates are detectable but after neuritic aggregates have appeared (Gutekunst et al., 1999; Sapp et al., 1999). In transgenic mice, the presence of aggregates in neurites is highly correlated with development of neuropathological symptoms (Li et al., 1999a), and expression of the abnormal protein alters synaptic function (Lee et al., 2003; Li et al., 2000a; Murphy et al., 2000; Usdin et al., 1999), indicating that the toxic effects of mutant Htt expression may not be limited to the nucleus and may also cause defects in non-nuclear cellular processes.

A clearer understanding of cellular dysfunction mediated by expansion of the polyQ repeat in Htt can be gained by generating transgenic animal models of HD. In particular, single-gene diseases such as the polyglutamine repeat disorders are well-suited for modeling in *Drosophila melanogaster*. The short generation time, small size, and genetic tractability of the organism, along with the high degree of conservation between mammalian and invertebrate cell biology, make *Drosophila* a powerful tool with which to explore basic molecular mechanisms of disease. A key strength of *Drosophila* disease models is the ability to perform either hypothesis-driven or unbiased screens on a large scale for modifiers of disease phenotypes, as well as the potential for rapid validation of candidate therapies before testing in mammalian models. Development of *Drosophila* HD models may thus facilitate the identification of both molecular pathways that lead to disease pathology and potential avenues for treatment.

To explore mutant Htt-mediated mechanisms of neuronal dysfunction, we generated transgenic *Drosophila* that express the first 548 amino acids of the human Htt gene with either a pathogenic polyglutamine tract of 128 repeats (Htt-Q128) or a nonpathogenic tract of 0 repeats (Htt-Q0). This N-terminal motif contains regions of strong homology between Htt isoforms from *Drosophila* to humans and is more likely to faithfully mimic endogenous Htt behavior than constructs containing only the first 81 amino acids, which display no sequence conservation between *Drosophila* and human Htt homologs. Our findings indicate the formation of cytoplasmic and neuritic Htt aggregates in our *Drosophila* HD model that sequester other non-nuclear polyQ-containing proteins and block axonal transport.

Materials and Methods

Drosophila Genetics and Generation of Htt Constructs

Drosophila melanogaster were maintained on standard medium at 22^{o}C. cDNAs for Htt-Q0 and Htt-Q128 were generously provided by M. R. Hayden and subsequently subcloned into pHS or pUAST vectors. Htt-Q0 was subcloned into 5' Hpa I and 3' Not I sites of pHS and into 5' Eco R1 (blunt end ligation) and 3' Not 1 sites of pUAST. Htt-Q128 was subcloned in 5' Eco RI and 3' Stu I (blunt end ligation) sites of pHS and into 5' Eco R1 and 3' Xba 1 (blunt end ligation) sites of pUAST. The cDNAs encompass the start of the ORF of human Htt and terminate after amino acid 548 of the published sequence. Transgenic animals were obtained through standard microinjection protocols. Confirmation of transgene expression was obtained by Western analysis with mouse anti-HD MAb2166 antibody against human Htt (Chemicon), which does not cross-react with the endogenous *Drosophila* Htt homolog. Transgenic *Drosophila* encoding Q127 were obtained from Parsi Kazemi-Esfarjani (Kazemi-Esfarjani and Benzer, 2000), SCA3-Q78 from Nancy Bonini (Warrick et al., 1998) and Dishevelled-Q108 from Leslie Thompson (Marsh et al., 2000). Transgenic lines expressing UAS-Htt and a second polyQ-encoding UAS transgene, or UAS-GFP, were generated through standard crosses.

Western Blot Analysis

To obtain samples for Western analysis of pHS-Htt lines, twenty *Drosophila* of each indicated genotype were collected and heat shocked at 37^{o}C for 40 minutes, allowed to recover at room temperature for one hour, heat shocked again for 40 minutes, and incubated at 28^{o}C overnight before processing. *Drosophila* were frozen in liquid nitrogen and vortexed in order to isolate 20 heads for each genotype. Heads were then homogenized in 50 μL of sample buffer and centrifuged for 5 seconds. 30 μL of the supernatant was added to 20 μL of sample buffer and boiled for 5 minutes. 10 μL per sample was loaded onto 10% SDS-PAGE gels and separated at 18 mA per gel. The gels were immunoblotted with mouse anti-HD MAb2166 (Chemicon) at 1:1000, and immunoreactive bands were visualized using ECL (Pierce).

Electroretinograms and DLM Flight Muscle Recordings

Extracellular recordings were made by placing a sharp glass electrode into the lateral thorax of adult animals, with the ground electrode in the *Drosophila* head. Basal activity was recorded for 2 minutes at 20ºC. Temperature shifts to 38ºC were then performed by heating the mounting clay in which the fly was embedded. Electroretinograms were performed as previously described (Littleton et al., 1998).

Larval Locomotion and Adult Behavioral Analysis

Wandering 3rd instar larvae grown at room temperature were collected, washed gently with distilled water, and placed individually on the center of a flat layer of 0.7% agar atop an evenly illuminated light box (Wang et al., 1997). Larval locomotion was recorded with a digital video camera (CanonXL1S) attached to a 16x zoom lens. Recording stopped at 2.5 minutes or when the larva reached the edge of the agar layer, with documentation of the recording time. Distance traveled by each larva was quantified by measuring the number of centimeters traversed by a crawling larva on the agar surface during the time recorded. Speed was calculated by dividing the distance traveled by the number of seconds of recording time. *Drosophila* viability assays were performed on elav-GAL4, Htt-Q128/white, Htt-Q128/elav-GAL4, and Htt-Q0/elav-GAL4 flies by daily quantification of lethality for 200 female flies of each genotype. Flies were aged at 25ºC, with twenty per food vial. Food vials were transferred every two days.

Microarray Analysis.

Microarray analysis was performed with Affymetrix® high-density oligonucleotide GeneChip® arrays using the laboratory methods described in the Affymetrix® Genechip Expression Manual. Transgenic flies were crossed to the elav-GAL4 driver, and RNA was isolated from adult male flies of the indicated genotypes aged for 3-4 days or 9-11 days post-eclosion at 25^{o}C. Flies were frozen in liquid nitrogen and vortexed to isolate heads. Circadian differences were minimized by processing tissue between 2 and 4pm. Total RNA was extracted from 600 pooled fly heads using the Trizol method (GIBCO-BRL), with two independent RNA samples prepared from both 3-4 day-old flies and 9-11 day-old flies for each genotype. From the total RNA, poly(A) mRNA was isolated using the Qiagen Oligotex mRNA extraction kit and used as a template for cDNA via the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen). Biotin-labeled cRNA was synthesized with biotinylated NTPs (Invitrogen), cleaned with the Qiagen RNEasy kit, and fragmented in fragmentation buffer (200mM Tris-acetate, pH8.1, 500 mM KOAc,

150 mM MgOAc) at 94°C for 35 minutes to produce 35-200 bp fragments for gene chip hybridization. Affymetrix® high-density oligonucleotide arrays were probed, hybridized, stained and washed in the MIT Biopolymers Facility. Microarray analysis was performed using the Affymetrix® Microarray Suite and Data Mining Tool statistics-based analysis software. 12 independent microarrays were analyzed (two each of RNA from 3-4 day-old control, Htt-Q0-, and Htt-Q128-expressing flies, and two each of RNA from 9-11 day-old control, Htt-Q0-, and Htt-Q128-expressing flies) for a total of eight pairwise comparisons between expression profiles from Htt-Q128-expressing flies and Htt-Q0-expressing flies or control flies for both the 3-4 day set and the 9-11 day set. Gene changes were called based upon the stringency criteria that differential regulation must be reported by Affymetrix® software for at least 75%, or twelve, of the sixteen pairwise comparisons for microarrays from both the 3-4 day set and the 9-11 day set. In addition, the fold change in gene expression was required to be greater than or equal to 1.5 with a statistical significance of $p<0.05$ for either the 3-4 day set of the 9-11 day set.

Morphological Analysis

Immunostaining was performed on wandering 3rd instar larvae reared at 25ºC. Larvae were dissected in HL3 buffer (Stewart et al., 1994) and fixed in 4% formaldehyde in HL3 for 40 minutes at room temperature. Primary antibodies used were mouse anti-HD MAb2166 against human Htt (Chemicon) at 1:500, rabbit anti-GFP sc8334 against green fluorescent protein (Santa Cruz Biotechnology) at 1:500, anti-HA to label Q127 and SCA3-Q78 at 1:1000, rabbit anti-Dishevelled (Willert et al., 1997) at 1:500, and rabbit anti-syt DSYT2 against *Drosophila* synaptotagmin (Littleton et al., 1993) at 1:500. FITC-HRP was used at 1:10,000 to label neuronal membranes. Visualization and quantification were performed on a Zeiss Pascal Confocal Microscope using Cy2-conjugated and Rhodamine Red-conjugated secondary antibodies (Molecular Probes, Chemicon, Jackson Labs). Htt-Q128 aggregate diameter was determined by digitally capturing images at 40x and computing diameters (in μm) using LSM 5 Pascal Analysis Software. All error measurements are STD unless otherwise indicated. Corneal pseudopupil analysis of adult ommatidia was performed as previously described (Van Vactor et al., 1991).

Electrophysiological analysis

Electrophysiological analysis of the neuromuscular junction in CNS-intact wandering 3rd instar larvae was performed in *Drosophila* HL3.1 saline solution (NaCl, 70 mM; KCl, 5 mM; $MgCl_2$, 4 mM; $NaHCO_3$, 10 mM; trehalose, 5 mM; sucrose, 115 mM; HEPES-NaOH, 5 mM; pH 7.2) (Stewart et al., 1994) with an extracellular Ca^{2+} concentration of 0.2 mM as previously described (Montana and Littleton, 2004). Recordings were performed in muscle 6 in larval segments A3-A5. All stimulus signals were generated through the pCLAMP v8.0 program (Axon Instruments) and data was analyzed using pCLAMP v9.0. EJP analysis was performed by averaging ten evoked events from each muscle as one measurement and then averaging all measurements for each genotype. Paired-pulse facilitation was analyzed by averaging ten first and second paired-pulse events from a single muscle, then dividing the average second EJP by the average first EJP for each timepoint. mEJP analysis was performed using event detection analysis in pCLAMP v9.0.

Results

Expression of a 548 aa N-terminal fragment of human huntingtin in a *Drosophila* model of Huntington's disease

To characterize neuronal defects that result from an expanded polyglutamine tract within the Htt gene, we generated transgenic *Drosophila* expressing N-terminal fragments of human Htt containing 0 (Htt-Q0) or 128 (Htt-Q128) glutamines. The Htt constructs were engineered to include the first 548 amino acids of the human Htt protein, which include and extend well beyond the 81 amino acid product encoded by the first exon of the gene. While several models of HD have focused on expression of the polyglutamine-containing first exon of Htt alone (Jackson et al., 1998; Mangiarini et al., 1996; Scherzinger et al., 1997; Steffan et al., 2001), the 548 amino acid fragment is truncated close to the site of cleavage by caspase-3, thought to be a crucial step in the generation of aggregate-forming Htt fragments (Kim et al., 2001; Wellington et al., 2002). This region also encompasses the highest stretch of homology between the *Drosophila* and human Htt proteins (Fig. 1A) (Li et al., 1999b), including protein interaction domains formed by HEAT repeats (Takano and Gusella, 2002a), providing a more accurate representation of the *in vivo* Htt protein context for the polyglutamine tract in our model. Two transgenic approaches were used to provide both temporal and spatial control of Htt gene expression. First, the Htt-Q0 and Htt-Q128 fragments were expressed using the UAS/GAL4 binary expression system, allowing for transgene expression in the presence of the yeast GAL4 transcriptional activator in a tissue or cell type-specific manner. In addition, Htt-Q0 and Htt-Q128 constructs were placed under control of the heat shock expression system, which enables temporal control of ubiquitous transgene expression following heat shock at 37^{o}C. We generated multiple independent transformant lines for each construct. To confirm transgene expression in these strains, Htt protein expression was compared between pHS-Htt *Drosophila* maintained at room temperature and following exposure to a heat shock paradigm (Fig. 1B). Western blotting with anti-human Htt antibodies detected no Htt protein in control Canton S or in pHS-Htt lines maintained at room temperature. In contrast, Htt-Q0 and Htt-Q128 lines showed abundant Htt expression following heat shock. As expected, the product detected in Htt-Q0 strains lacking the expanded polyglutamine tract is smaller than that in Htt-Q128 strains. Similar results were obtained from pUAST-Htt strains crossed to a neuronal GAL4 driver (Fig. 1C).

Figure 1

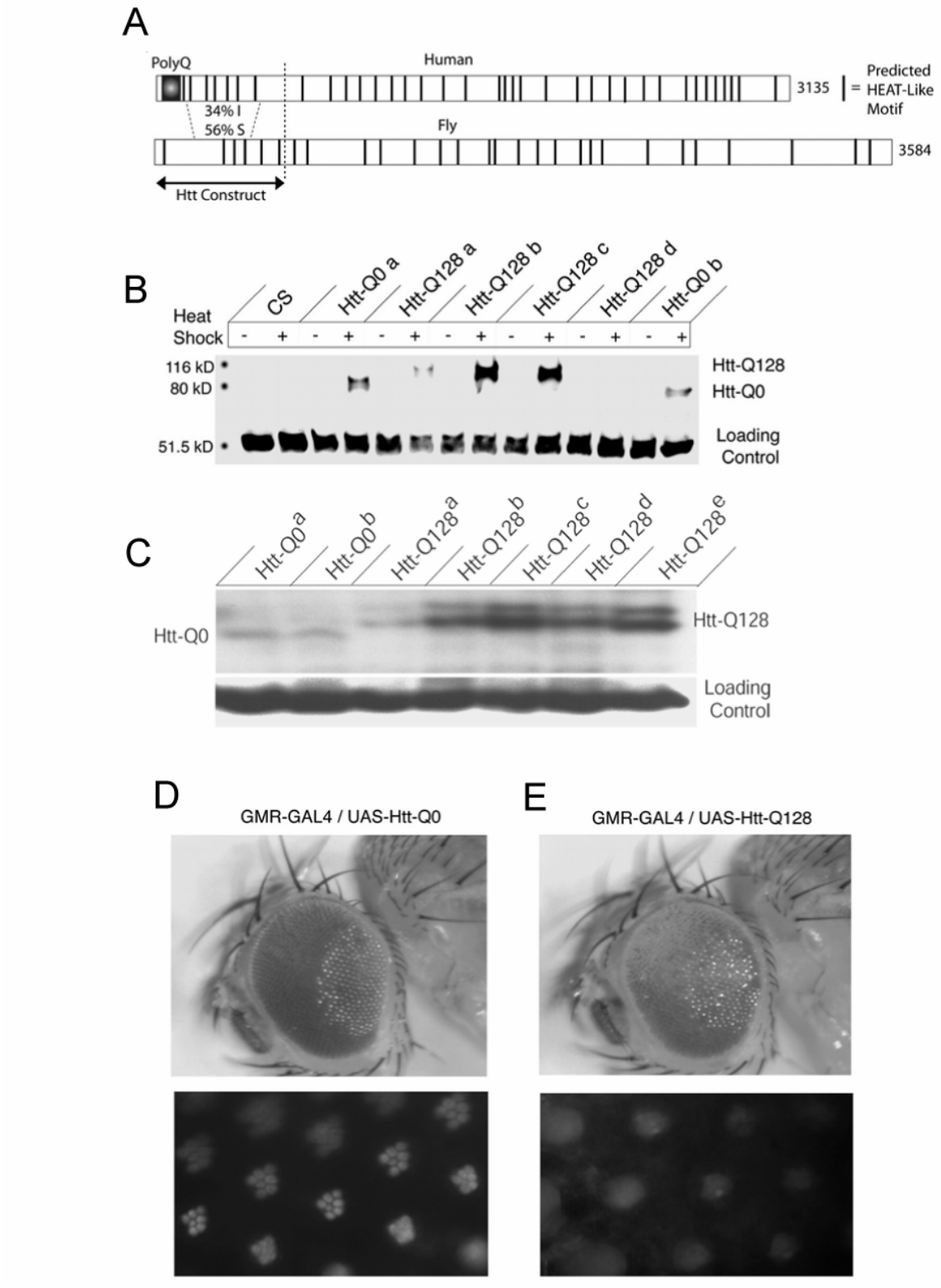
A
PolyQ
Human
3135
= Predicted HEAT-Like Motif
34% I
56% S
Fly
3584
Htt Construct
B
CS
Htt-Q0 a
Htt-Q128 a
Htt-Q128 b
Htt-Q128 c
Htt-Q128 d
Htt-Q0 b
Heat Shock
116 kD
80 kD
51.5 kD
Htt-Q128
Htt-Q0
Loading Control
C
Htt-Q0a
Htt-Q0b
Htt-Q128a
Htt-Q128b
Htt-Q128c
Htt-Q128d
Htt-Q128e
Htt-Q0
Htt-Q128
Loading Control
D
GMR-GAL4 / UAS-Htt-Q0
E
GMR-GAL4 / UAS-Htt-Q128

FIGURE 1. **Generation of a *Drosophila* transgenic model of Huntington's disease.** (A) Domain structure of the human and *Drosophila* Htt homologs with predicted HEAT-like motifs indicated. The 548 amino acid N-terminus of human Htt used for transgenic construction is indicated. (B) Heat shock induction of Htt in Q0 and Q128 pHS strains. Western blotting was performed with an antibody generated to the N-terminus of human Htt that recognizes both the Q0 and Q128 variants. (C) Expression of UAS-Htt-Q0 and UAS-Htt-Q128 with the pan-neuronal elav-GAL4 driver. Western blotting was performed with an antibody against the N-terminus of human Htt. (D, E). External morphology and pseudopupil analysis of transgenic *Drosophila* expressing either Htt-Q0 (C) or Htt-Q128 (D) driven by the eye-specific GMR-GAL4 driver. Flies were aged for 2-4 days at 25ºC prior to analysis. Htt-Q128 causes a rough-eye phenotype with loss of pigmentation, abnormal bristle pattern and photoreceptor degeneration.

To determine the functional consequences of Htt-Q128 expression on neuronal activity and morphology, we first examined effects in the visual system. Previous *Drosophila* models of polyglutamine diseases have demonstrated that eye-specific expression of expanded polyQ proteins leads to a rough-eye phenotype and photoreceptor degeneration (Fernandez-Funez et al., 2000; Jackson et al., 1998; Kazemi-Esfarjani and Benzer, 2000; Marsh et al., 2000). To determine if the 548 amino acid Htt transgene caused similar effects, Htt-Q0 and Htt-Q128 were expressed using the eye-specific GMR-GAL4 driver and the resulting eye phenotypes were observed by external morphology and by the corneal pseudopupil method, an optical technique that allows assessment of photoreceptor patterns in the ommatidia. While expression of Htt-Q0 did not perturb external eye appearance or ommatidial morphology (Fig. 1D), expression of Htt-Q128 caused a rough-eye phenotype with corresponding photoreceptor degeneration (Fig. 1E). These results suggest that polyglutamine expansion in the context of a larger Htt fragment results in neurodegeneration, as observed in other polyglutamine disease models.

Mutant Htt induces electrophysiological defects in the eye and the giant fiber flight circuit

To characterize the physiological effects of mutant Htt expression, we recorded electroretinograms (ERGs) from transgenic animals (Fig. 2A). ERGs are extracellular recordings of photoreceptor depolarization and on/off transients thought to represent synaptic transmission in response to light. A normal electrical response to light was seen in *Drosophila* expressing the GMR-GAL4 driver alone, Htt-Q0 with GMR-GAL4, or Htt-Q128 without the GMR-GAL4 driver. In contrast, *Drosophila* expressing Htt-Q128 with the GMR-GAL4 driver showed reduced photoreceptor depolarization and complete abolishment of on/off transients in response to light. Similar abnormal ERGs were observed in heat-shocked pHS-Htt-Q128 lines following a developmental heat shock paradigm, but not with control pHS-Htt-Q0 strains (Fig. 2A). The defective ERGs confirm that Htt-Q128 disrupts photoreceptor viability and function, and suggest abnormal synaptic transmission in the visual system.

To further analyze defects in neuronal activity, electrophysiological analysis was performed in the giant fiber flight circuit, a pathway important in escape responses and flight initiation. The pathway can be activated by stimulation of the brain and extracellular recordings can be made from the dorsal longitudinal flight muscles (DLMs).

Wild type *Drosophila* display little to no spontaneous activity when the temperature is raised to 38ºC. In contrast, previously isolated neurodegenerative mutants (Palladino et al., 2002) have demonstrated robust spontaneous seizure activity in the DLM flight muscles at 38ºC. Similar seizure activity was recorded in Htt-Q128 flies at 38ºC following a developmental heat shock paradigm (Fig. 2B). In contrast, no seizure activity was recorded in Htt-Q0 flies at 38^{o}C (Fig. 2B). Together, these results suggest that Htt-Q128 expression results in neurodegeneration, accompanied by widespread defects in membrane excitability and brain activity.

Mutant Htt causes behavioral phenotypes in *Drosophila* at larval and adult stages

To establish whether neuronal Htt-Q128 transgene expression causes defects at earlier stages of *Drosophila* development, we performed quantitative locomotion assays to examine the function of the motor central pattern generator in 3rd instar larvae. *Drosophila* larvae move by performing rhythmic waves of body wall muscle contraction and elongation, resulting in peristaltic propagation. Locomotor behavior depends on central pattern generators that organize and produce specific patterns of activation and inhibition of motor neurons. To determine if Htt-Q128 altered larval locomotion, we assayed larval crawling speed in transgenic animals. When Htt transgenes were expressed with the pan-neuronal elav-GAL4 driver C155, Htt-Q128 larvae showed a significant decrease in distance traveled, corresponding to a >25% reduction in locomotor speed from 1.23 mm/sec in control Htt-Q0 animals to 0.92 mm/sec in Htt-Q128 larvae (Student's t-test, $p<0.001$, Fig. 2C). These behavioral abnormalities suggest that Htt-Q128 has significant effects on motor output, consistent with abnormal neuronal function secondary to Htt-Q128 expression.

Adult transgenic flies also display abnormal motor behavior caused by pan-neuronal expression of the Htt-Q128 protein. While expression of Htt-Q128 with the C155 neuronal GAL4 driver causes pharate adult lethality with no viable adult escapers, Htt-Q128 driven by a weaker 2nd chromosome elav-GAL4 driver results in fully viable adults. However, several days after eclosion, flies expressing Htt-Q128, but not Htt-Q0, begin to display uncoordinated movement and abnormal grooming behaviors. The behavioral defects worsen with age, eventually resulting in premature death. To quantify the reduction in viability, lifespan curves were generated for control adults, Htt-Q128 adults without elav-GAL4, or adults expressing Htt-Q0 or Htt-Q128 with elav-GAL4 (Fig. 2D). Compared to controls, Htt-Q128/elav-GAL4 animals showed a dramatic reduction

Figure 2

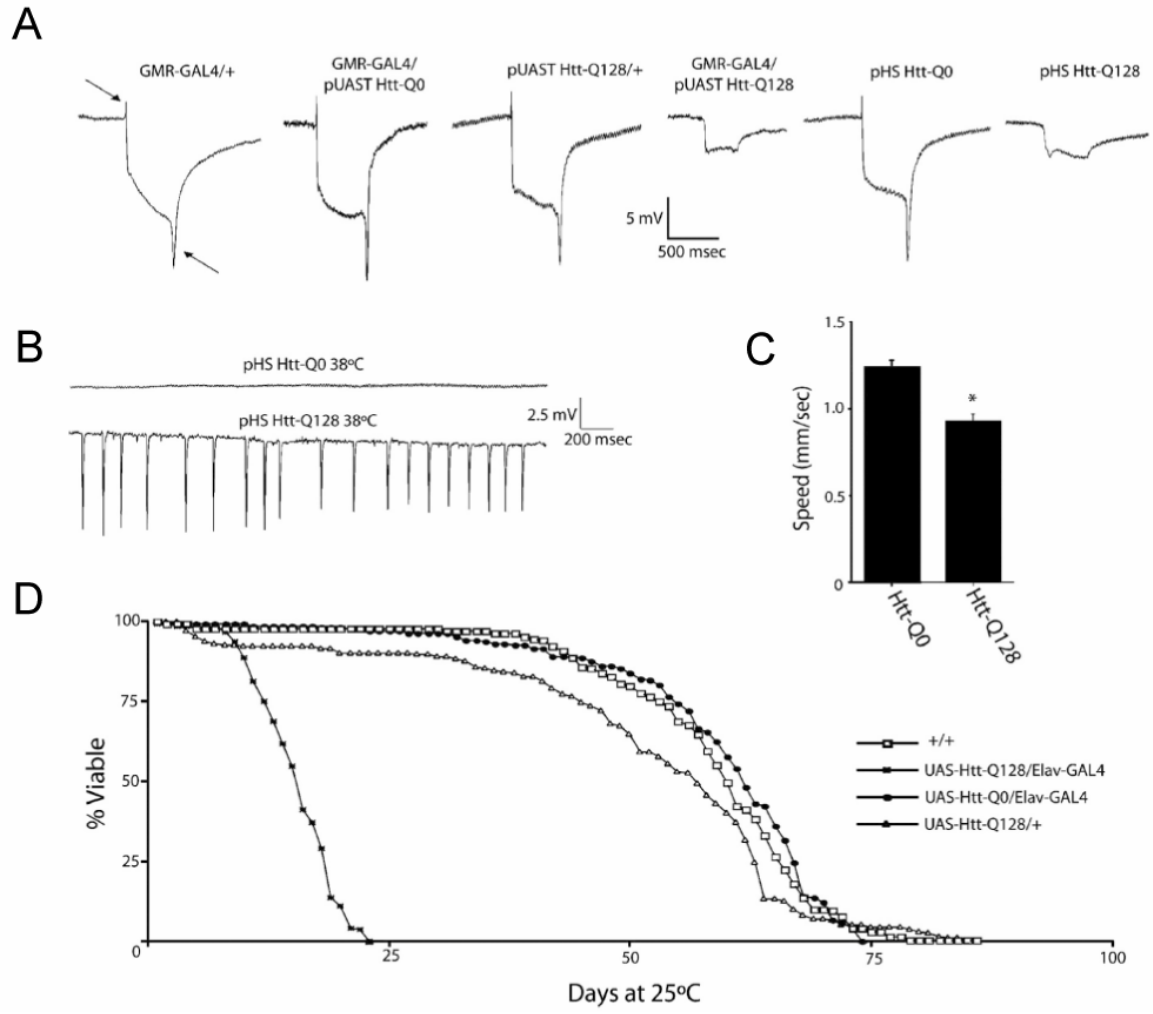
A
GMR-GAL4/+
GMR-GAL4/ pUAST Htt-Q0
pUAST Htt-Q128/+
GMR-GAL4/ pUAST Htt-Q128
pHS Htt-Q0
pHS Htt-Q128
5 mV
500 msec
B
pHS Htt-Q0 38°C
pHS Htt-Q128 38°C
2.5 mV
200 msec
C
Speed (mm/sec)
1.5
1.0
0.5
0
*
Htt-Q0
Htt-Q128
D
% Viable
100
75
50
25
0
25
50
75
100
Days at 25°C
+/+
UAS-Htt-Q128/Elav-GAL4
UAS-Htt-Q0/Elav-GAL4
UAS-Htt-Q128/+

FIGURE 2. **Physiological and behavioral analysis of Htt-Q128-expressing transgenic *Drosophila*.** (A) Both UAS-Htt-Q128/GMR-GAL4 and pHS-Htt-Q128 exhibit reduced photoreceptor depolarization and loss of on/off transients (arrows), suggesting abolished synaptic transmission, in electroretinogram recordings. (B) Extracellular recordings from the DLM flight muscles of Htt-Q0 and Htt-Q128 *Drosophila* are shown. A developmental heat-shock paradigm that induces Htt-Q128 expression results in abnormal seizure activity in the flight circuit at 38ºC. For panels A and B, *Drosophila* were aged for 2-4 days at 25ºC. (C) Quantitative analysis of wandering 3rd instar larval crawling behavior indicates that pan-neuronal expression of Htt-Q128 disrupts motor pattern generation, resulting in a significant decrease in locomotor speed. 30 larvae were analyzed for each genotype. (D) Viability analysis of *Drosophila* maintained at 25ºC indicates that pan-neuronal expression of Htt-Q128 with a second chromosome elav-GAL4 driver results in decreased lifespan compared to control strains. Expression of Htt-Q128 with the stronger X-chromosome elav-GAL4 driver C155 leads to 100% pharate adult lethality.

in lifespan, with a decrease in T_{50} (age at which 50% of the culture has died) of 70%, indicating a highly significant effect of Htt-Q128 expression on lifespan in *Drosophila* (Fig. 2D).

Altered gene expression in *Drosophila* expressing mutant Htt

Microarray analysis has been used to identify mutant Htt-mediated changes in expression profile in several models of HD (Chan et al., 2002; Hodges et al., 2006; Luthi-Carter et al., 2002a). To characterize gene expression changes in our *Drosophila* HD model, expression profiling was performed with Affymetrix® high-density oligonucleotide GeneChip® arrays containing representative sequences for most of the 14,000 genes encoded by the *Drosophila* genome. RNA was isolated from *Drosophila* head extracts from control strains or strains expressing Htt-Q0 or Htt-Q128 with the pan-neuronal elav-GAL4 driver. Two distinct stages were chosen for analysis: RNA was isolated at 3-4 days post-eclosion, before the onset of a noticeable behavioral phenotype, and at 9-11 days post-eclosion, when a severe behavioral phenotype was observed but just before a dramatic decrease in viability. A total of 12 independent microarrays were analyzed (two each of RNA from 3-4 day-old control, Htt-Q0-, and Htt-Q128-expressing flies, and two each of RNA from 9-11 day-old control, Htt-Q0-, and Htt-Q128-expressing flies) for a total of eight pairwise comparisons between expression profiles from Htt-Q128-expressing flies and Htt-Q0-expressing flies or control flies for both the 3-4 day set and the 9-11 day set. Gene changes were called based upon the stringency criteria that differential regulation must be reported by Affymetrix® software for at least 75%, or twelve, of the sixteen pairwise comparisons for microarrays from both the 3-4 day set and the 9-11 day set. In addition, the fold change in gene expression was required to be greater than or equal to 1.5 with a statistical significance of $p<0.05$. Under these criteria, 34 genes were found to be upregulated and 29 were downregulated in flies expressing mutant Htt as compared to control flies and flies expressing normal Htt. In contrast, only 7 genes were observed to be differentially regulated between normal Htt-expressing lines and control lines, indicating that expression of mutant Htt induced a significant change in gene transcription in *Drosophila*.

Upregulated or downregulated genes were subjected to BLAST analysis in order to identify sequence and domain similarities to known proteins, and were categorized according to known or putative function (Tables 1 and 2). While behavioral dysfunction is mild in Htt-Q128-expressing animals at 3-4 days post-eclosion, a robust

downregulation of eye-specific transcripts was observed, indicating significant degeneration of photoreceptors before the onset of behavioral defects. In addition, a strong decrease in expression was seen for two metabolic genes, Sorbitol dehyrogenase 1 and CG14576 (a UDP glucose 4-epimerase), at 3-4 days, and became more pronounced at 9-11 days, suggesting potential progressive metabolic defects in mutant Htt-expressing animals. Surprisingly, few other categories of genes were shown to be downregulated, perhaps due to the stringency of the statistical criteria. Upregulated categories included a number of immune response genes, which may indicate a strong immune response to the transgenic mutant Htt protein, and a small number of metabolic genes. A robust increase in expression was seen for chaperone genes, especially CG6489, a Hsp70 homologue.

In summary, microarray analysis of mutant Htt-expressing flies reveals a neurodegenerative response early in disease progression before the appearance of obvious behavioral defects. Upregulation of immune genes and chaperones provides evidence for a cellular stress response mounted specifically against the mutant protein, while changes in expression patterns for genes involved in metabolism suggest potential metabolic dysfunction. Future studies may relax stringency criteria in order to identify genes that undergo more subtle changes in expression level, or may compare expression levels between control and experimental lines for candidate genes believed to undergo transcriptional dysregulation in HD.

The 548 aa N-terminal fragment of mutant Htt forms cytoplasmic aggregates in neuronal and non-neuronal cells in *Drosophila*

A hallmark of HD is the formation of Htt-immunopositive intracellular aggregates in neurons. To determine whether intracellular aggregates are formed in transgenic Htt *Drosophila*, both Htt-Q128 and Htt-Q0 strains were crossed to flies containing C155 elav-GAL4 to direct expression of Htt within the nervous system. Htt-immunopositive staining was visualized in both central and peripheral neurons of dissected 3rd instar larvae. While Htt staining remained diffuse throughout the cytoplasm of neurons in *Drosophila* expressing Htt-Q0 (Fig. 3A), distinct accumulations, or aggregates, of Htt were observed in the cytoplasm and processes of neurons in lines expressing Htt-Q128 (Fig. 3B). It is unclear whether the accumulations seen in our *Drosophila* model are structurally identical to the aggregates found in HD; however, the term “aggregate” is used here to describe microscopically visible accumulations of Htt protein. Contrary to

Table 1. Upregulated genes in mutant Htt-expressing flies at 3-4 days or 9-11 days

Gene	*Description*	*Fold Change (3-4 Days)*	*Fold Change (9-11 Days)*
Immune Response Genes			
CG6639	immune response-linked endopeptidase	5.69	4.98
Cecropin A2	antibacterial response protein	5.42	1.22
TotM	humoral defense mechanism protein	4.62	2.25
Drosomycin	antifungal response protein	4.35	2.79
CG10794	defense/immunity protein	2.99	1.17
Metchnikowin	antibacterial response protein	2.91	1.77
Attacin-A	antibacterial response protein	2.78	1.51
CG18372 (Attacin-B)	antibacterial response protein	2.72	1.89
Drosocin	antibacterial response protein	2.7	1.51
CG4740	attacin A-like defense/immunity protein	2.43	1.29
Immune induced protein 2	defense/immunity protein	2.09	2.15
Diptericin	antibacterial response protein	1.89	-1.13
CG18108 (Immune induced protein 1)	defense/immunity protein	1.84	1.55
Metabolic Genes			
GH05741	carboxylesterase	3.01	1.99
Nmdmc	NAD-dependent methylenetetrahydrofolate dehydrogenase	2.08	-1.08
CG14935	maltase 2-like	1.72	1.97
Chaperones			
CG6489	Hsp70 homologue	4.27	6.93
lethal (2) essential for life	chaperone	1.87	2.04
CG7015	has cold-shock DNA-binding domain	1.85	1.28
Other			
EG:39E1.3	novel	3.19	3.7
CG11797 (Obp56A)	pheromone/odorant binding protein	2.65	1.04
Ca-alpha1D	voltage-gated calcium channel, type D, alpha1-subunit	2.51	1.43
CG12846 (Tsp42ED)	putative integral membrane protein	2.5	1.73
CG15066	novel	2.39	1.58
CG7695	structural protein	2	1.43
CG9080	novel	1.98	1.07
polyA-binding protein	poly(A) binding protein	1.91	-1.15
CG10126	calcium binding protein-like	1.88	1.79
CG5791	novel	1.77	1.52
CG18279	novel	1.75	1.61
CG14619	ubiquitin-specific protease	1.62	1.22
Smad on X	transcription factor	1.6	1.23
CG15825	intracellular protein transport	1.54	1.91
CG17836	protein dimerization activity	1.54	1.24

Table 2. Downregulated genes in mutant Htt-expressing flies at 3-4 days or 9-11 days

Gene	*Description*	*Fold Change (3-4 Days)*	*Fold Change (9-11 Days)*
Phototransduction Genes			
inaD	light-activated voltage-gated calcium channel, regulatory-subunit	-2.97	-2.72
Rhodopsin 3	light-sensitive visual pigment	-2.49	-2.33
bride of sevenless	sevenless receptor ligand	-2.38	-2.87
Calphotin	calcium-binding protein	-2.25	-2.24
inaF	calcium channel regulator activity	-2.19	-1.51
ninaA	cyclophilin	-2.12	-2.23
transient receptor potential	light-activated voltage-gated calcium channel, alpha-subunit	-1.93	-1.78
Rhodopsin 6	light-sensitive visual pigment	-1.88	-1.74
Rhodopsin 4	light-sensitive visual pigment	-1.88	-1.71
CG18511 (Gγ30A)	heterotrimeric G-protein GTPase activity	-1.87	-1.68
Arrestin A	arrestin	-1.86	-1.79
chaoptic	cell adhesion protein	-1.81	-1.93
G protein beta-subunit 76C	heterotrimeric G-protein GTPase activity	-1.8	-1.86
Rhodopsin 5	light-sensitive visual pigment	-1.73	-2.35
ninaC	calmodulin binding serine/threonine kinase	-1.68	-2.07
trp-like	calmodulin binding	-1.64	-1.68
Metabolic Genes			
CG14576	UDP glucose 4-epimerase	-2.81	-3.29
Sorbitol dehydrogenase 1	L-iditol 2-dehydrogenase	-1.94	-3.05
Other			
CG11706	transposable element "springer"	-3.54	-3.37
CG12120	novel	-3.15	-3.13
CG18674 (Sox100B)	transcription factor	-3.1	-3.89
CG6656	acid phosphatase-like	-2.54	-3.97
msta	novel	-2.28	-2.14
CG8889	serine/threonine specific protein phosphatase	-2.04	-1.9
CG2080	novel	-1.84	-2.69
CG1497	endopeptidase	-1.78	-3.17
CG9119	novel	-1.72	-4.15
CG18598	novel	-1.7	-3.08
Slowpoke binding protein	signal transduction	-1.58	-2.06

Figure 3

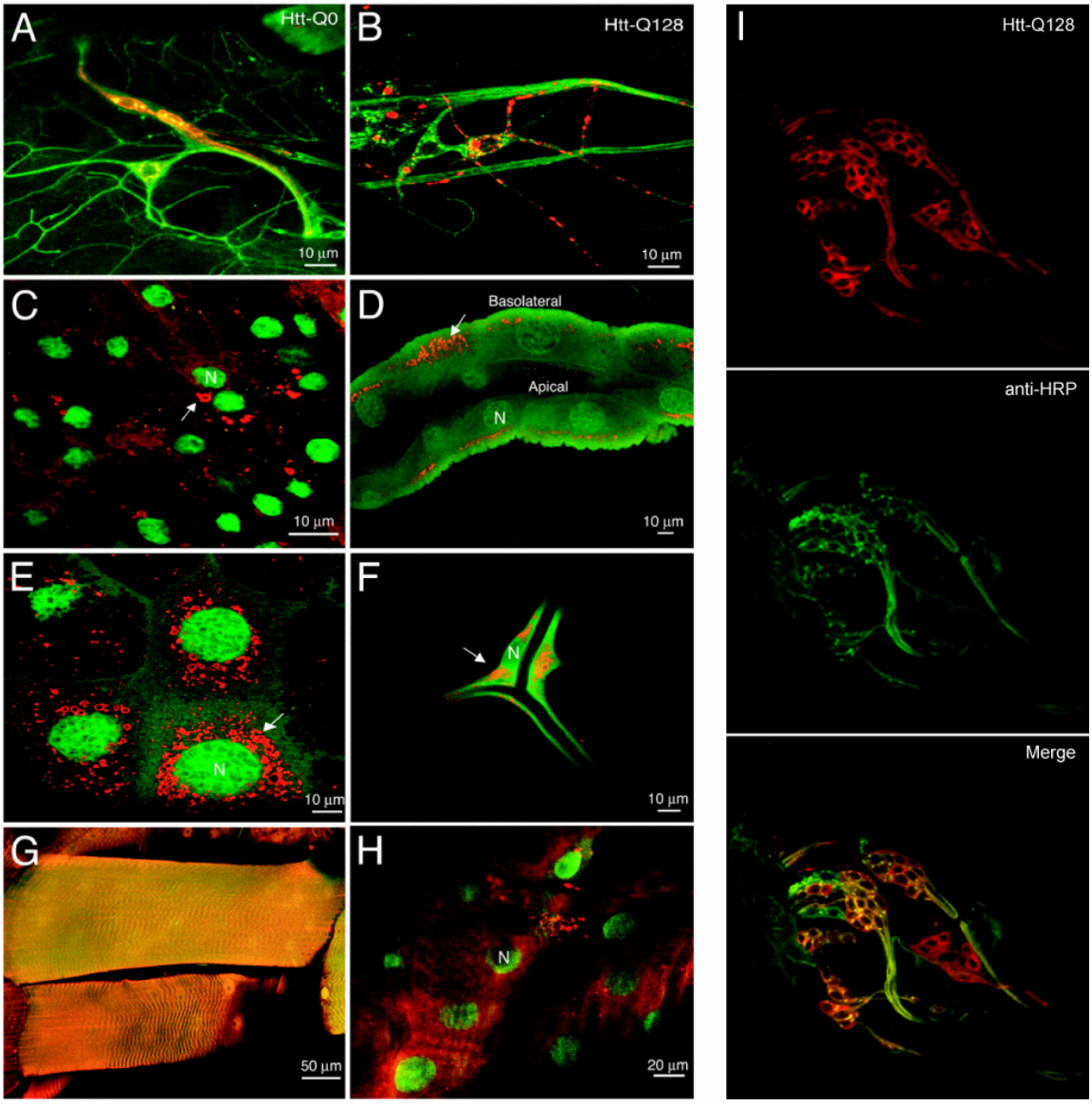
A
Htt-Q0
10 µm
B
Htt-Q128
10 µm
I
Htt-Q128
C
N
10 µm
D
Basolateral
Apical
N
10 µm
anti-HRP
E
N
10 µm
F
N
10 µm
Merge
G
50 µm
H
N
20 µm

FIGURE 3. **Cytoplasmic aggregation of Htt-Q128 in neuronal and non-neuronal tissues.** (A) Immunocytochemical detection of Htt (red) and HRP (green) in multi-dendritic neurons of Htt-Q0-expressing 3rd instar larvae. Htt-Q0 is found diffusely throughout the cytoplasm. (B) Immunocytochemical detection of Htt (red) and HRP (green) in multi-dendritic neurons of Htt-Q128-expressing 3rd instar larvae. Unlike Htt-Q0, Htt-Q128 is found in cytoplasmic aggregates throughout the cell body and neurites. (C-H) Expression of UAS-Htt-Q128 (red) and UAS-GFP-nls (green) by tubP-GAL4 in the CNS (C), gut (D), salivary gland (E), trachea (F), muscle (G) and epidermis (H) of 3rd instar larvae. In all cases, cytoplasmic aggregates are observed. However, muscle and epidermis form far fewer aggregates than other tissues. In polarized cells like the gut, basolateral transport of Htt aggregates is observed, with a complete absence of aggregates in the apical domain. The nucleus is indicated by N, and Htt aggregates are highlighted with arrows. (I) Immunocytochemical detection of Htt-Q128 (red) and HRP (green) in *Drosophila* embryos. High levels of Htt are visible in the embryonic CNS and PNS, but few aggregates are observed in comparison to the 3rd instar larval stage (B), indicating that aggregate-formation is time-dependent.

what has been observed in several exon 1 HD models, we found no evidence for nuclear aggregate localization. To verify that Htt aggregation is based on the length of the polyglutamine tract and not on protein concentration, Htt levels were quantified for several Htt-Q0 and Htt-Q128 transgenic lines crossed to elav-GAL4. Levels of Htt protein were generally higher in Htt-Q128 lines than in Htt-Q0 lines, likely due to sequestration of the mutant protein into stable aggregates. However, low-expressing Htt-Q128 lines that produced transgenic protein at a level comparable to that in Htt-Q0 strains still exhibited aggregates, while Htt-Q0 lines did not (data not shown), indicating that polyglutamine tract expansion and not protein concentration alone is necessary for the formation of aggregates. Aggregate formation was also time-dependent. Although Htt levels were visibly high in the central and peripheral nervous system of Htt-Q128/elav-GAL4 embryos, the protein remained largely diffuse in the cytoplasm with rare occurrence of aggregates (Fig. 3I). By the 3rd instar larval stage, essentially all Htt was observed in aggregates, with relatively little non-aggregate staining (Fig. 3B). We conclude that Htt-Q128 forms cytoplasmic neuronal aggregates in a time-dependent manner.

Although the causative proteins for many of the polyglutamine repeat diseases are expressed ubiquitously, aggregate formation and cell death occur in subsets of neurons that differ between the diseases. A central question in polyglutamine disease research is why certain cell types are affected in each disorder while others are spared, and why neurons are affected in every case. To examine the effect of cellular context on aggregate formation, the Htt-Q128 protein was expressed in different tissues using the tubP-GAL4 driver. We generated transgenic lines containing both the UAS-Htt-Q128 construct and UAS-GFP fused to a nuclear localization signal, allowing for co-visualization of Htt-immunopositive aggregates and GFP-stained cell nuclei in expressing cells. Immunocytochemical analysis demonstrated the formation of cytoplasmic aggregates in both neuronal and non-neuronal tissues, including CNS neurons (Fig. 3C), gut (Fig. 3D), salivary glands (Fig. 3E), and trachea (Fig. 3F). Interestingly, Htt aggregates were differentially distributed in polarized cells such as the gut, with transport of Htt aggregates to the basolateral domain and exclusion from the apical surface (Fig. 3D). Similar aggregate transport was found in neurons (see below), indicating that Htt undergoes a cytoskeletal association that allows for directed transport. This suggests that the mutant protein may partially retain the ability of normal huntingtin to interact with microtubules and undergo transport (Block-Galarza et al., 1997). Also

noteworthy is the fact that the Htt-Q128 protein was found in a more diffuse, non-aggregated state in certain cell types, including muscle and epidermis (Fig. 3G & H), suggesting that some tissues may be more resistant to polyglutamine-mediated protein aggregation. In all cell types where aggregate formation occurred, only cytoplasmic aggregates (as opposed to nuclear aggregates) were observed, suggesting unique differences between the larger Htt fragments used in our study compared to smaller Htt fragments that form nuclear aggregates in other HD models.

The protein context of the polyglutamine repeat controls aggregate formation and localization

Although the polyglutamine repeat diseases all result from CAG repeat expansion in the causative gene, the pattern of neurodegeneration and behavioral dysfunction is distinct for each disorder, indicating that the protein context for expanded polyglutamine tracts is critical to disease manifestation. The protein context may determine aggregation potential, subcellular localization, toxicity, and potential binding partners. To examine the importance of protein context in the subcellular localization of polyglutamine-containing proteins, immunocytochemical analysis was performed on larvae expressing either an expanded polyglutamine tract alone (Q127) (Kazemi-Esfarjani and Benzer, 2000), the mutant polyglutamine protein responsible for Machado Joseph disease (SCA3-Q78) (Warrick et al., 1998) or an expanded polyglutamine tract (Q108) previously engineered into the non-pathogenic *dishevelled* gene (Marsh et al., 2000). In contrast to the cytoplasmic localization of Htt aggregates (Fig. 4A), both Q127 and SCA3-Q78 aggregates localized exclusively to the nucleus (Fig. 4B & C). Very few Dishevelled-immunopositive aggregates were observed (Fig. 4D), and the protein was present diffusely in the cytoplasm. These results demonstrate that the protein context in which the polyglutamine tract is found exquisitely controls both aggregate localization and aggregate formation.

To test whether Htt-Q128 can interact and co-aggregate with other polyglutamine repeat proteins, we generated double transgenic Htt-Q128;Q127 and Htt-Q128;SCA3-Q78 strains expressing both transgenes. When the Htt-Q128 and Q127 proteins were co-expressed, both central and peripheral neurons showed localization of Htt-Q128 aggregates to the cytoplasm while Q127 aggregates were restricted to the nucleus (Fig. 4E & H). Likewise, in strains expressing Htt-Q128 and SCA3-Q78, aggregates were segregated independently in the cytoplasm and nucleus (respectively) of both neuronal

Figure 4

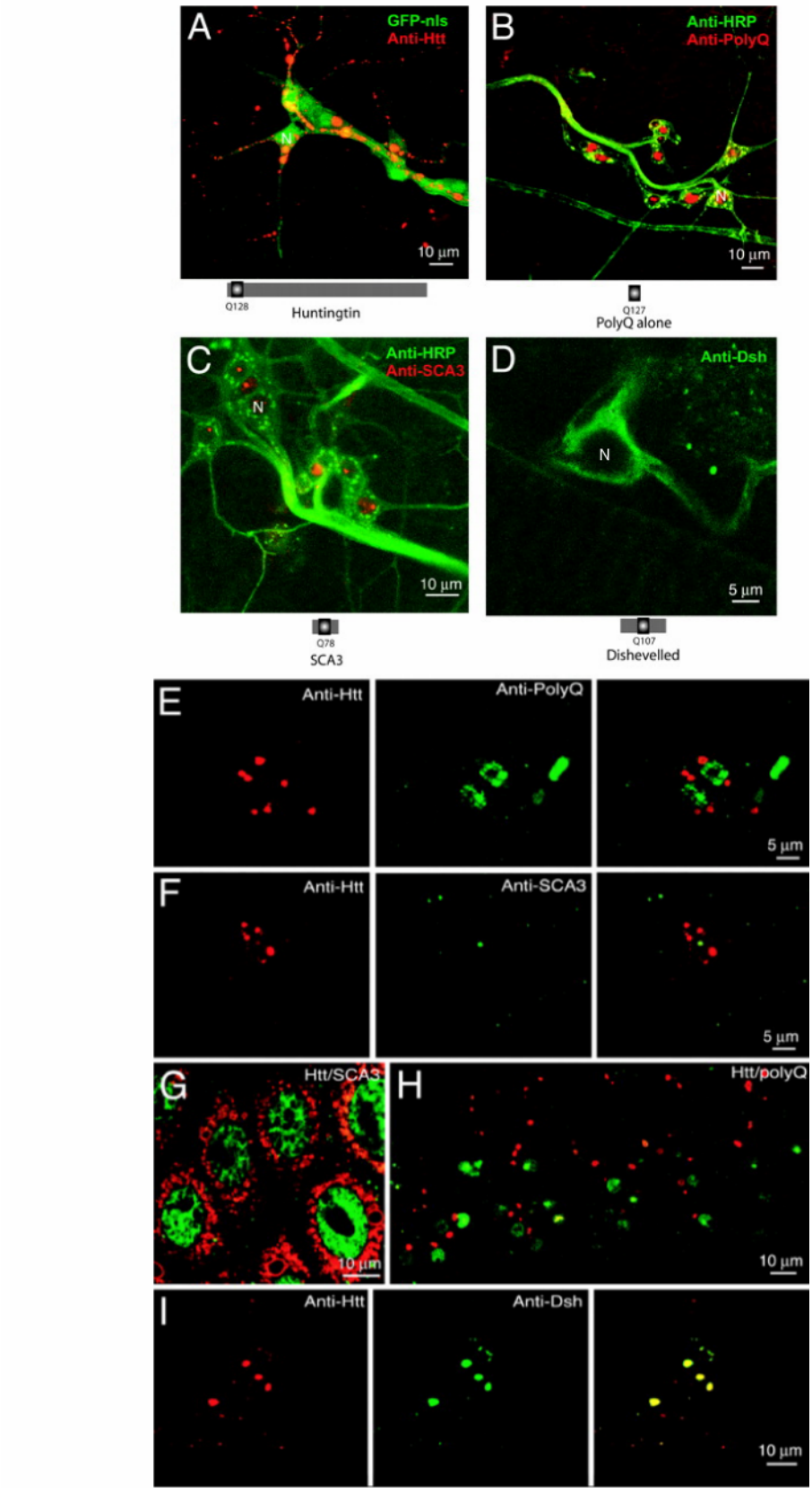
A
GFP-nls
Anti-Htt
N
10 µm
Q128
Huntingtin
B
Anti-HRP
Anti-PolyQ
N
10 µm
Q127
PolyQ alone
C
Anti-HRP
Anti-SCA3
N
10 µm
Q78
SCA3
D
Anti-Dsh
N
5 µm
Q107
Dishevelled
E
Anti-Htt
Anti-PolyQ
5 µm
F
Anti-Htt
Anti-SCA3
5 µm
G
Htt/SCA3
10 µm
H
Htt/polyQ
10 µm
I
Anti-Htt
Anti-Dsh
10 µm

FIGURE 4. **Protein context is important for polyglutamine-mediated aggregation and aggregate localization.** (A-D) Immunolocalization of aggregates in 3rd instar larvae expressing Htt-Q128 (A), Q127 (B), SCA3-Q78 (C) and Dsh-Q108 (D) in multi-dendritic neurons with the C155 elav-GAL4 driver. Htt-Q128 aggregates are exclusive to the cytoplasm, while Q127 and SCA3-Q78 aggregates are found only in the nucleus. Dsh-Q108 is mostly diffuse in the cytoplasm, with few aggregates visible. The nucleus is represented by N. (E, F) Double transgenic 3rd instar larvae expressing Htt-Q128 and Q127 (E) or Htt-Q128 and SCA3-Q78 (F) with the elav-GAL4 driver were dissected and immunostained for Htt (red) or SCA3/Q127 (green). Multi-dendritic sensory neurons were identified as in panels A-D, and assayed for aggregate colocalization. No colocalization of aggregates is observed, indicating distinct nuclear and cytoplasmic aggregation pathways. Similar results are observed in the CNS (H) and in non-neuronal tissues such as the salivary glands (G). (I) In contrast, in double transgenic larvae expressing Htt-Q128 and Dsh-Q108 with elav-GAL4, Htt-Q128 (red) is able to completely sequester Dsh-Q108 (green) into aggregates as indicated by yellow colocalization in the merged panel.

(Fig 4F) and non-neuronal (Fig. 4G) cells. These results suggest that the trafficking and aggregation of nuclear and cytoplasmic aggregates are independently regulated.

To determine whether Htt-Q128 might interact with cytoplasmic proteins containing an expanded polyglutamine tract, double transgenic strains were made containing Htt-Q128 and Dishevelled-Q108 (Dsh-Q108). As demonstrated above (Fig. 4D), Dsh-Q108 formed few aggregates when expressed alone; however, when co-expressed with Htt-Q128, the subcellular distribution of Dsh-Q108 shifted from a diffuse cytoplasmic pattern to a complete sequestration into aggregates that colocalized with Htt-Q128 (Fig. 4I). Similar sequestration of endogenous cytoplasmic proteins by Htt aggregates might be predicted to play an important role in disease pathology.

Mutant Htt aggregates block axonal transport

As described above (Fig. 3D), Htt-Q128 forms cytoplasmic aggregates that are associated with cytoskeletal transport systems. It is important to define if and how transport of aggregates contributes to disease pathology. When Htt-Q128 was expressed with the eye-specific driver GMR-GAL4, Htt aggregates were abundantly transported along axons entering the CNS of the developing visual system and accumulated in pathfinding photoreceptor growth cones (Fig. 5A & B). Similarly, when Htt-Q128 was expressed with the C155 driver, aggregates were transported in larval motor axons and accumulated at presynaptic neuromuscular junction terminals (Fig. 5D & F). No aggregates were observed in axons from animals expressing Htt-Q0 (Fig. 5C & E). Axonal transport of aggregates was not observed in transgenic animals that produce exclusively nuclear aggregates (Q127 and SCA3-Q78) (Fig. 5G), suggesting that axonal and synaptic defects that may occur downstream of cytoplasmic aggregate formation are likely to be specific to HD. Additionally, no Dsh-Q108 aggregates were observed in axon bundles (Fig. 5H). However, when Dsh-Q108 was co-expressed with Htt-Q128, Dsh-Q108 protein trapped by Htt-Q128 was also transported along axons (Fig. 5I). Similar recruitment of endogenous cytoplasmic polyQ proteins may sequester them away from their natural cellular locations and contribute to neuronal dysfunction.

In observing axonal aggregates in Htt-Q128 expressing animals, it was noted that the diameter of Htt aggregate accumulations often appeared to exceed that of normal larval axons. This suggested that large Htt aggregates might physically block axonal transport, as would be manifested by axonal swellings at the sites of such blockages. We tested this hypothesis in Htt-Q128-expressing animals by observing the

localization of synaptotagmin I, a synaptic vesicle protein exclusively localized to synapses in *Drosophila*. Normal transport of the synaptotagmin protein along axons is below the threshold for immunocytochemical detection (Littleton et al., 1993). This standard pattern of synaptotagmin trafficking, as observed in Htt-Q0 expressing animals (Fig. 5J), is abruptly altered in Htt-Q128-expressing *Drosophila*. Instead of the normal diffuse localization along axonal tracts, synaptotagmin became concentrated at specific points along axons that corresponded to large areas of Htt-immunopositive aggregate accumulation, suggesting sites of axonal blockage (Fig. 5K). These synaptotagmin-rich areas of Htt aggregate accumulation were quantified in 100 μm segments along peripheral nerves and were observed at a density of 6.1 $\pm$ 2.6 sites per 100 μM. In contrast, synaptotagmin-immunopositive accumulations alone without Htt aggregate colocalization were only observed at a density of 1.3 $\pm$ 0.9 per 100 μM. The average diameter of Htt-Q128 aggregate accumulations at putative axonal blockage sites was 2.4 $\pm$ 0.6 μM. Aggregate accumulations smaller than 2.0 μM rarely resulted in colocalized synaptogtagmin concentration. These results indicate that axonal segments may be obstructed by a concentration of Htt aggregates that exceeds a critical threshold, leading to a specific block in axonal transport at these sites. Over time, the cumulative blockage of axons and synaptic terminals in post-mitotic neurons is likely to contribute to the progressive physiological defects and neuronal dysfunction we have documented in Htt-Q128 expressing *Drosophila*, as well as to late-onset neurodegeneration in HD patients.

Electrophysiological analysis of synaptic function in *Drosophila* expressing mutant Htt

Blockage of axons and synaptic terminals by aggregates of mutant Htt may prevent proper localization of synaptic components, leading to synaptic pathology. Indeed, studies have shown defects in neurotransmitter release in transgenic HD mice (Nicniocaill et al., 2001; Usdin et al., 1999), indicating that expression of mutant Htt induces dysfunction at the synapse. To characterize the physiological consequences of mutant Htt expression at the 3rd instar larval neuromuscular junction (NMJ) of transgenic *Drosophila*, intracellular recordings were performed on control larvae and larvae expressing Htt-Q0 or Htt-Q128 with the pan-neuronal C155 elav-GAL driver. Analysis of basal synaptic transmission, short-term synaptic facilitation, and spontaneous vesicle fusion events was performed to quantify mutant Htt-induced alterations in synaptic function.

Figure 5

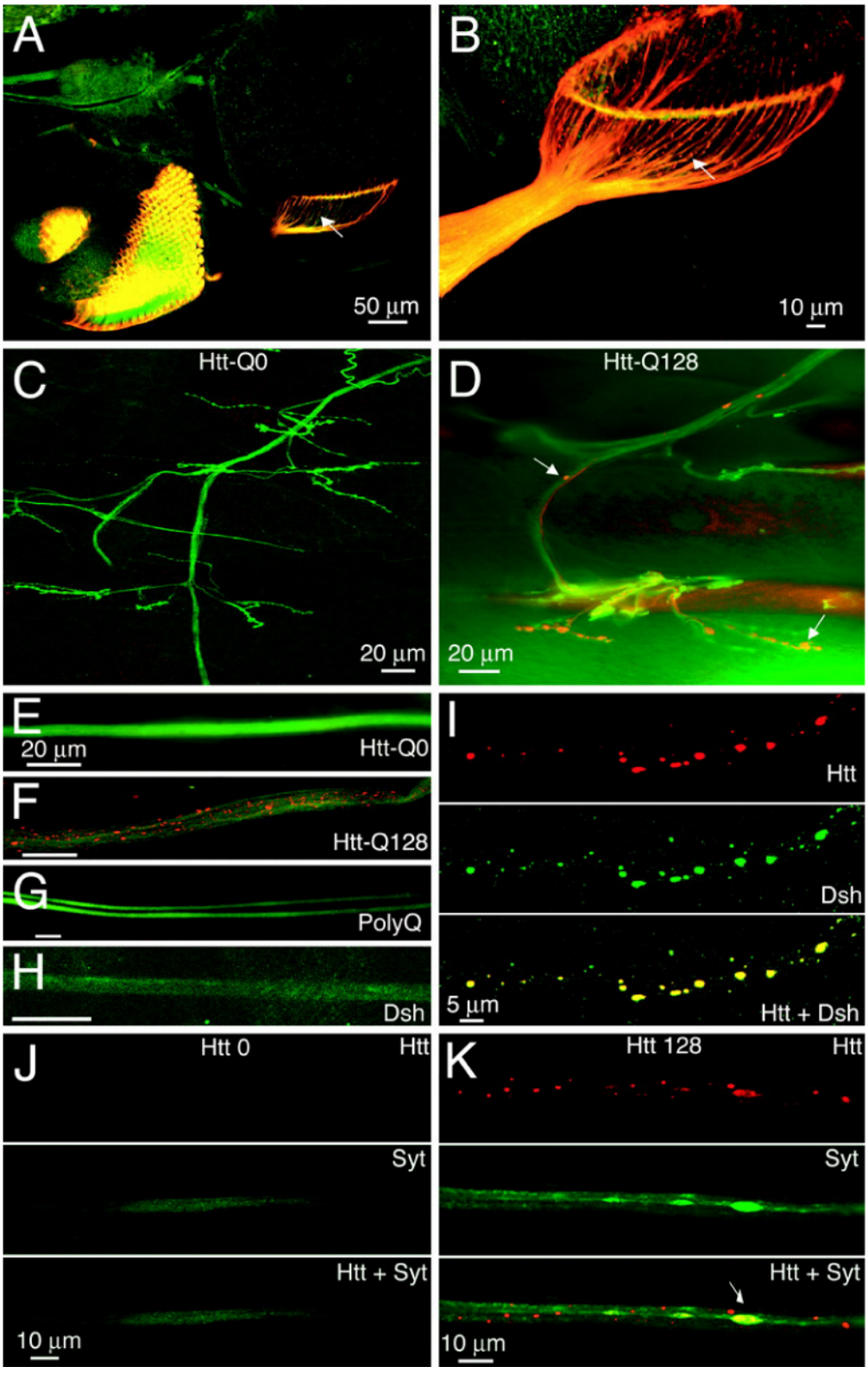
A
50 µm
B
10 µm
C
Htt-Q0
20 µm
D
Htt-Q128
20 µm
E
20 µm
Htt-Q0
F
Htt-Q128
G
PolyQ
H
Dsh
I
Htt
Dsh
5 µm
Htt + Dsh
J
Htt 0
Htt
Syt
Htt + Syt
10 µm
K
Htt 128
Htt
Syt
Htt + Syt
10 µm

FIGURE 5. **Htt-Q128 aggregates block axonal transport.** (A, B) Expression of Htt-Q128 by GMR-GAL4 results in axonal transport of Htt aggregates (arrows) in the developing visual system. (C, D) Expression of Htt-Q128 (D), but not Htt-Q0 (C), by elav-GAL4 results in axonal transport and synaptic accumulation of Htt aggregates at neuromuscular junctions in 3rd instar larvae. Axonal transport of aggregates is abundant in Htt-Q128-expressing 3rd instar larvae (F), but absent in animals expressing Htt-Q0 (E), Q127 (G) or Dsh-Q108 (H). Nerves are stained green by anti-HRP, and polyglutamine proteins are visualized in red. (I) Consistent with the trapping of Dsh-Q108 by Htt-Q128 aggregates in the cell bodies of multi-dendritic sensory neurons, Htt-Q128 also traps and transports Dsh-Q108 in peripheral nerves. (J, K) Expression of Htt-Q128 (K), but not Htt-Q0 (J), results in an accumulation of the synaptic protein synaptotagmin I in sites of axonal swelling, colocalizing with large accumulations of Htt-Q128 aggregates. Synaptotagmin I is not trapped in Htt-Q128 aggregates, as indicated by the lack of protein colocalization in smaller Htt-Q128 aggregates, but rather concentrates specifically at sites where larger aggregate accumulations result in swollen axons, indicating blockage of axonal transport.

No change was seen in resting membrane potential between control and Htt-Q128-expressing animals (Fig. 6A), suggesting that the resting status of postsynaptic muscle cells at the NMJ is not affected by presynaptic expression of mutant Htt. To determine whether mutant Htt induces a change in basal synaptic transmission, evoked excitatory junctional potentials (EJPs) were recorded in the presence of low external Ca^{2+} (0.2 mM), allowing for sensitive detection of alterations in neurotransmitter release. No statistical differences were observed in EJP amplitude between control white flies, Htt-Q0-expressing flies, and Htt-Q128-expressing flies (Fig. 6B), indicating a lack of gross mutant Htt-induced defects in vesicle docking or fusion machinery.

To detect potential defects in calcium handling at NMJ synapses of mutant Htt-expressing animals, paired pulse-facilitation was performed. Paired-pulse facilitation is used to measure short-term plasticity on a timescale of milliseconds, and is performed by evoking two action potentials within a set time interval under low calcium conditions. At close time intervals, residual intracellular Ca2+ from the first evoked potential will induce a larger second release event (Zucker and Regehr, 2002); alterations in cellular calcium management will thus manifest as changes in facilitation of the second evoked action potential. No statistical changes in paired-pulse facilitation were observed between control and Htt-Q128-expressing lines (Fig. 6C), indicating a lack of observable defects in calcium buffering at the NMJ of transgenic 3rd instar larvae expressing mutant Htt.

Miniature excitatory junctional potentials (mEJPs) were then analyzed to monitor defects in spontaneous synaptic vesicle fusion and vesicle neurotransmitter content. mEJP frequency was slightly increased in animals expressing Htt-Q128 (Fig. 7A), suggesting that spontaneous vesicle fusion may be altered in the presence of mutant Htt. No change was observed in mEJP mean amplitude between control and Htt-Q128-expressing animals (Fig. 7B), indicating that neurotransmitter content of individual vesicles is not altered; however, multiple spontaneous vesicle fusion events (simultaneous release from more than one vesicle) are increased in a subset of NMJ synapses from Htt-Q128-expressing animals, implying potential facilitation of spontaneous fusion in the presence of mutant Htt.

Future studies will include further analysis of the subset of synapses producing irregular mEJPS; while no overall difference is observed in mean EJP amplitude or paired-pulse facilitation between control and mutant Htt-expressing lines, statistical analysis may reveal significant alterations in synaptic function that occur in only this

subset of synapses. Studies may also be performed to visualize the synaptic morphology at these NMJs in comparison to NMJs in mutant Htt-expressing animals that show no evidence of synaptic dysfunction. In addition, future work will include study of defects in vesicle endocytosis and recycling through stimulation under high calcium, high frequency conditions to quantitate changes in activity-dependent reduction in evoked currents over time. Impairment of synaptic function in HD may contribute to neuronal pathology seen in the disease. It will be of interest to determine if these pathological mechanisms can be modeled in *Drosophila*.

Figure 6

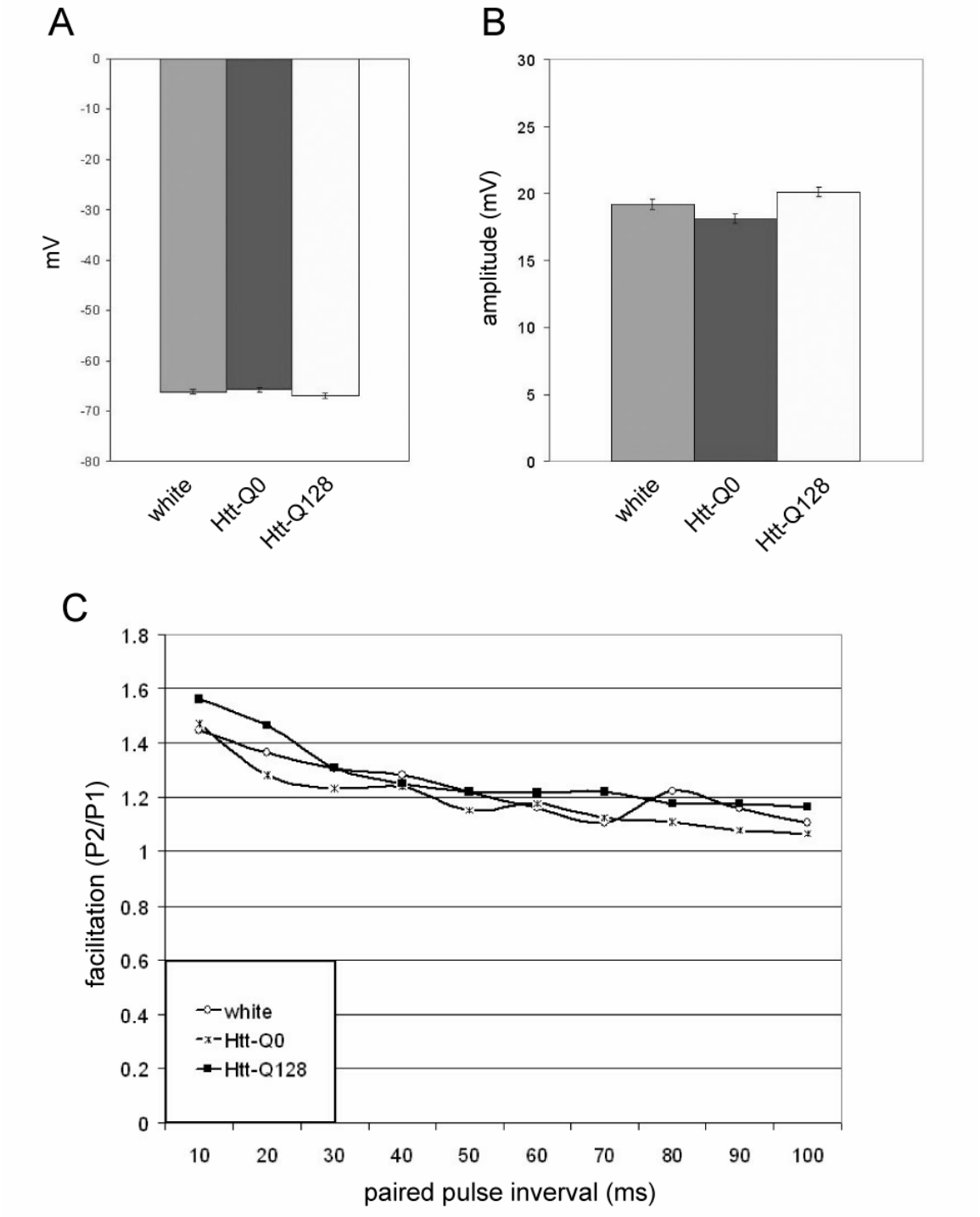
A
mV
0
-10
-20
-30
-40
-50
-60
-70
-80
white
Htt-Q0
Htt-Q128
B
amplitude (mV)
30
25
20
15
10
5
0
white
Htt-Q0
Htt-Q128
C
facilitation (P2/P1)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
white
Htt-Q0
Htt-Q128
10
20
30
40
50
60
70
80
90
100
paired pulse inverval (ms)

FIGURE 6. **Basal synaptic transmission and paired pulse facilitation are unchanged in *Drosophila* expressing Htt-Q128**. (A) Average muscle resting potentials are unchanged at the NMJ of wandering 3rd instar larvae expressing Htt-Q128 compared to Htt-Q0 or controls. Error bars are SEM. (B) Mean evoked EJP amplitudes recorded in 0.2 mM extracellular calcium for the indicated genotypes. No change is seen in EJP amplitude for larvae expressing Htt-Q128 compared to Htt-Q0 or controls. Error bars are SEM. (C) Quantification of paired-pulse facilitation for the indicated genotypes. Recordings were performed in 0.2 mM extracellular calcium. Facilitation of the second response is unaltered in Htt-Q128-expressing larvae compared to Htt-Q0 or controls.

Figure 7

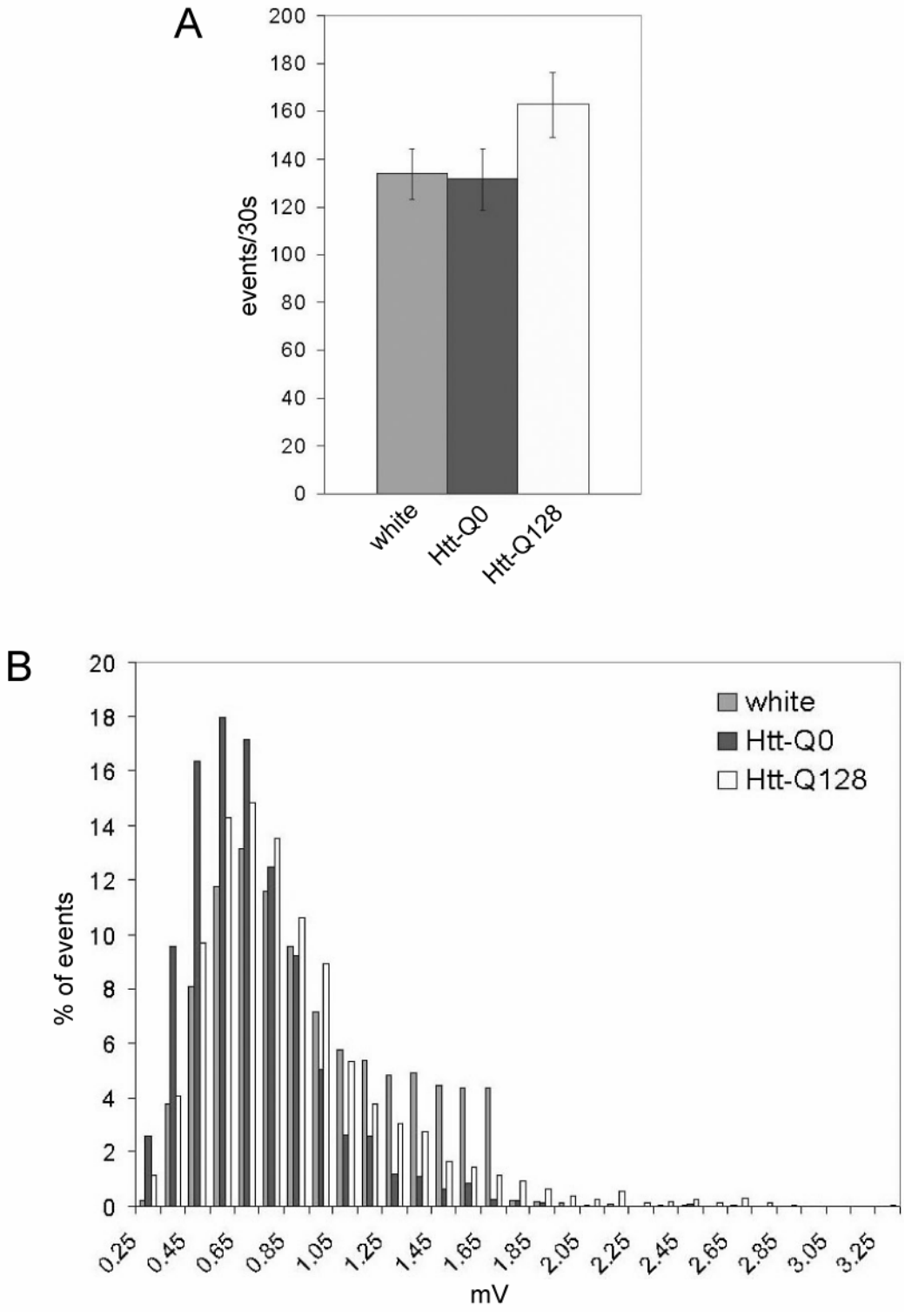
A
200
180
160
140
120
100
80
60
40
20
0
events/30s
white
Htt-Q0
Htt-Q128
B
20
18
16
14
12
10
8
6
4
2
0
% of events
white
Htt-Q0
Htt-Q128
0.25
0.45
0.65
0.85
1.05
1.25
1.45
1.65
1.85
2.05
2.25
2.45
2.65
2.85
3.05
3.25
mV

FIGURE 7. **mEJP frequency and multiple spontaneous fusion events are increased in Htt-Q128-expressing *Drosophila*.** (A) Frequency of mEJP events per 30 seconds recorded at the 3rd instar larval NMJ of control, Htt-Q0-expressing, or Htt-Q128-expressing animals. A slight increase in mEJP frequency is seen in larvae expressing Htt-Q128. Error bars are SEM. (B) Histogram of mEJP events recorded at the NMJ of control, Htt-Q0-expressing, or Htt-Q128-expressing 3rd instar larvae. No change is seen in amplitude of individual mEJP events. However, animals expressing Htt-Q128 exhibit a slight increase in multiple mEJP events.

Discussion

Many neurodegenerative diseases, including Parkinson's disease (Feany and Bender, 2000), Alzheimer's disease (Wittmann et al., 2001), and spinocerebellar ataxia type 1 (Fernandez-Funez et al., 2000) and type 3 (Warrick et al., 1998) have been successfully modeled in *Drosophila* with replication of key neuropathological features of the human diseases, including late onset and progressive neurodegeneration. Development of such disease models may facilitate the identification of molecular pathways that lead to neurodegeneration in the corresponding human disorders.

Several existing *Drosophila* models of HD (Muqit and Feany, 2002) target expression of the first exon of the mutant Htt protein to the fly retina, either through use of an eye-specific promoter (Jackson et al., 1998) or the GAL4/UAS system (Steffan et al., 2001). In both HD models, expression of Htt with a pathogenic number of glutamine repeats results in nuclear accumulation of aggregates and progressive neurodegeneration of photoreceptor cells, suggesting a role for nuclear aggregates in disease pathology. Indeed, nuclear aggregate-mediated impairment of transcription has become a favored hypothesis to explain polyglutamine-mediated neurodegeneration (Ross, 2002). *Drosophila* HD models expressing Htt transgenes with a pan-neuronal GAL4 driver (Gunawardena et al., 2003) suggest that non-nuclear pathology associated with cytoplasmic and neuritic aggregates is likely to play an essential role in disease progression as well. Given that *Drosophila* polyglutamine disease models with either nuclear-restricted (expanded polyQ alone, mutant SCA-3) or cytoplasm-restricted (Htt-Q128) aggregates both exhibit neurodegeneration, it is likely that multiple pathways for polyglutamine-mediated dysfunction exist. Indeed, we have been unable to rescue adult lethality in Htt-Q128-expressing *Drosophila* with any of the previously published genetic suppressors of *Drosophila* transgenic polyglutamine models (Chan et al., 2000; Fernandez-Funez et al., 2000; Kazantsev et al., 2002; Kazemi-Esfarjani and Benzer, 2000). These negative results likely reflect distinct modes of toxicity between nuclear and cytoplasmic aggregates, and suggest that preventing polyglutamine-mediated Htt toxicity may require more research into the role of non-nuclear aggregates.

We have demonstrated a potential role for cytoplasmic and neuritic aggregates in the sequestration of cytoplasmic polyQ proteins and in the blockage of axonal transport. Consistent with our hypothesis that Htt-Q128 expression causes neurodegeneration secondary to axonal transport impairment, several recent studies

have found that neurodegeneration is a primary consequence of axonal transport defects in non-polyglutamine diseases as well (Hafezparast et al., 2003; LaMonte et al., 2002; Lee and Cleveland, 1994; Puls et al., 2003), including Alzheimer's disease (Gunawardena and Goldstein, 2001). In summary, our results indicate that cytoplasmic aggregate formation in HD sequesters endogenous polyglutamine proteins and blocks axonal transport, contributing to neuronal dysfunction and neurodegeneration.

Acknowledgments

We thank Leslie Thompson, Nancy Bonini, and Parsi Kazemi-Esfarjani for *Drosophila* strains, Michael Hayden for Htt cDNAs, Paul Garrity for help with the technique of pseudopupil analysis, Barry Ganetzky, in whose lab this project was initiated, and Ling-Ling Ho and Jessica Slind for help with generation of transgenic *Drosophila*. This work was supported by grants from the NIH, the David and Lucile Packard Foundation, and the Merck/MIT Collaboration Program.

References

Becher, M.W., Kotzuk, J.A., Sharp, A.H., Davies, S.W., Bates, G.P., Price, D.L. and Ross, C.A. (1998) Intranuclear neuronal inclusions in Huntington's disease and dentatorubral and pallidoluysian atrophy: correlation between the density of inclusions and IT15 CAG triplet repeat length. *Neurobiol Dis*, **4**, 387-397.

Block-Galarza, J., Chase, K.O., Sapp, E., Vaughn, K.T., Vallee, R.B., DiFiglia, M. and Aronin, N. (1997) Fast transport and retrograde movement of huntingtin and HAP 1 in axons. *Neuroreport*, **8**, 2247-2251.

Chan, E.Y., Luthi-Carter, R., Strand, A., Solano, S.M., Hanson, S.A., DeJohn, M.M., Kooperberg, C., Chase, K.O., DiFiglia, M., Young, A.B., Leavitt, B.R., Cha, J.H., Aronin, N., Hayden, M.R. and Olson, J.M. (2002) Increased huntingtin protein length reduces the number of polyglutamine-induced gene expression changes in mouse models of Huntington's disease. *Hum Mol Genet*, **11**, 1939-1951.

Chan, H.Y., Warrick, J.M., Gray-Board, G.L., Paulson, H.L. and Bonini, N.M. (2000) Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in *Drosophila*. *Hum Mol Genet*, **9**, 2811-2820.

DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P. and Aronin, N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science*, **277**, 1990-1993.

Feany, M.B. and Bender, W.W. (2000) A *Drosophila* model of Parkinson's disease. *Nature*, **404**, 394-398.

Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J., McCall, A., Canal, I., Orr, H.T., Zoghbi, H.Y. and Botas, J. (2000) Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature*, **408**, 101-106.

Gunawardena, S. and Goldstein, L.S. (2001) Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in *Drosophila*. *Neuron*, **32**, 389-401.

Gunawardena, S., Her, L.S., Brusch, R.G., Laymon, R.A., Niesman, I.R., Gordesky-Gold, B., Sintasath, L., Bonini, N.M. and Goldstein, L.S. (2003) Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in *Drosophila*. *Neuron*, **40**, 25-40.

Gutekunst, C.A., Li, S.H., Yi, H., Mulroy, J.S., Kuemmerle, S., Jones, R., Rye, D., Ferrante, R.J., Hersch, S.M. and Li, X.J. (1999) Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. *J Neurosci*, **19**, 2522-2534.

Hafezparast, M., Klocke, R., Ruhrberg, C., Marquardt, A., Ahmad-Annuar, A., Bowen, S., Lalli, G., Witherden, A.S., Hummerich, H., Nicholson, S., Morgan, P.J., Oozageer, R., Priestley, J.V., Averill, S., King, V.R., Ball, S., Peters, J., Toda, T.,

Yamamoto, A., Hiraoka, Y., Augustin, M., Korthaus, D., Wattler, S., Wabnitz, P., Dickneite, C., Lampel, S., Boehme, F., Peraus, G., Popp, A., Rudelius, M., Schlegel, J., Fuchs, H., Hrabe de Angelis, M., Schiavo, G., Shima, D.T., Russ, A.P., Stumm, G., Martin, J.E. and Fisher, E.M. (2003) Mutations in dynein link motor neuron degeneration to defects in retrograde transport. *Science*, **300**, 808-812.

HDCRG. (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell*, **72**, 971-983.

Hodges, A., Strand, A.D., Aragaki, A.K., Kuhn, A., Sengstag, T., Hughes, G., Elliston, L.A., Hartog, C., Goldstein, D.R., Thu, D., Hollingsworth, Z.R., Collin, F., Synek, B., Holmans, P.A., Young, A.B., Wexler, N.S., Delorenzi, M., Kooperberg, C., Augood, S.J., Faull, R.L., Olson, J.M., Jones, L. and Luthi-Carter, R. (2006) Regional and cellular gene expression changes in human Huntington's disease brain. *Hum Mol Genet*, **15**, 965-977.

Jackson, G.R., Salecker, I., Dong, X., Yao, X., Arnheim, N., Faber, P.W., MacDonald, M.E. and Zipursky, S.L. (1998) Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron*, **21**, 633-642.

Kazantsev, A., Walker, H.A., Slepko, N., Bear, J.E., Preisinger, E., Steffan, J.S., Zhu, Y.Z., Gertler, F.B., Housman, D.E., Marsh, J.L. and Thompson, L.M. (2002) A bivalent Huntingtin binding peptide suppresses polyglutamine aggregation and pathogenesis in *Drosophila*. *Nat Genet*, **30**, 367-376.

Kazemi-Esfarjani, P. and Benzer, S. (2000) Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science*, **287**, 1837-1840.

Kim, Y.J., Yi, Y., Sapp, E., Wang, Y., Cuiffo, B., Kegel, K.B., Qin, Z.H., Aronin, N. and DiFiglia, M. (2001) Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. *Proc Natl Acad Sci U S A*, **98**, 12784-12789.

Kopito, R.R. (2000) Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol*, **10**, 524-530.

LaMonte, B.H., Wallace, K.E., Holloway, B.A., Shelly, S.S., Ascano, J., Tokito, M., Van Winkle, T., Howland, D.S. and Holzbaur, E.L. (2002) Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. *Neuron*, **34**, 715-727.

Lee, J.A., Lim, C.S., Lee, S.H., Kim, H., Nukina, N. and Kaang, B.K. (2003) Aggregate formation and the impairment of long-term synaptic facilitation by ectopic expression of mutant huntingtin in Aplysia neurons. *J Neurochem*, **85**, 160-169.

Lee, M.K. and Cleveland, D.W. (1994) Neurofilament function and dysfunction: involvement in axonal growth and neuronal disease. *Curr Opin Cell Biol*, **6**, 34-40.

Li, H., Li, S.H., Cheng, A.L., Mangiarini, L., Bates, G.P. and Li, X.J. (1999a) Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. *Hum Mol Genet*, **8**, 1227-1236.

Li, H., Li, S.H., Johnston, H., Shelbourne, P.F. and Li, X.J. (2000) Amino-terminal fragments of mutant huntingtin show selective accumulation in striatal neurons and synaptic toxicity. *Nat Genet*, **25**, 385-389.

Li, Z., Karlovich, C.A., Fish, M.P., Scott, M.P. and Myers, R.M. (1999b) A putative *Drosophila* homolog of the Huntington's disease gene. *Hum Mol Genet*, **8**, 1807-1815.

Littleton, J.T., Bellen, H.J. and Perin, M.S. (1993) Expression of synaptotagmin in *Drosophila* reveals transport and localization of synaptic vesicles to the synapse. *Development*, **118**, 1077-1088.

Littleton, J.T., Chapman, E.R., Kreber, R., Garment, M.B., Carlson, S.D. and Ganetzky, B. (1998) Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. *Neuron*, **21**, 401-413.

Luthi-Carter, R., Hanson, S.A., Strand, A.D., Bergstrom, D.A., Chun, W., Peters, N.L., Woods, A.M., Chan, E.Y., Kooperberg, C., Krainc, D., Young, A.B., Tapscott, S.J. and Olson, J.M. (2002) Dysregulation of gene expression in the R6/2 model of polyglutamine disease: parallel changes in muscle and brain. *Hum Mol Genet*, **11**, 1911-1926.

Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W. and Bates, G.P. (1996) Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell*, **87**, 493-506.

Marsh, J.L., Walker, H., Theisen, H., Zhu, Y.Z., Fielder, T., Purcell, J. and Thompson, L.M. (2000) Expanded polyglutamine peptides alone are intrinsically cytotoxic and cause neurodegeneration in *Drosophila*. *Hum Mol Genet*, **9**, 13-25.

Montana, E.S. and Littleton, J.T. (2004) Characterization of a hypercontraction-induced myopathy in *Drosophila* caused by mutations in Mhc. *J Cell Biol*, **164**, 1045-1054.

Muqit, M.M. and Feany, M.B. (2002) Modelling neurodegenerative diseases in *Drosophila*: a fruitful approach? *Nat Rev Neurosci*, **3**, 237-243.

Murphy, K.P., Carter, R.J., Lione, L.A., Mangiarini, L., Mahal, A., Bates, G.P., Dunnett, S.B. and Morton, A.J. (2000) Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. *J Neurosci*, **20**, 5115-5123.

Nicniocaill, B., Haraldsson, B., Hansson, O., O'Connor, W.T. and Brundin, P. (2001) Altered striatal amino acid neurotransmitter release monitored using microdialysis in R6/1 Huntington transgenic mice. *Eur J Neurosci*, **13**, 206-210.

Palladino, M.J., Hadley, T.J. and Ganetzky, B. (2002) Temperature-sensitive paralytic mutants are enriched for those causing neurodegeneration in *Drosophila*. *Genetics*, **161**, 1197-1208.

Persichetti, F., Trettel, F., Huang, C.C., Fraefel, C., Timmers, H.T., Gusella, J.F. and MacDonald, M.E. (1999) Mutant huntingtin forms *in vivo* complexes with distinct context-dependent conformations of the polyglutamine segment. *Neurobiol Dis*, **6**, 364-375.

Puls, I., Jonnakuty, C., LaMonte, B.H., Holzbaur, E.L., Tokito, M., Mann, E., Floeter, M.K., Bidus, K., Drayna, D., Oh, S.J., Brown, R.H., Jr., Ludlow, C.L. and Fischbeck, K.H. (2003) Mutant dynactin in motor neuron disease. *Nat Genet*, **33**, 455-456.

Ross, C.A. (2002) Polyglutamine pathogenesis: emergence of unifying mechanisms for Huntington's disease and related disorders. *Neuron*, **35**, 819-822.

Rubinsztein, D.C., Leggo, J., Coles, R., Almqvist, E., Biancalana, V., Cassiman, J.J., Chotai, K., Connarty, M., Crauford, D., Curtis, A., Curtis, D., Davidson, M.J., Differ, A.M., Dode, C., Dodge, A., Frontali, M., Ranen, N.G., Stine, O.C., Sherr, M., Abbott, M.H., Franz, M.L., Graham, C.A., Harper, P.S., Hedreen, J.C., Hayden, M.R. and et al. (1996) Phenotypic characterization of individuals with 30-40 CAG repeats in the Huntington disease (HD) gene reveals HD cases with 36 repeats and apparently normal elderly individuals with 36-39 repeats. *Am J Hum Genet*, **59**, 16-22.

Sapp, E., Penney, J., Young, A., Aronin, N., Vonsattel, J.P. and DiFiglia, M. (1999) Axonal transport of N-terminal huntingtin suggests early pathology of corticostriatal projections in Huntington disease. *J Neuropathol Exp Neurol*, **58**, 165-173.

Saudou, F., Finkbeiner, S., Devys, D. and Greenberg, M.E. (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell*, **95**, 55-66.

Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G.P., Davies, S.W., Lehrach, H. and Wanker, E.E. (1997) Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and *in vivo*. *Cell*, **90**, 549-558.

Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M., Kurokawa, R., Housman, D.E., Jackson, G.R., Marsh, J.L. and Thompson, L.M. (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature*, **413**, 739-743.

Stewart, B.A., Atwood, H.L., Renger, J.J., Wang, J. and Wu, C.F. (1994) Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J Comp Physiol [A]*, **175**, 179-191.

Takano, H. and Gusella, J.F. (2002) The predominantly HEAT-like motif structure of huntingtin and its association and coincident nuclear entry with dorsal, an NF-kB/Rel/dorsal family transcription factor. *BMC Neurosci*, **3**, 15.

Usdin, M.T., Shelbourne, P.F., Myers, R.M. and Madison, D.V. (1999) Impaired synaptic plasticity in mice carrying the Huntington's disease mutation. *Hum Mol Genet*, **8**, 839-846.

Van Vactor, D.L., Jr., Cagan, R.L., Kramer, H. and Zipursky, S.L. (1991) Induction in the developing compound eye of *Drosophila*: multiple mechanisms restrict R7 induction to a single retinal precursor cell. *Cell*, **67**, 1145-1155.

Wang, J.W., Sylwester, A.W., Reed, D., Wu, D.A., Soll, D.R. and Wu, C.F. (1997) Morphometric description of the wandering behavior in *Drosophila* larvae: aberrant locomotion in Na+ and K+ channel mutants revealed by computer-assisted motion analysis. *J Neurogenet*, **11**, 231-254.

Warrick, J.M., Paulson, H.L., Gray-Board, G.L., Bui, Q.T., Fischbeck, K.H., Pittman, R.N. and Bonini, N.M. (1998) Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell*, **93**, 939-949.

Wellington, C.L., Ellerby, L.M., Gutekunst, C.A., Rogers, D., Warby, S., Graham, R.K., Loubser, O., van Raamsdonk, J., Singaraja, R., Yang, Y.Z., Gafni, J., Bredesen, D., Hersch, S.M., Leavitt, B.R., Roy, S., Nicholson, D.W. and Hayden, M.R. (2002) Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J Neurosci*, **22**, 7862-7872.

Willert, K., Brink, M., Wodarz, A., Varmus, H. and Nusse, R. (1997) Casein kinase 2 associates with and phosphorylates dishevelled. *Embo J*, **16**, 3089-3096.

Wittmann, C.W., Wszolek, M.F., Shulman, J.M., Salvaterra, P.M., Lewis, J., Hutton, M. and Feany, M.B. (2001) Tauopathy in *Drosophila*: neurodegeneration without neurofibrillary tangles. *Science*, **293**, 711-714.

Xuereb, J.H., MacMillan, J.C., Snell, R., Davies, P. and Harper, P.S. (1996) Neuropathological diagnosis and CAG repeat expansion in Huntington's disease. *J Neurol Neurosurg Psychiatry*, **60**, 78-81.

Zucker, R.S. and Regehr, W.G. (2002) Short-term synaptic plasticity. *Annu Rev Physiol*, **64**, 355-405.

CHAPTER 3

Mutant huntingtin blocks axonal transport through aggregate-dependent and -independent means in a *Drosophila* model of Huntington's disease

Wyan-Ching Mimi Lee[1], Rosemarie V. Barkus[2], Ray Truant[3], William M. Saxton[2], and J.Troy Littleton[1]

[1]Picower Institute for Learning and Memory, Departments of Biology and Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, [2]Department of Biology, Indiana University, Bloomington, Indiana 47405, [3]Department of Biochemistry, McMaster University, Health Sciences Centre Rm 4H45, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

The majority of the work described in these studies was performed by Wyan-Ching Mimi Lee. mRFP- and eGFP-tagged HttQ15 and HttQ138 cDNAs were provided by Ray Truant. HttQ96-GFP cDNA was kindly donated by David Housman, and Katie Lynch provided help with the subcloning of HttQ96-GFP into the pUAST vector. S2 cell experiments were performed by Rupali Avasare and Albert Su. The glue secretion assay was designed and performed by J. Troy Littleton. Htt transport rate measurement and analysis were performed by Rosemarie Barkus.

Abstract

To explore axonal transport defects in Huntington's disease, we generated *Drosophila* transgenic strains expressing 588 aa N-terminal fragments of human huntingtin encoding pathogenic (HttQ138) or nonpathogenic (HttQ15) proteins tagged with mRFP and/or eGFP. These transgenic lines allow *in vivo* imaging of Htt aggregation and trafficking in live *Drosophila*, providing a unique resource for tracking Htt in real time. Expression of pathogenic Htt proteins results in the formation of neuritic aggregates that are immobile and physically block axonal transport of various cargoes, including synaptic and dense core vesicles, clathrin-coated vesicles, endosomes, cytosolic proteins, and the normal Htt protein. In addition, the number of mitochondria and dense core vesicles is reduced in axons of mutant Htt-expressing animals, suggesting a potential mutant Htt-mediated disruption of interactions between specific transport cargoes and axonal transport machinery. Our findings indicate that expression of mutant Htt may impair axonal transport through both aggregate-dependent and -independent means.

Introduction

Huntington's disease (HD) is one of nine known neurological disorders caused by a polyglutamine (polyQ) repeat expansion. The disease is characterized by neurodegeneration and formation of neuronal intracellular inclusions primarily in the striatum and cortex, leading to personality changes, motor impairment, and dementia (Vonsattel et al., 1985). The causative mutation in HD is expansion of a polyQ tract in exon 1 of the gene encoding huntingtin (Htt) (HDCRG, 1993), a 348 kDa protein hypothesized to be involved in vesicular transport (Block-Galarza et al., 1997; DiFiglia et al., 1995; Gauthier et al., 2004). Expansion of the polyQ repeat in Htt is thought to promote abnormal protein conformation(s) that lead to the formation of aggregates and are central to disease pathogenesis (Persichetti et al., 1999; Scherzinger et al., 1997). However, the molecular mechanisms that underlie the neurodegenerative process remain to be defined.

While several studies suggest that intranuclear aggregates are the primary cause of HD (Becher et al., 1998; Davies et al., 1997), recent evidence points to a pathological role for the mutant protein in the cytoplasm and neurites (Li et al., 1999a; Sapp et al., 1999) and possibly at the synapse (Li et al., 2003c; Usdin et al., 1999). In particular, recent studies indicate that disruption of Htt function may impair axonal transport, either through toxic effects of the mutant protein (Gunawardena et al., 2003; Lee et al., 2004; Szebenyi et al., 2003; Trushina et al., 2004) or through loss of the normal protein (Gunawardena et al., 2003; Trushina et al., 2004). Axonal transport is essential for delivery of proteins from the neuronal cell body to the synapse, and to convey retrograde signals from the synapse to the cell body. In addition to Huntingon's disease, impairment of axonal transport has been implicated in other late-onset neurodegenerative disorders including amyotrophic lateral sclerosis (Kieran et al., 2005; Williamson and Cleveland, 1999), spinal bulbar muscular atrophy (Piccioni et al., 2002), Parkinson's disease (Saha et al., 2004), and Alzheimer's disease (Chee et al., 2006; Praprotnik et al., 1996).

Expression of the mutant Htt protein has been shown to disrupt axonal transport of exogenous markers (Li et al., 2001), synaptic vesicles (Lee et al., 2004), neurotrophic factors (Gauthier et al, 2004), and organelles (Chang et al., 2006). In particular, impaired axonal transport may lead to defects in trafficking and distribution of mitochondria. Proper mitochondrial distribution is necessary for calcium buffering,

sequestration of apoptotic signals, and production of ATP in areas of high metabolic need; defects in trafficking of mitochondria can both decrease the number of healthy mitochondria in areas of need and increase the number of old or damaged mitochondria that can cause neuronal damage. Recent studies suggest that defective mitochondrial transport may be a factor in HD pathology. A 50% decrease in wild-type Htt results in a decrease in the motility of mitochondria (Trushina et al., 2004). Additionally, in flies lacking Milton, the *Drosophila* homologue of HAP1 (a known Htt interactor), mitochondria are prevented from entering the axon and remain in the cell body (Stowers et al., 2002), further suggesting a link between Htt function and mitochondrial transport in the axon. Expansion of the polyQ repeat in Htt reduces mitochondrial movement in HD cortical neurons (Chang et al., 2006) and in striatal neurons from transgenic HD mice (Trushina et al., 2004), indicating that mutant Htt may impair transport of mitochondria in HD. Defects in mitochondrial transport, and resulting mitochondrial insufficiency and dysfunction, may contribute to the excitotoxicity, oxidative damage, and neuronal cell death observed in Huntington's disease.

To characterize axonal transport defects in Huntington's disease, we generated transgenic *Drosophila* that express 588 aa N-terminal fragments of the human Htt gene with either a pathogenic polyQ tract of 138 repeats (HttQ138) or a non-pathogenic tract of 15 repeats (HttQ15). These fragments were fused to mRFP or eGFP at the N-terminus, or to eGFP at the N-terminus and mRFP at the C-terminus, to allow for *in vivo* imaging. We also generated a transgenic strain expressing exon 1 of the human Htt protein with a 96Q repeat, fused to GFP at the C-terminus (HttQ96-GFP). Our findings indicate that expression of either the 588 aa or exon 1 mutant Htt fragment leads to the formation of non-nuclear aggregates in both neuronal and non-neuronal cell types, and can induce cellular dysfunction. In neurons, Htt-immunopositive aggregates occur in both the axon and dendrites, and larger aggregates are immobile during development. These aggregates physically block the axon, capturing axonal transport cargoes such as synaptic and dense core vesicles, clathrin-coated vesicles, endosomes, and cytosolic proteins, as well as the normal Htt protein. A subset of mitochondria colocalize and are transported with the normal Htt protein. However, this association is reduced between mitochondria and mutant Htt, suggesting that impairment of the normal role of Htt in mitochondrial transport may contribute to disease pathogenesis. Defects in axonal transport and corresponding neurodegeneration in post-mitotic neurons may underlie many symptoms of HD pathology. In *Drosophila* models of HD, these axonal transport

defects arise from both a physical blockage in transport secondary to neuritic aggregate formation, and a non-aggregate-dependent reduction in axonal cargo, including mitochondria.

Materials and Methods

Drosophila Genetics and Generation of Htt Constructs

Drosophila melanogaster were maintained on standard medium at 25^{o}C. cDNAs for mRFP-HttQ15, mRFP-HttQ138, eGFP-HttQ15, eGFP-HttQ138 were subcloned into the EcoRI (blunt end ligation) and KpnI sites of the pUAST expression vector. cDNA for eGFP-HttQ138-mRFP was subcloned into the XbaI site of the pUAST vector. cDNA for HttQ96-GFP was kindly provided by David Housman (Center for Cancer Research, MIT) and was subcloned into the KpnI and XbaI sites of the pUAST vector. Microinjection of mRFP-HttQ15, mRFP-HttQ138, eGFP-HttQ15, eGFP-HttQ138, and eGFP-HttQ138-mRFP constructs into *Drosophila* embryos was performed by the Duke University Model Systems Genomics Group, while microinjection of HttQ96-GFP was performed by Genetic Services, Inc.

S2 Cell Transfection and Analysis

cDNAs for mRFP-HttQ15 and mRFP-HttQ138 were subcloned into the BamH1 and EcoRI (blunt end ligation) sites of the pSR11 vector. To generate constructs expressing mRFP-HttQ15-eGFP and mRFP-HttQ138-eGFP, mRFP-HttQ15 and mRFP-HttQ138 cDNAs were PCR amplified with a forward primer containing an EcoRI restriction site and a reverse primer containing a 3' Sal I site. The added restriction sites were then used to subclone the PCR products into the pPL17 vector. Constructs were transfected with 50 μL cytofectene (BioRad) into *Drosophila* S2 cells using the BioRad Liposome Mediated Transfection Protocol. 4 μG of construct DNA were used per 5 mL S2 cell culture for single transfections while 4 μG of each construct were used for double transfections. After 72 hours, 20 μL cell suspensions were fixed with 3.7% formaldehyde in 1 x PBT, then mounted on slides with 50% glycerol in 1 x PBS. Visualization of slides was performed on a Pascal confocal microscope (Zeiss).

Western Blot Analysis

For the HttQ96-GFP Western blot, *Drosophila* were frozen in liquid nitrogen and vortexed. 20 heads for each indicated genotype were isolated and homogenized in sample buffer, and proteins were separated on 10% SDS-PAGE gels. The gels were immunoblotted with rabbit anti-GFP sc8334 (Santa Cruz Biotechnology) at 1:1,000, and immunoreactive bands were visualized by using ECL (Pierce). For the eGFP-Htt and

mRFP-Htt Western blot, 5 larvae for each indicated genotype were homogenized in sample buffer and proteins were separated on 10% SDS-PAGE gels. The gels were immunoblotted with mouse anti-Htt MAb2166 (Chemicon) at 1:1,000, and immunoreactive bands were visualized by using ECL (Pierce).

Adult Viability Analysis

Drosophila viability assays were performed on white/C155, HttQ96-GFP/C155, mRFP-HttQ15 /C155, mRFP-HttQ138/C155, and mRFP-HttQ138 B/C155 flies by daily quantification of lethality for 100 males and 100 females of each genotype. Flies were aged at 25^{o}C, with 20 flies per food vial, and were transferred every 2-3 days.

Morphological Analysis

Wandering 3rd instar larvae reared at 25^{o}C were dissected as described (Rieckhof et al., 2003). For Alexa Fluor® 488 phalloidin-staining, larvae were fixed for 15 minutes in 4% formaldehyde in HL3.1 solution (Stewart et al., 1994), washed for 15 minutes in phosphate-buffered saline (PBS), and stained for 15 minutes with Alexa Fluor® 488 phalloidin (Invitrogen) at 1:500. Immunostaining was performed on larvae fixed for 40 minutes in 4% formaldehyde solution in HL3.1 solution at room temperature. Primary antibody against *Drosophila* complexin (Littleton Lab, unpublished) was used at 1:500 and visualized with Cy2-conjugated secondary antibody (Jackson Labs). Visualization and quantification were performed on a Pascal confocal microscope (Zeiss).

Glue Secretion Assay

Pupae reared at 25^{o}C were isolated shortly after pupariation and adhered to glass slides with ventral sides facing up using double-sided tape. For salivary gland analysis, salivary glands were dissected from wandering 3rd instar larvae and mounted in 70% glycerol in phosphate-buffered saline (PBS) on glass slides. Visualization and quantification were performed on a Pascal confocal microscope (Zeiss).

Axonal Transport Rate Analysis

Time-lapse imaging of GFP and RFP particle movement in *Drosophila* motor axons was performed as described (Horiuchi et al, 2005). NIH Image version 1.62b7 was used with an object tracking macro based on software designed by Kurt Anderson

and Rob Cross (http://mc11.mcri.ac.uk/motorhome.html) to mark the positions of individual RFP or GFP particles. Anterograde movements were given a positive designation and retrograde displacements were given a negative designation. To describe transport behavior, particle motility was modeled as a three-state system consisting of plus-end and minus-end runs, based on periods of uninterrupted motion, or pauses, based on lack of motion. Mean velocities, durations, and lengths were calculated for plus- and minus-end runs, as well as mean duration for pauses. The "duty cycles" of transport represent the percentage of time spent by particles in each of the three states. Graphing, modeling, and statistical analysis of organelle tracking data were done using SPSS Base 10.0 (SPSS Inc., Chicago, IL) and Microsoft Excel.

Results

The 588 aa N-terminal fragment of mutant human Htt reduces *Drosophila* lifespan

To explore axonal transport defects in Huntington's disease, we generated transgenic *Drosophila* that express 588 aa N-terminal fragments of human Htt gene with either a pathogenic polyQ tract of 138 repeats (HttQ138) or a non-pathogenic tract of 15 repeats (HttQ15). While several models of HD have focused on expression of the polyglutamine-containing first exon of Htt alone, the 588 amino acid fragment is truncated near a number of well-characterized sites of caspase cleavage, thought to be a crucial step in the generation of aggregate-forming Htt fragments (Kim et al., 2001; Wellington et al., 2002). Additionally, many sites of protein interaction that are lost in exon 1 constructs are conserved in the 588 aa fragment, including a region of well-conserved HEAT repeats known to be involved in Htt binding to interaction partners such as HIP1, HAP1, and HIP14 (Harjes and Wanker, 2003). The 588 aa fragment also encompasses the highest stretch of homology between the *Drosophila* and human Htt proteins (Li et al., 1999b), providing a more accurate representation of the *in vivo* Htt protein context for the polyglutamine tract. The fragments were fluorescently tagged with mRFP or eGFP at the N-terminus, or tagged at both ends with eGFP at the N-terminus and mRFP at the C-terminus. For comparison, we also created a transgenic strain expressing exon 1 (81 aa) of the human Htt protein with a pathogenic 96Q repeat, fused to GFP at the C-terminus (HttQ96-GFP). All constructs are expressed using the UAS-GAL4 system, which allows for temporal and tissue-specific control of transgene expression. These transgenic lines allow *in vivo* imaging of Htt aggregation and trafficking in live *Drosophila*, providing a unique resource for tracking Htt in real time (Fig. 1A).

To confirm transgene expression, strains were crossed to the neuronal GAL4 driver C155 elav-GAL4, and Htt expression in offspring was assessed through Western blot analysis with anti-human Htt antibodies (Fig. 1B). No Htt expression is detected in control lines crossed to the neuronal GAL4 driver, while mRFP-Htt, eGFP-Htt, and HttQ96-GFP lines all demonstrate abundant Htt expression. As expected, the product detected in HttQ15 strains lacking the expanded polyglutamine tract is smaller than that in HttQ138 or HttQ96 strains.

Pan-neuronal expression of mRFP-HttQ138 with the C155 elav-GAL4 driver causes pharate adult lethality with less than 1% viable adult escapers. Expression of

Figure 1

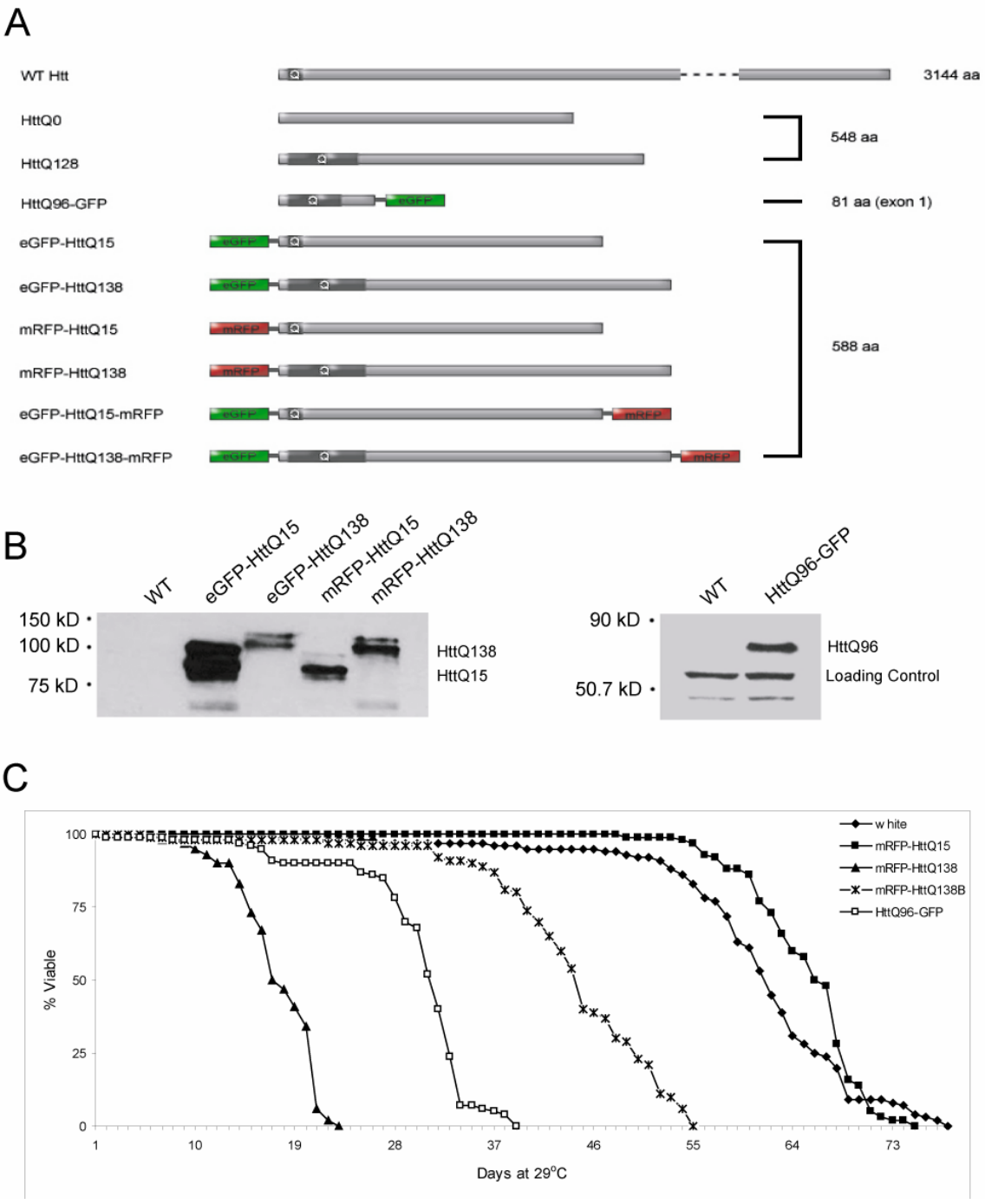
A
WT Htt
3144 aa
HttQ0
548 aa
HttQ128
HttQ96-GFP
81 aa (exon 1)
eGFP-HttQ15
eGFP-HttQ138
mRFP-HttQ15
588 aa
mRFP-HttQ138
eGFP-HttQ15-mRFP
eGFP-HttQ138-mRFP
B
WT
eGFP-HttQ15
eGFP-HttQ138
mRFP-HttQ15
mRFP-HttQ138
150 kD
100 kD
75 kD
HttQ138
HttQ15
WT
HttQ96-GFP
90 kD
50.7 kD
HttQ96
Loading Control
C
w hite
mRFP-HttQ15
mRFP-HttQ138
mRFP-HttQ138B
HttQ96-GFP
% Viable
100
75
50
25
0
1
10
19
28
37
46
55
64
73
Days at 29°C

FIGURE 1. **Generation of a *Drosophila* transgenic model of HD.** (A) The N-terminal fragments of human Htt used for transgenic construction. Polyglutamine tracts (Q) and fluorescent tags (mRFP, eGFP) are indicated. The full-sized Htt protein is depicted for comparison. (B) Expression of Htt in control, mRFP-Htt, eGFP-Htt, and HttQ96-GFP strains with transgene expression driven by the C155 elav-GAL4 driver. Western blotting was performed with an antibody to the N-terminus of human Htt (mRFP-Htt and eGFP-Htt blot) or an antibody to GFP (HttQ96-GFP blot). (C) Reduced viability of transgenic strains expressing mutant Htt. T_{50} is decreased by over 70% in strains expressing mRFP-HttQ138, 30% in strains expressing mRFP-HttQ138B (a lower expression strain), and 50% in strains expressing HttQ96-GFP, in comparison to controls.

mutant Htt with a weaker elav-GAL4 driver results in viable adults that appear behaviorally normal at the time of eclosion. However, several days after eclosion, mRFP-HttQ138-expressing flies begin to exhibit motor coordination defects and abnormal grooming behaviors, worsening with age and resulting in premature death. Similar defects occur at a later timepoint in a separate mRFP-HttQ138 insertion line expressing mutant Htt at a lower level, as well as in flies expressing the mutant HttQ96-GFP exon 1 protein. These behaviors are not observed in mRFP-HttQ15-expressing flies or control flies. To quantify the reduction in viability of mutant Htt-expressing flies, lifespan curves were generated for control adults and adults expressing mRFP-HttQ15, mRFP-HttQ138, mRFP-HttQ138B (a line expressing a lower level of mRFP-HttQ138), or HttQ96-GFP. The T_{50} (age at which 50% of the culture has died) for mRFP-HttQ138 lines is dramatically decreased by over 70% in comparison to controls. A lower expression strain (mRFP-HttQ138B) shows a 30% decrease in T_{50}, indicating that viability is inversely correlated with the level of expression of the mutant protein. HttQ96-GFP lines also demonstrate a decrease in T_{50} of 50%, suggesting that expression of the expanded polyQ-containing first exon of Htt is also toxic (Fig. 1C). Decreases in T_{50} for all lines expressing fragments of the mutant Htt protein, but not the normal protein, indicate that expression of mutant Htt significantly reduces lifespan in *Drosophila*.

Mutant Htt forms cytoplasmic aggregates in neuronal and non-neuronal cells *in vivo*

A hallmark of HD is the formation of intracellular aggregates immunopositive for the mutant Htt protein. To determine the effect of transgene expression at the cellular level, *Drosophila* S2 cells were transiently transfected with the mRFP-HttQ15 or mRFP-HttQ138 constructs and imaged through confocal microscopy. While mRFP-HttQ15 demonstrated diffuse cytoplasmic localization, mRFP-HttQ138 formed large, distinct cytoplasmic aggregates (Fig. 2A).

To assess whether intracellular aggregates are also formed *in vivo* by the transgenic proteins, mRFP-Htt strains were crossed to lines expressing the pan-neuronal C155 elav-GAL4 driver, and 3rd instar larval offspring were imaged using confocal microscopy. As observed in S2 cells, mRFP-HttQ15 remained diffuse throughout the cytoplasm and neurites of neurons in both the CNS (Fig. 2B) and PNS (Fig. 2D). In contrast, distinct Htt aggregates were observed throughout the cytoplasm

and neurites in lines expressing mRFP-HttQ138 (Fig. 2C & E). mRFP-HttQ15 is also diffusely localized in the cytoplasm of non-neuronal cells such as epidermis (Fig. 2E) and salivary glands (Fig. 2G), while mRFP-HttQ138 forms cytoplasmic aggregates (Fig. 2F & H). Nuclear aggregates were not observed in any cell types.

Mutant Htt causes defects in salivary gland glue secretion in *Drosophila*

To determine whether the abundant presence of Htt aggregates in larval salivary glands (Fig. 2G) causes cellular dysfunction, salivary gland secretion of GFP-tagged glue was compared between control animals and animals expressing mRFP-HttQ138. During normal pupariation, *Drosophila* pupae secrete a glue-like substance from the salivary glands to attach the pupal case to a surface (Fig. 3A). While glue secretion is evident in both control and mRFP-HttQ15-expressing pupae (Fig. 3B & C), secretion is dramatically decreased in pupae expressing mRFP-HttQ138 (Fig. 3D), suggesting severe salivary gland dysfunction. Normal 3rd instar larval salivary gland cells are filled with glue (Fig. 3E) that is depleted during pupariation (Fig. 3F); however, the pupal salivary glands of mRFP-HttQ138-expressing larvae retain glue (Fig. 3G), further indicating salivary gland dysfunction mediated by mutant Htt in the cytoplasm.

The 588 aa fragment of mutant human Htt does not show evidence of cleavage in *Drosophila*

Many previous studies of HD have focused on nuclear aggregates, as opposed to the cytoplasmic aggregates observed in our model. Htt is known to undergo cleavage by caspases and calpains into N-terminal fragments that are found in both the nucleus and the cytoplasm (Gafni et al., 2004; Kim et al., 2001; Lunkes et al., 2002; Wellington et al., 2002). Cleavage is thought to be an essential step in the generation of toxic Htt fragments (Qin and Gu, 2004); however, the size(s) of truncated Htt fragments responsible for HD pathology remain to be identified. To determine whether cleavage of the N-terminal 588 aa of Htt occurs in our fly model, S2 cells were transiently transfected with 588 aa Htt constructs labeled with eGFP at the N-terminus and mRFP at the C-terminus. Complete colocalization of the eGFP and mRFP signals is seen for both the normal eGFP-HttQ15-mRFP fragment and the mutant eGFP-HttQ138-mRFP fragment (Fig. 4A), suggesting that cleavage of the proteins does not occur in the context of *Drosophila*.

Figure 2

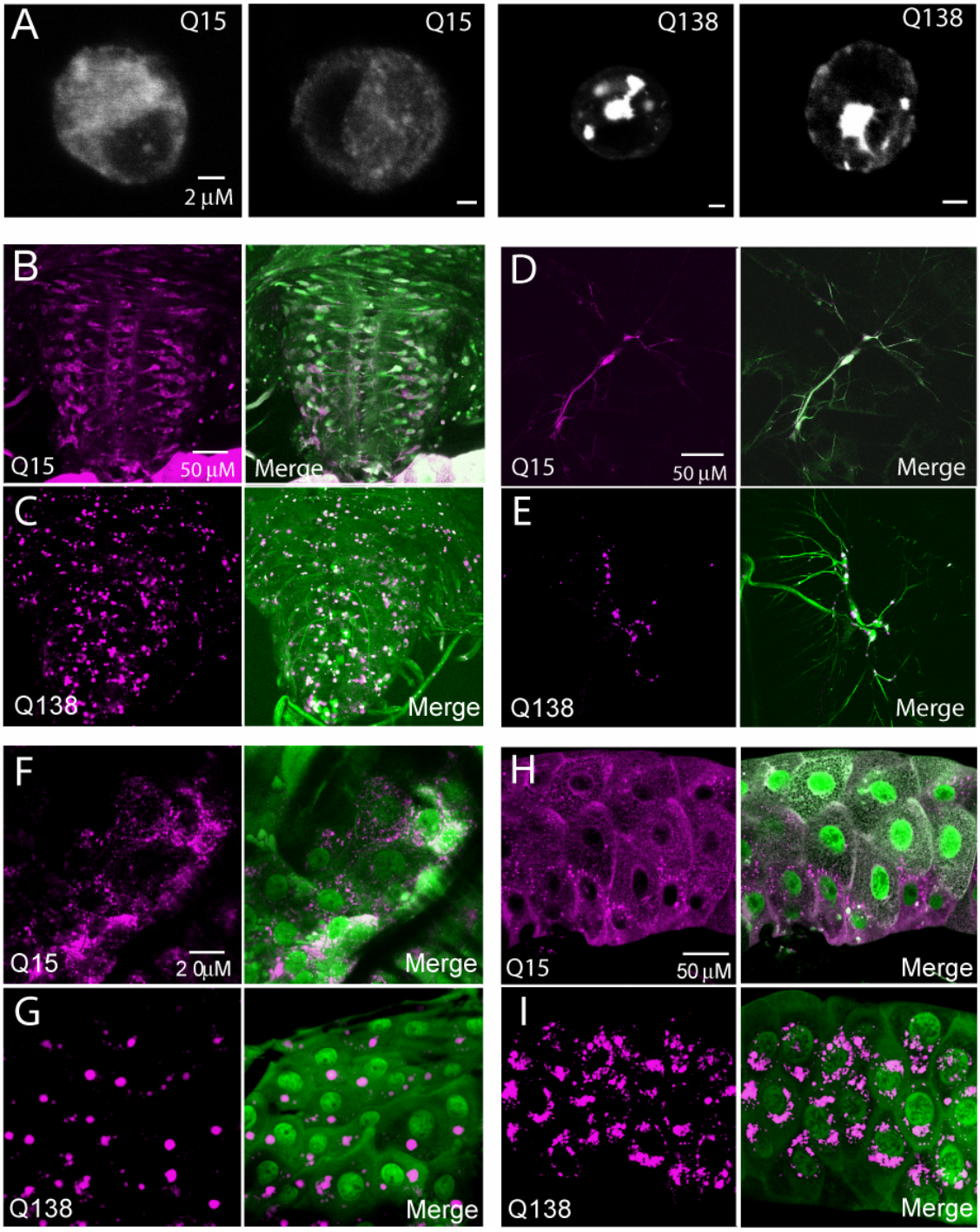
A
Q15
2 µM
Q15
Q138
Q138
B
Q15
50 µM
Merge
D
Q15
50 µM
Merge
C
Q138
Merge
E
Q138
Merge
F
Q15
2 0µM
Merge
H
Q15
50 µM
Merge
G
Q138
Merge
I
Q138
Merge

FIGURE 2**. Cytoplasmic aggregation of mRFP-HttQ138 in neuronal and non-neuronal tissues.** (A) Htt localization in *Drosophila* S2 cells transiently transfected with mRFP-HttQ15 or mRFP-HttQ138. mRFP-HttQ15 is found diffusely throughout the cytoplasm, while mRFP-HttQ138 forms cytoplasmic aggregates. (B) Visualization of mRFP-HttQ15 (magenta) and GFP with a nuclear localization signal (nls) (green) in 3rd instar larvae with transgene expression driven by the C155 elav-GAL4 driver. mRFP-HttQ15 is diffusely localized in the cytoplasm of CNS neurons in the ventral nerve cord. (C) Visualization of mRFP-HttQ138 (magenta) and GFP-nls (green) in CNS neurons of 3rd instar larvae with transgene expression driven by the C155 elav-GAL4 driver. Unlike mRFP-HttQ15, mRFP-HttQ138 forms cytoplasmic aggregates throughout the cell bodies of ventral nerve cord neurons. (D, E) Visualization of mRFP-Htt in peripheral MD neurons. While mRFP-HttQ15 exhibits diffuse cytoplasmic localization, mRFP-HttQ138 is found in cytoplasmic aggregates throughout the cell body and neurites. (F-I) Expression of mRFP-Htt (magenta) and GFP-nls (green) driven by the tubP-GAL4 driver in the epidermis (F, G) and salivary gland (H, I). In all cases, mRFP-HttQ15 is diffuse throughout the cytoplasm, while mRFP-HttQ138 forms cytoplasmic aggregates.

Figure 3

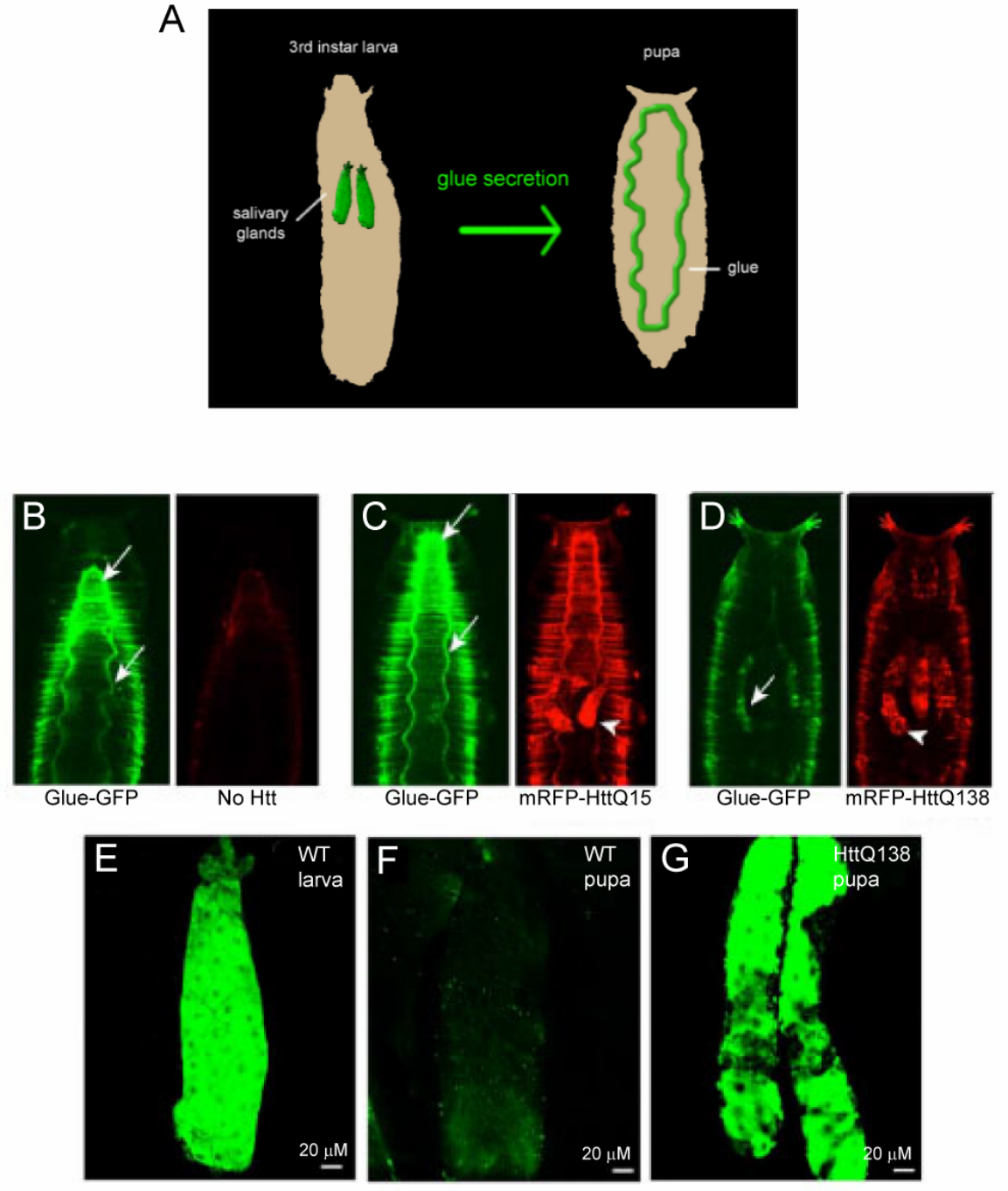
A
3rd instar larva
pupa
glue secretion
salivary glands
glue
B
Glue-GFP
No Htt
C
Glue-GFP
mRFP-HttQ15
D
Glue-GFP
mRFP-HttQ138
E
WT larva
20 μM
F
WT pupa
20 μM
G
HttQ138 pupa
20 μM

FIGURE 3**. Salivary gland glue secretion is defective in *Drosophila* expressing mRFP-HttQ138.** (A) Diagram of normal glue secretion during pupariation. Expression of the glue protein fused to GFP enables *in vivo* imaging of secreted glue. (B) Secretion of glue (green) is normal in control pupae. (C) Secretion of glue (green) is also normal in pupae expressing mRFP-HttQ15 (red). Glue-GFP is indicated by arrows, while mRFP-HttQ15-filled salivary glands are indicated by arrowheads. Expansion of the polyQ tract in Htt is necessary to induce glue secretion defects, as Htt is abundantly present in the salivary glands of mRFP-HttQ15-expressing animals, but does not impair secretion of glue-GFP. (D) Secretion of glue (green) is strikingly decreased in pupae expressing mRFP-HttQ138 (red). Arrows indicate the presence of glue-GFP in the salivary glands where mRFP-HttQ138 is also expressed (indicated by arrowheads). (E) Normal 3rd instar salivary gland cells contain glue that is depleted during pupariation (F). However, the pupal salivary glands of mRFP-HttQ138-expressing animals retain glue (G).

Figure 4

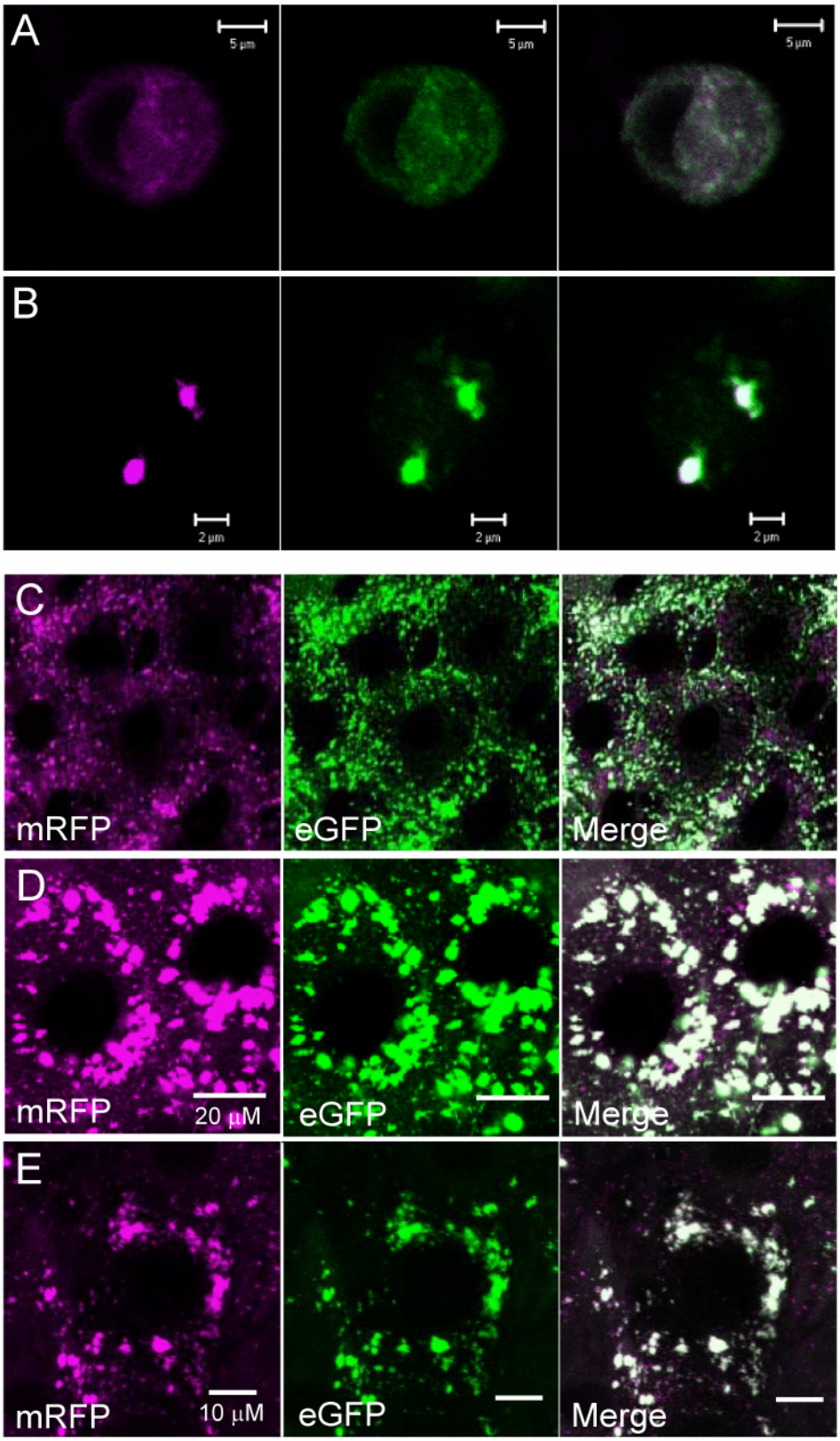
A
5 µm
5 µm
5 µm
B
2 µm
2 µm
2 µm
C
mRFP
eGFP
Merge
D
mRFP
20 µM
eGFP
Merge
E
mRFP
10 µM
eGFP
Merge

FIGURE 4. **The 588 aa fragment of mutant human Htt does not show evidence of cleavage in *Drosophila***. (A) Transient transfection of *Drosophila* S2 cells with eGFP-HttQ138-mRFP demonstrates no separation of eGFP (green) and mRFP (magenta) signals, indicating that the ends of the 588 aa fragment of mutant Htt protein colocalize and that there is no evidence for cleavage of the fragment. Visualization of signal localization in 3rd instar larvae with expression of eGFP-HttQ138-mRFP driven by the C155 elav-GAL4 driver shows no separation of eGFP (green) and mRFP (magenta) signals in brain lobe CNS neurons (B), salivary gland cells (C), or epidermal cells (D).

To assess whether cleavage occurs *in vivo*, we generated transgenic strains expressing the double-labeled mutant Htt fragment eGFP-HttQ138-mRFP. As observed in the S2 cell model, eGFP and mRFP signals colocalized in all tissues studied, including CNS neurons (Fig. 4B), salivary gland cells (Fig. 4C), and epidermal cells (Fig. 4D), demonstrating colocalization of both ends of the mutant Htt protein and suggesting that cleavage of the mutant Htt protein does not occur *in vivo* in *Drosophila.* No fluorescent signal is seen in the nucleus, indicating that even if cleavage does occur, the mutant Htt-mediated toxicity observed in our 588 aa transgenic lines reflects a specific effect of mutant Htt on cytoplasmic processes.

Exon 1 of mutant Htt forms cytoplasmic and neuritic aggregates

Upon cleavage of Htt in HD tissue, the smallest N-terminal fragments are thought to enter the nucleus and form neuronal intranuclear inclusions (NIIs) (DiFiglia et al., 1997; Sieradzan et al., 1999). These intranuclear inclusions are postulated to play a role in HD pathology (Becher et al., 1998; Davies et al., 1997), and are found in many HD models expressing exon 1 of the mutant Htt protein. (Davies et al., 1997; Jackson et al., 1998; Krobitsch and Lindquist, 2000; Tagawa et al., 2004). To determine whether the *in vivo* subcellular localization of the 81 aa exon 1 fragment of mutant human Htt (HttQ96-GFP) differs from that of the 588 aa mutant Htt fragment, HttQ96-GFP-expressing 3rd instar larvae were imaged using confocal microscopy. In both neuronal and non-neuronal cell types, HttQ96-GFP formed distinct cytoplasmic aggregates identical in appearance and localization to those formed by the 588 aa mRFP-HttQ138 protein. GFP-labeled aggregates are found in the cytoplasm of salivary gland cells (Fig. 5B) and epidermal cells (Fig. 5G), as well as in CNS (Fig. 5D) and PNS (Fig. 5F) neurons. As observed with the 588 aa fragment, the exon 1 fragment is also observed in axons (Fig. 5E) and localizes at nerve terminals. The non-nuclear localization of mutant Htt in mRFP-HttQ138 and HttQ96-GFP flies provides an ideal system to characterize the consequences of aggregate formation in the cytoplasm and neurites on neuronal function.

Mutant Htt causes physical blockage of axonal transport and is differentially transported compared to normal Htt

The presence of mutant Htt aggregates in the axon may impair neuronal function by sequestering axonal or synaptic proteins or by physically blocking transport in

individual axons. In order to determine whether the diameter of accumulations of aggregates within the axon is sufficient to block axonal transport, we expressed the mutant HttQ96-GFP protein with a motor axon GAL4 driver, allowing the visualization of UAS-HttQ96-GFP aggregates in single axons in 3rd instar larvae. By co-expressing UAS-RFP in the motor axon to define its structure, it is clear that large aggregate accumulations exceed the diameter of individual axons and cause the axon to swell around the aggregates, in concordance with physical axonal blockage (Fig. 6A-C). While wild-type Htt undergoes both anterograde and retrograde transport (Block-Galarza et al., 1997), long-term imaging of aggregates in live 3rd instar larvae demonstrated that large aggregate accumulations are immobile within the axon, indicating that they may block traffic in both directions (Fig. 6D). Driving expression of transgenes in aCC neurons with the eve-GAL4 driver allows for visualization of both axons and dendrites. In mRFP-HttQ138-expressing animals, mutant Htt aggregates are observed in the dendritic arbor as well as in axons (Fig. 6E), suggesting that Htt aggregates may cause deleterious effects on trafficking in both dendrites and axons.

To characterize the effects of Htt on axonal transport, we analyzed the trafficking of normal and mutant Htt *in vivo*. Movement of mRFP-HttQ15 and mRFP-HttQ138 transport particles can readily be visualized *in vivo* in the motor axons of 3rd instar larvae. Within these axons, transport particles of Htt move in a saltatory manner, with short runs in both anterograde and retrograde directions. Several different variables of movement for each direction, including percentage of time spent traveling in each direction, mean velocity, mean duration of runs, and mean length of runs, were measured for both mRFP-HttQ15- and mRFP-HttQ138-expressing animals (Table 1). An analysis of mRFP-HttQ15 and mRFP-HttQ138 movement revealed striking differences in axonal transport of normal and mutant Htt. The most significant difference observed was an increase in the duration of retrograde runs and the length of axon traveled per retrograde run for mutant Htt particles compared to normal Htt. In addition, anterograde velocity of mRFP-HttQ138 particles was decreased, with a ~6-fold increase in the duration of pauses during anterograde movement compared to mRFP-HttQ15 particles. Mutant Htt is known to undergo an increased interaction with huntingtin-associated protein 1 (HAP1) (Li et al., 1995), which binds to p150glued, an accessory protein for dynein that is involved in retrograde transport (Engelender et al., 1997; Li et al., 1998). Our findings suggest that increased binding of mutant Htt to retrograde transport molecules may increase the duration and length of retrograde runs of mutant

Figure 5

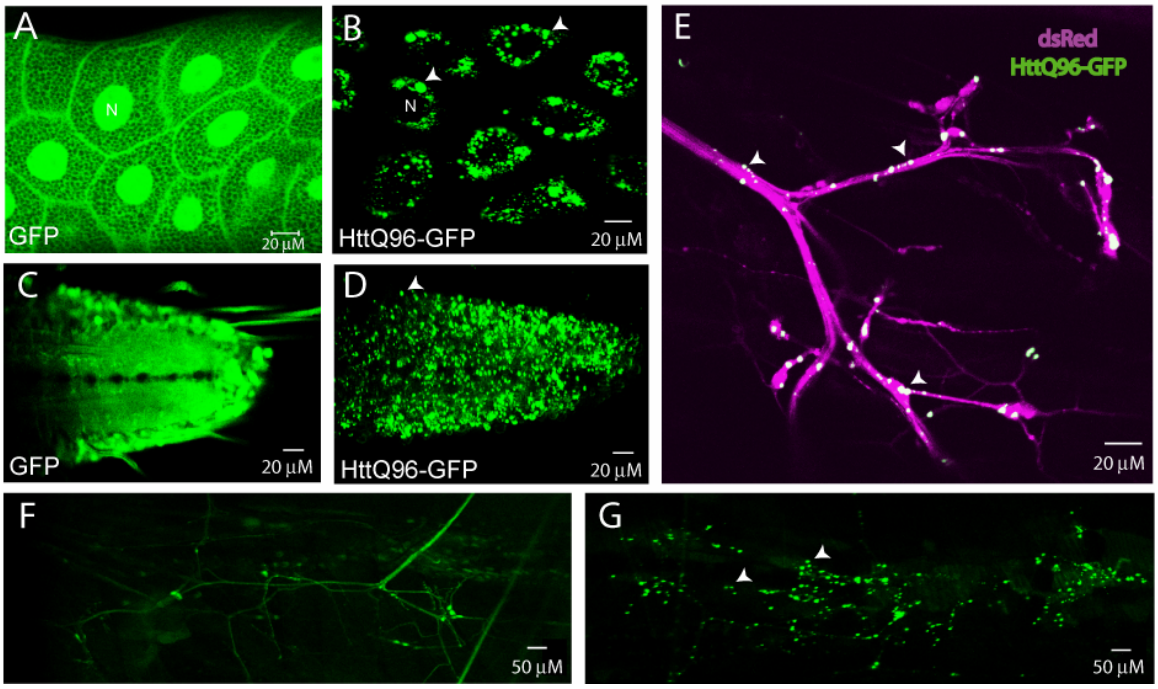
A
N
GFP
20 µM
B
N
HttQ96-GFP
20 µM
E
dsRed
HttQ96-GFP
20 µM
C
GFP
20 µM
D
HttQ96-GFP
20 µM
F
50 µM
G
50 µM

FIGURE 5. **Cytoplasmic aggregation of HttQ96-GFP in neuronal and non-neuronal tissues.** (A) Visualization of GFP (green) in the salivary gland. GFP is found in both the cytoplasm and the nucleus. The nucleus is indicated by N. (B) Visualization of HttQ96-GFP (green) in the salivary gland. Unlike GFP alone, HttQ96-GFP forms cytoplasmic aggregates in the salivary gland. The nucleus is indicated by N and aggregates are indicated by arrowheads. (C, D) Visualization of GFP and HttQ96-GFP in CNS neurons of the ventral nerve cord. GFP is diffusely localized in ventral nerve cord cells, while HttQ96-GFP forms aggregates. Aggregates are indicated by arrowheads. (E) HttQ96-GFP aggregates (green, indicated by arrowheads) are found in axons labeled with dsRed (magenta). (F) In epidermal cells, GFP (green) is diffuse in the cytoplasm. (G) HttQ96-GFP (green) is found in cytoplasmic aggregates (indicated by aggregates) in epidermal cells.

Figure 6

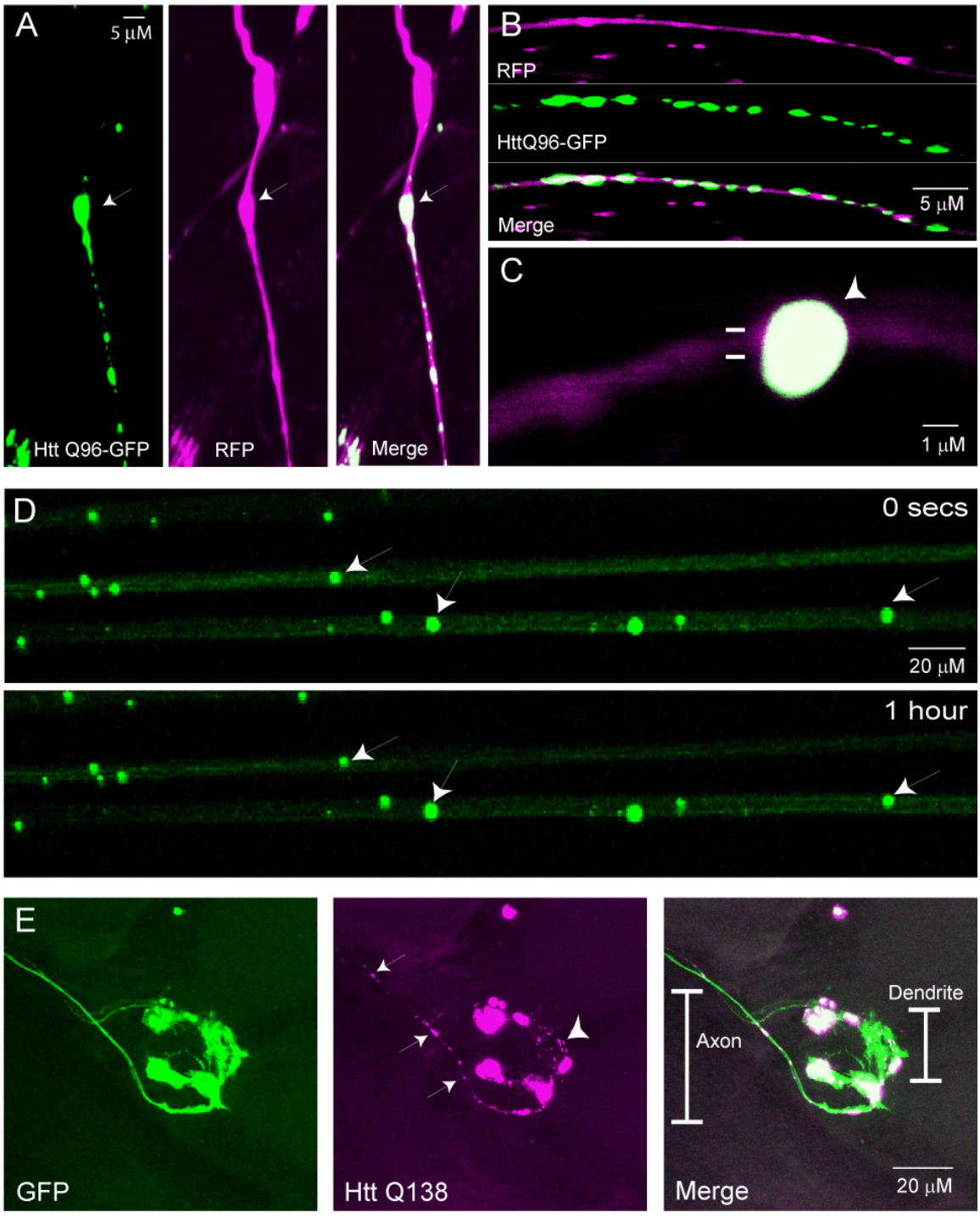
A
5 μM
Htt Q96-GFP
RFP
Merge
B
RFP
HttQ96-GFP
Merge
5 μM
C
1 μM
D
0 secs
20 μM
1 hour
E
GFP
Htt Q138
Axon
Dendrite
Merge
20 μM

FIGURE 6. **Blockage of axons by mutant Htt aggregates.** (A-C) Blockage of axons by mutant Htt aggregates. With transgene expression driven in individual motor axons by the RRa-GAL4 driver, the accumulation of HttQ96-GFP aggregates (green, indicated by the arrowhead) exceeds the diameter of the axon (magenta) and causes it to swell outwards. (D) mRFP-HttQ138 aggregates are immobile in the axon. In live 3rd instar larvae with transgene expression driven by the C155 elav-GAL4 driver, HttQ96-GFP aggregates (green, indicated by arrows) are located in the same area of the axon at 0 seconds and at 1 hour, indicating that the aggregates are immobile. (E) Mutant Htt aggregates are found in both axons and dendrites. Expression of GFP (green) and mRFP-HttQ138 (magenta) with the eve-GAL4 driver demonstrates localization of mRFP-HttQ138 aggregates in both axons (indicated by small arrowheads) and dendrites (indicated by the large arrowhead) in RP2 neurons.

Table 1

	HttQ15		HttQ138	
Particle Class	**Anterograde**	**Retrograde**	**Anterograde**	**Retrograde**
n=# Larvae [objects]	5[25]	5[25]	5[25]	5[25]
Anterograde Runs				
% Tot. Time	**76.34 +/- 3.53**	10.61+/-1.64	**76.06 +/- 4.35**	7.49+/-1.86
Velocity (μm/s)	**0.77 +/- 0.06**	0.38+/-0.02	**0.59 +/- 0.06**	0.37+/-0.03
Duration (s/run)	**13.40 +/- 1.47**	2.31+/-0.08	**17.04 +/- 2.80**	2.79+/-0.24
Length (μm/run)	**14.99 +/- 2.24**	1.14+/-0.14	**17.17 +/- 3.71**	1.08+/-0.63
Retrograde Runs				
% Total Time	4.80+/-1.28	**55.87+/-4.08**	4.70+/-1.26	**63.22+/-5.05**
Velocity (μm/s)	-0.50 +/-0.04	**-0.70+/-0.03**	-0.52+/-0.06	**-0.84+/-0.06**
Duration (s/run)	2.75+/-0.25	**5.64+/-0.36**	2.24+/-0.13	**10.32+/-1.96**
Length (μm/run)	0.16+/-0.10	**4.31+/-0.38**	1.61+/-0.22	**11.41+/-3.11**
Pauses				
% Total Time	**18.86+/-3.30**	**33.53+/-3.45**	**19.25+/-3.89**	**29.29+/-4.14**
Duration (s)	**0.67+/-0.18**	**3.48+/-0.69**	**3.60+/-0.97**	**3.49+/-0.58**

Htt-associated cargoes, while decreasing anterograde velocity and increasing the duration of anterograde pauses. These defects raise the possibility of non-aggregate-dependent alterations in axonal transport, in addition to physical blocks from large, non-motile aggregates.

Axonal transport cargoes are trapped by accumulations of mutant Htt aggregates

If aggregates are causing a physical blockage of axonal transport, axonal cargoes should be obstructed by sites of larger Htt aggregate accumulations that approach or exceed the diameter of the axon, but not by smaller accumulations. We coexpressed several GFP-tagged proteins that normally undergo axonal trafficking in the context of either the normal mRFP-HttQ15 protein or the mutant mRFP-HttQ138 protein to observe their localization within the axon. While all of the cargoes display diffuse or punctuate staining when coexpressed with mRFP-HttQ15, cargoes clearly colocalize with larger areas of mRFP-HttQ138 aggregate accumulation in the case of the dense core vesicle marker ANF (Fig. 7A), synaptic vesicle markers synaptotagmin (Fig. 7B) and synaptobrevin (Fig. 7C), clathrin-coated vesicle marker Chc (Fig. 7D), and the endosomal marker Rab11 (Fig. 7E). mRFP-HttQ138 aggregates are also able to sequester cytosolic proteins such as *Drosophila* complexin (Fig. 7F). Axonal swellings seen around accumulations of aggregates suggest that large Htt accumulations block and distort axons, sequestering a variety of cargoes required for normal synaptic function.

Aggregate-dependent versus –independent axonal transport defects vary with specific cargo

Mutant Htt may cause defects in axonal transport through aggregate-independent means, including loss of normal Htt function (Gunawardena et al., 2003; Trushina et al., 2004) or sequestration of key transport proteins (Trushina et al., 2004). To address this possibility in our *Drosophila* model, we imaged sites of synaptotagmin accumulation, marking areas of impaired axonal transport, in 100 μM axon segments of mRFP-HttQ15- and mRFP-HttQ138-expressing 3rd instar larvae. Sites of colocalization between synaptotagmin accumulations and Htt can be differentiated from sites of Htt-independent synaptotagmin accumulation, providing a method to compare aggregate-dependent and aggregate-independent blockages. If expression of mutant Htt results in general, aggregate-independent transport defects, the number of aggregate-

Figure 7

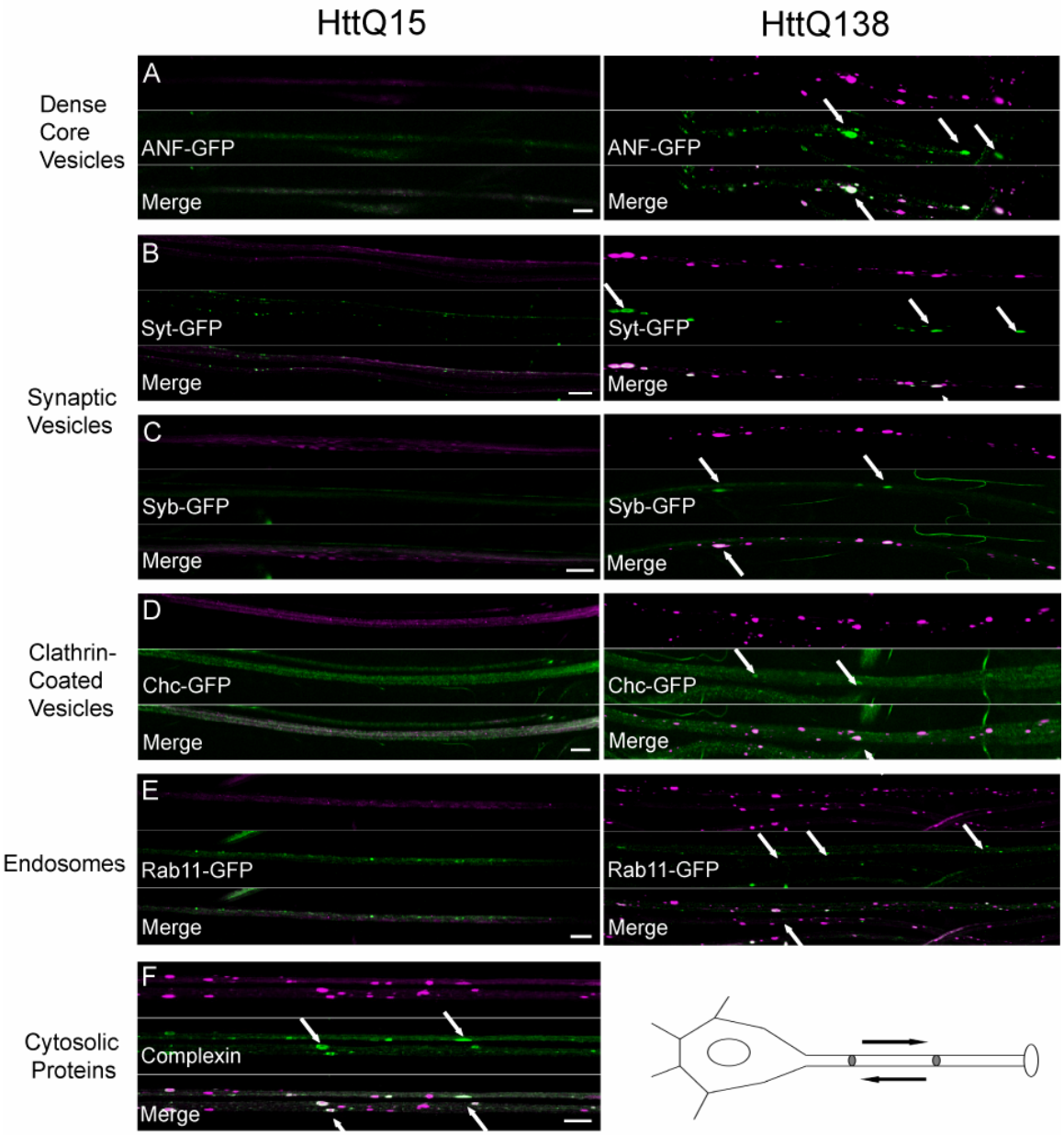
HttQ15
HttQ138
Dense Core Vesicles
A
ANF-GFP
Merge
ANF-GFP
Merge
Synaptic Vesicles
B
Syt-GFP
Merge
Syt-GFP
Merge
C
Syb-GFP
Merge
Syb-GFP
Merge
Clathrin-Coated Vesicles
D
Chc-GFP
Merge
Chc-GFP
Merge
Endosomes
E
Rab11-GFP
Merge
Rab11-GFP
Merge
Cytosolic Proteins
F
Complexin
Merge

FIGURE 7. **Mutant Htt aggregates trap axonal transport cargo**. Coexpression of mRFP-HttQ15 or mRFP-HttQ138 (magenta) with various axonal transport cargoes (green) was driven by the C155 elav-GAL4 driver. (A) Dense core vesicles, labeled by ANF-GFP (green), colocalize with mRFP-HttQ138 aggregates (magenta) in areas indicated by the arrows. (B-C) Synaptic vesicles, labeled by Syt1-GFP or Syb-GFP (green), colocalize with mRFP-HttQ138 aggregates (magenta) in areas indicated by the arrows. Colocalization with mRFP-HttQ138 aggregates (magenta) is also seen for clathrin-coated vesicles, labeled by Chc-GFP (green) (D), endosomes, labeled by Rab11-GFP (green) (E), and cytosolic proteins such as complexin (green) (F).

Figure 8

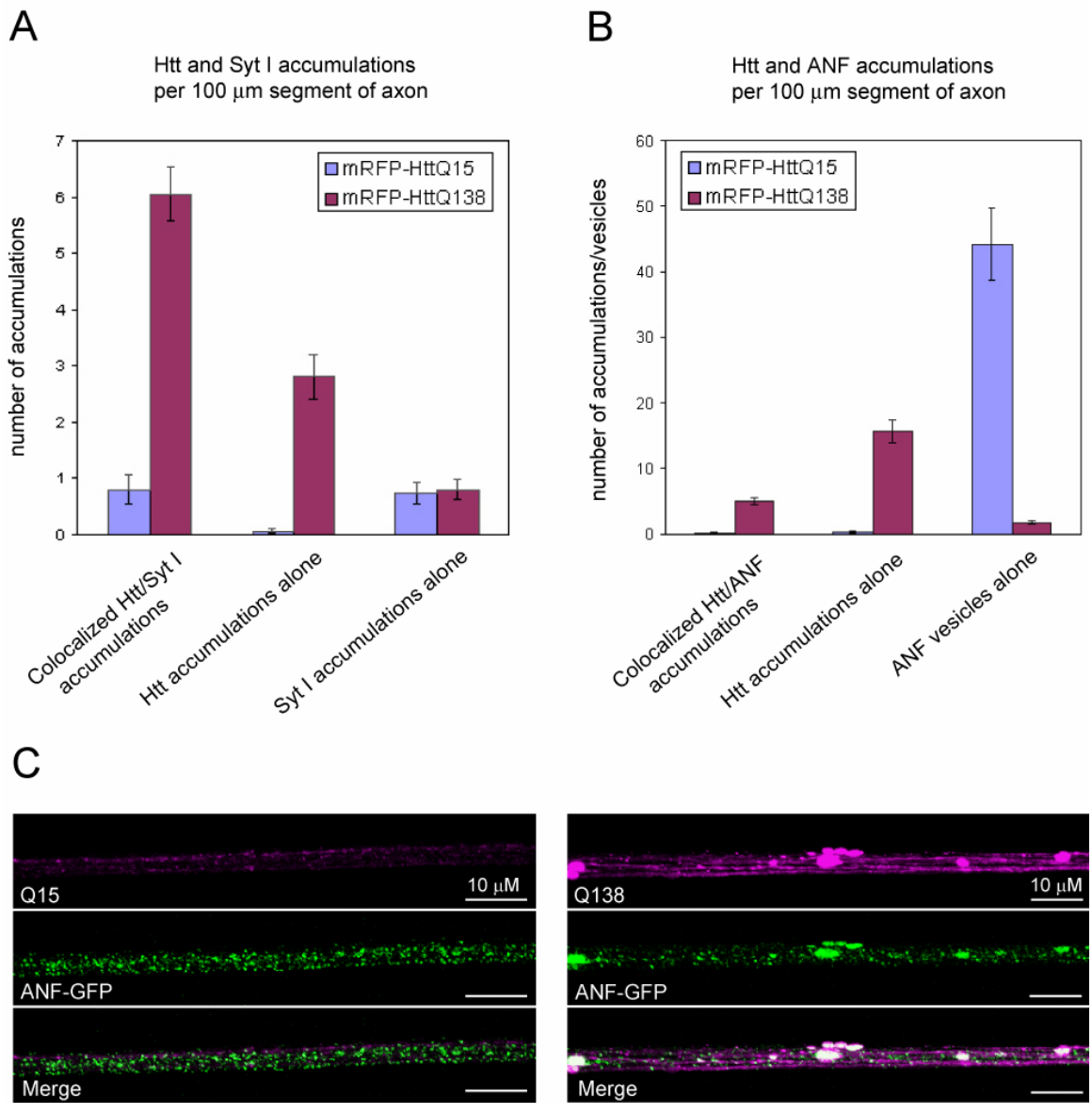
A
Htt and Syt I accumulations
per 100 μm segment of axon
number of accumulations
mRFP-HttQ15
mRFP-HttQ138
Colocalized Htt/Syt I accumulations
Htt accumulations alone
Syt I accumulations alone
B
Htt and ANF accumulations
per 100 μm segment of axon
number of accumulations/vesicles
mRFP-HttQ15
mRFP-HttQ138
Colocalized Htt/ANF accumulations
Htt accumulations alone
ANF vesicles alone
C
Q15
10 μM
ANF-GFP
Merge
Q138
10 μM
ANF-GFP
Merge

FIGURE 8. **Effects of mutant Htt expression on vesicular transport.** (A) To determine the effect of mutant Htt expression of synaptic vesicle transport, areas of accumulation for mRFP-HttQ138 alone, Syt1-GFP alone, and areas where mRFP-HttQ138 and Syt1-GFP accumulations colocalized were quantified per 100 μm motor axon segment for 30 segments. Bars indicate SEM. (B) To determine the effect of mutant Htt expression on dense core vesicle transport, areas of accumulation for mRFP-HttQ138 alone, ANF-GFP alone, and areas of colocalization between mRFP-HttQ138 and ANF-GFP accumulations were quantified per 100 μm motor axon segment for 30 segments. Bars indicate standard error. (C) Visualization of ANF-GFP (green) in motor axon segments. Non-aggregate-associated ANF-GFP levels are visibly decreased in motor axons of animals expressing mRFP-HttQ138 (magenta) compared to animals expressing mRFP-HttQ15 (magenta).

independent synaptotagmin accumulations in mRFP-HttQ138-expressing animals should exceed that of mRFP-HttQ15-expressing animals. However, no difference is seen between numbers of non-Htt-colocalized synaptotagmin accumulations between mRFP-HttQ15 and mRFP-HttQ138 (Fig. 8A), suggesting that impairment of axonal transport of synaptic vesicle proteins is predominately due to aggregate-dependent physical blockage of the axon.

In contrast to results observed with the synaptic vesicle marker synaptotagmin, visualization of the dense core vesicle marker ANF in 100 μM segments of axon suggests that expression of mutant Htt may also affect axonal transport in an aggregate-independent manner. A striking decrease in the number of dense core vesicles per 100 μM segment of axon is observed in mRFP-HttQ138-expressing lines as compared to mRFP-HttQ15-expressing lines (Fig. 8B & C). There is no significant increase in the number of aggregate-independent blockage sites, suggesting that the decrease in vesicle number is not due to a general defect in axonal transport, but may instead be due to mutant Htt-mediated disruption of interactions between dense core vesicles and transport machinery within the axon.

Mitochondrial transport is disrupted in mRFP-HttQ138-expressing animals

In addition to defects in dense core vesicle transport, mRFP-HttQ138-expressing animals also exhibit defects in mitochondrial transport. Mitochondria continuously attach to and detach from axonal transport systems in order to localize to sites where they are needed for calcium buffering or ATP production in the axon or synapse (Hollenbeck and Saxton, 2005). Thus, regulation of mitochondrial transport is essential to the health of the neuron. To observe the localization of mitochondria in axons of mutant Htt-expressing animals, mRFP-HttQ138-expressing flies were crossed to flies expressing mitochondria tagged with GFP (mito-GFP). Mitochondria were observed to colocalize more with mRFP-HttQ15 than with mRFP-HttQ138 (Fig. 9A & B), suggesting that normal Htt may interact with mitochondria and that mutation of Htt may reduce this interaction. In concordance with a role for normal Htt in mitochondrial trafficking, observation of *in vivo* transport in the motor axons of 3rd instar larvae revealed colocalized movement between mito-GFP and a subset of mRFP-HttQ15 transport particles (unpublished data). However, most mRFP-HttQ15 particles move independently of mito-GFP, suggesting that mitochondria may represent only a subset of normal Htt-dependent cargo. Interestingly, a significant reduction in the number of axonal mitochondria was seen in

mRFP-HttQ138-expressing lines in comparison to mRFP-HttQ15-expressing lines (Fig. 9C & D), indicating that disruption of the interaction between normal Htt and mitochondria may reduce mitochondrial transport within the axon.

Mutant Htt aggregates sequester normal Htt

If normal Htt plays a role in axonal transport of mitochondria or other transport cargoes, Htt mutation may cause trafficking defects by decreasing the amount of normally functioning Htt within the axon. This could occur through haploinsufficiency and/or through interference of mutant Htt with normal Htt function. To determine whether mutant Htt is able to recruit normal Htt into aggregates, 3rd instar larvae expressing both mRFP-HttQ138 and eGFP-HttQ15 were imaged to visualize the localization of mutant and normal Htt coexpressed within the same tissue. While eGFP-HttQ15 expressed by itself exhibits a diffuse cytoplasmic localization pattern, eGFP-HttQ15 expressed in the presence of mRFP-HttQ138 becomes partially recruited into mutant Htt aggregates in salivary glands (Fig. 10A) and, importantly, in axons (Fig. 10B), where sequestration of normal Htt could interfere with its putative role in axonal transport. This suggests that mutant Htt may disrupt normal Htt function in a dominant negative manner and that HD may in part be caused by loss of function of the normal Htt protein.

Figure 9

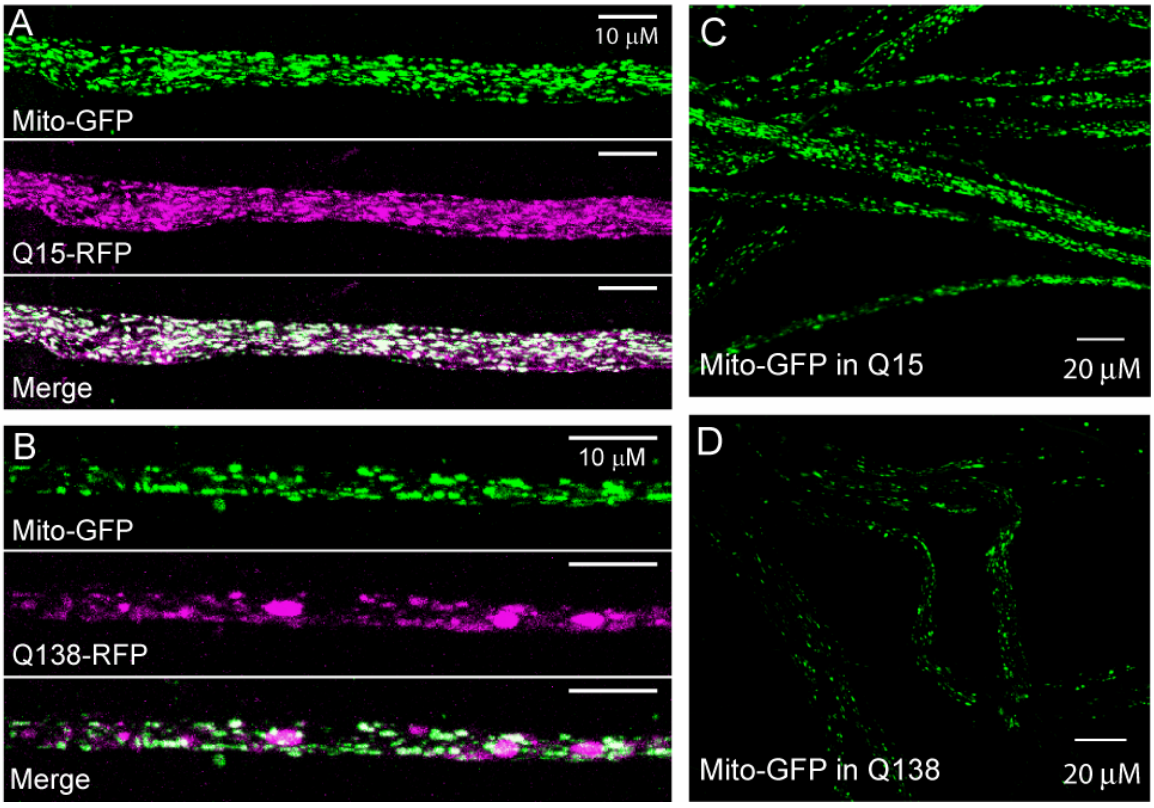
A
10 μM
Mito-GFP
Q15-RFP
Merge
B
10 μM
Mito-GFP
Q138-RFP
Merge
C
Mito-GFP in Q15
20 μM
D
Mito-GFP in Q138
20 μM

FIGURE 9. **Mutant Htt affects mitochondrial localization.** (A) *In vivo* visualization of mitochondria in motor axons of 3rd instar larvae coexpressing mRFP-HttQ15 (magenta) with GFP-tagged mitochondria (mito-GFP, green) driven by the C155 elav-GAL4 driver. Partial colocalization of mRFP-HttQ15 with mitochondria is seen. (B) In 3rd instar larve coexpressing mRFP-HttQ138 (magenta) and mito-GFP (green), less colocalization is seen. (C) In mRFP-HttQ15-expressing animals, mito-GFP (green) is abundant within motor axons. (D) In comparison, mRFP-HttQ138-expressing animals exhibit a lower concentration of mito-GFP in motor axons.

Figure 10

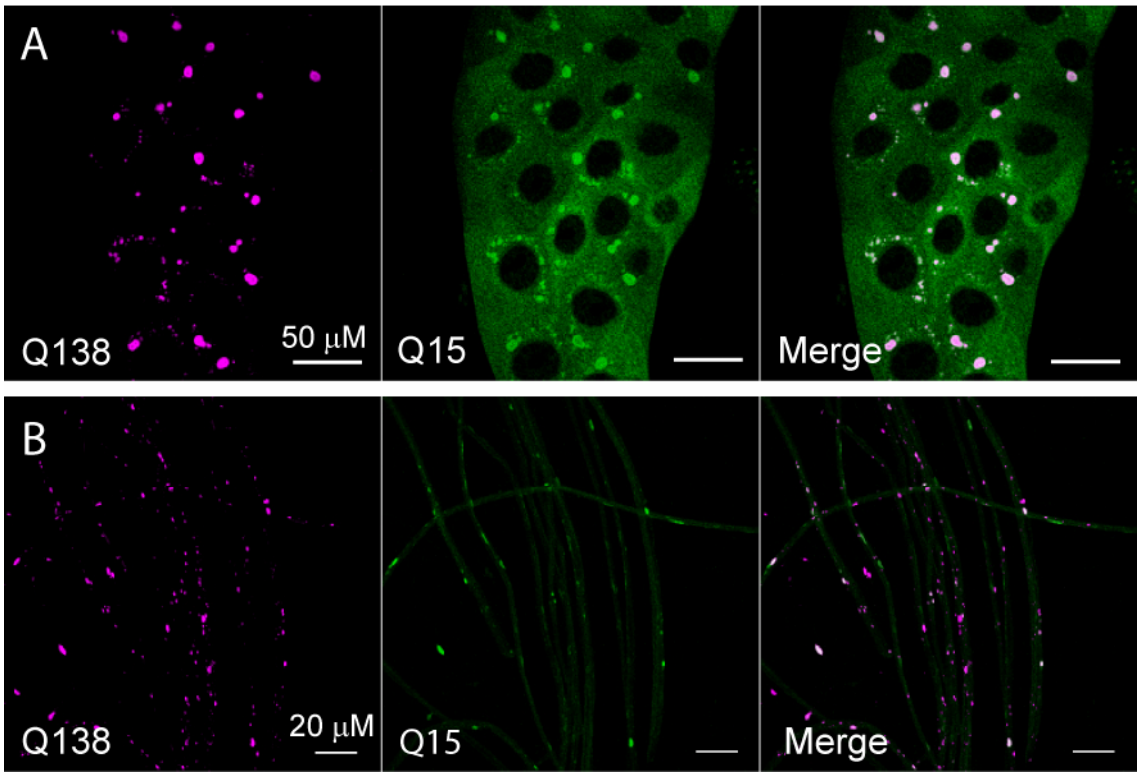

FIGURE 10. **Mutant Htt aggregates sequester normal Htt.** In 3rd instar larvae coexpressing mRFP-HttQ138 and eGFP-HttQ15 with the C155 elav-GAL4 driver, eGFP-HttQ15 (green) colocalizes with mRFP-HttQ138 aggregates (rmagenta) in salivary gland cells (A) and motor axons (B).

Discussion

Overview

Many neurodegenerative diseases caused by protein misfolding have been modeled in *Drosophila*, including Parkinson's disease (Feany and Bender, 2000), Alzheimer's disease (Wittmann et al., 2001), spinocerebeller ataxia type 1 (Fernandez-Funez et al., 2000) and type 3 (Warrick et al., 1999), and Huntington's disease (Gunawardena et al., 2003; Jackson et al., 1998; Lee et al., 2004; Steffan et al., 2001). These models replicate neuropathological features characteristic of the diseases, such as late onset, progressive neurodegeneration, and formation of inclusions containing the mutant protein. Development of such disease models may facilitate the identification of molecular pathways that lead to neurodegeneration in the corresponding human disorders.

We have demonstrated formation of non-nuclear aggregates in *Drosophila* HD models expressing either 588 aa or 81 aa N-terminal fragments of mutant Htt, providing an ideal *in vivo* environment in which to study the role of Htt aggregates in the cytoplasm and neurites and the effects of mutant Htt expression on axonal transport. Accumulations of mutant Htt aggregates exceed the diameter of motor axons, indicating a physical blockage of axonal transport. Visualization of both vesicular and cytosolic GFP-tagged axonal transport cargoes reveals abnormal accumulation of cargoes at sites where aggregates physically block the axon. In the case of synaptic vesicles, aggregate-independent cargo accumulations do not increase in the presence of mutant Htt as compared to normal Htt, suggesting that accumulation of synaptic vesicles at sites colocalizing with Htt aggregates results from physical blockage by aggregates rather than from general, blockage-independent effects of mutant Htt on transport.

In addition to physical blockage of axonal trafficking through accumulation of Htt aggregates in the axon, mutant Htt also decreases the association of certain transport cargoes with the axonal transport machinery. The concentration of both dense core vesicles and mitochondria is reduced in motor axons of mutant Htt-expressing animals, suggesting that mutation of Htt disrupts the ability of these cargoes to interact with motor protein complexes and undergo transport from the cytosol into the axon. This may be caused by loss of wild-type Htt function in axonal transport or by abnormal properties of mutant Htt. Coexpression of normal with mutant Htt demonstrates that normal Htt can

be sequestered by mutant Htt aggregates, suggesting that pathology may be caused by aggregation of Htt and by loss of normal Htt function through recruitment into aggregates.

Axonal transport defects in HD

In HD, aggregates of mutant Htt are found in both the nucleus and cytoplasm of neurons in the striatum and cortex. While nuclear aggregate-mediated impairment of transcription is proposed to play a key role in polyQ disease neuropathology (Ross, 2002), several lines of evidence indicate that the presence of aggregates in the nucleus does not correlate with neurodegeneration (Klement et al., 1998; Saudou et al., 1998). Indeed, in HD, formation of neuropil aggregates is more highly correlated with neuronal dysfunction (Li et al., 1999a). In addition, neuritic degeneration precedes cell body degeneration in cultured striatal neurons (Li et al., 2001), and is observed in presymptomatic HD patients (Albin et al., 1990), indicating a potential role for neuropil aggregates in early HD pathology.

Axons may be particularly vulnerable to aggregate-mediated pathology. Defects in axonal transport have been implicated in a number of neurodegenerative diseases, including HD, Alzheimer's disease, Parkinson's disease, and motor neuron diseases (Raff et al., 2002). Neurons rely on axonal trafficking over long distances to support the axon and the synapse and to deliver survival signals from the synapse to the cell body; bidirectional blockage of transport by aggregates may result in synaptic dysfunction, axonal degeneration, and cell death. Indeed, loss of axonal motor proteins, including kinesin, dynein, and dynactin, can cause neurodegenerative phenotypes (Hafezparast et al., 2003; LaMonte et al., 2002; Reid et al., 2002; Zhao et al., 2001). Htt aggregates in the axon have been shown to block transport of exogenous markers (Li et al., 2001), synaptic vesicles (Lee et al., 2004), and organelles (Chang et al., 2006). Our data provides evidence for aggregate-dependent blockage of synaptic vesicles, dense core vesicles, clathrin-coated vesicles, endosomes, and cytoplasmic proteins, indicating that Htt aggregates within the axon cause widespread impairment of axonal trafficking. While aggregates may not be toxic in the cell body, and may indeed serve a neuroprotective role (Arrasate et al., 2004), aggregates in the axon may block axonal transport and contribute to HD pathogenesis.

In addition to aggregate-dependent impairment of axonal transport, loss of wild-type Htt may also result in axonal trafficking defects. Although the precise function of Htt

has yet to be determined, the protein is enriched in membrane-containing compartments (DiFiglia et al., 1995), is transported in both anterograde and retrograde directions (Block-Galarza et al., 1997), and interacts with microtubules (DiFiglia et al., 1995) and the p150glued component of dynactin (via HAP1) (Engelender et al., 1997; Li et al., 1998), suggesting that it may be vital for axonal trafficking. Decreasing normal Htt function has been shown to impair axonal trafficking in *Drosophila* (Gunawardena et al., 2003) and mammalian neurons (Trushina et al., 2004), and recent data suggests that wild-type Htt may play a role in transport of BDNF, a neurotrophic support molecule (Gauthier et al., 2004). We have shown that mutant Htt aggregates are able to sequester normal Htt, indicating that axonal transport defects seen in HD may be due not only to physical blockage by mutant Htt aggregates and disrupted interactions between mutant Htt and Htt binding partners, but also mutant Htt-mediated loss of normal Htt function.

Effects of impaired axonal transport can be severe. In mice expressing mutant Htt, a decrease of density in synaptic vesicles is seen at synapses in the brain, resulting in decreased neurotransmitter release (Li et al., 2003a). Such synaptic dysfunction may play a role in the acute motor and cognitive deficits seen in HD before widespread neuronal loss. Additionally, defects in axonal transport can result in a lack of axonal support and maintenance. Axonal damage and subsequent degeneration are seen in a variety of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, motor neuron disease, and Huntington's disease (Raff et al., 2002). Degeneration of the axon prevents the transport of neurotrophic factors from the synapse to the cell body, leading to neuronal death, and may be responsible for the neurodegenerative phenotypes seen in these diseases.

Mitochondrial transport defects in HD

One specialized role of axonal transport is the trafficking of mitochondria to and from the axon and synapse. The dynamic distribution of mitochondria is carefully regulated in order to ensure that the organelles are targeted to areas of the neuron with energy or calcium buffering needs. Recent studies suggest that normal Htt may play a role in axonal trafficking of mitochondria; a 50% decrease in wild-type Htt results in a decrease in the motility of mitochondria (Trushina et al., 2004). Additionally, in *Drosophila* lacking Milton, the *Drosophila* homologue of HAP1, mitochondria fail to enter the axon and remain in the cell body (Stowers et al., 2002), suggesting a link between

Htt and mitochondrial transport in the axon. In *Drosophila* expressing mutant Htt, we have observed a defect in mitochondrial transport in motor axons. While normal Htt is seen to colocalize with mitochondria in the axon, colocalization of mitochondria with mutant Htt is reduced, indicating that expansion of the polyglutamine repeat in the Htt protein may disrupt a potential interaction of Htt with mitochondria. We have also observed a decrease in the density of mitochondria within the axon in mutant Htt-expressing animals. A similar decrease was observed in the number of mitochondria entering Htt aggregate-containing segments of axon in cortical neurons (Chang et al., 2006). Mutation of Htt may limit or abolish its normal function in axonal transport. Alternatively, aggregates of mutant Htt could disrupt transport by sequestering mitochondria (Chang et al., 2006), or by titrating normal Htt or other mitochondrial trafficking proteins in the axon, leading to pathological defects in mitochondrial distribution in the axon and at the synapse.

Proper distribution of mitochondria in the axon is essential to neuronal health. Mitochondria with high membrane potential necessary for ATP production move towards the synapse, while old or damaged mitochondria are transported back to the cell body for repair or autophagy (Miller and Sheetz, 2004). Defects in mitochondrial transport may have a twofold effect: first, lack of healthy mitochondria at sites of high energy demand or tight calcium regulation, such as the axon and the synapse, will keep the metabolic and calcium buffering needs of the neuron from being met. Sufficient ATP production is necessary for maintenance of ionic and voltage gradients and for the activity of many cellular components, while proper calcium buffering is important for many neuronal functions and plays an essential role in synaptic plasticity; defects in calcium homeostasis caused by impaired mitochondrial buffering may contribute to cognitive defects seen in HD. Second, failure to recycle aged or damaged mitochondria may lead to mitochondrial production of reactive oxygen species and release of cytochrome C, resulting in apoptotic death of neurons (Lee and Wei, 2000). Indeed, mice expressing the mutant Htt protein exhibit degenerated mitochondria in axons (Li et al., 2001) and at axon terminals (Li et al., 2003a), and several studies indicate that mitochondrial function is impaired in mutant Htt-expressing models (Grunewald and Beal, 1999), providing evidence that aged/damaged mitochondria are not properly processed in HD neurons. Additionally, systemic administration of mitochondrial toxins to rodents and primates causes HD-like pathology, including striatal lesions and choreiform movement disorders (Browne and Beal, 2004), further suggesting a role for

mitochondrial dysfunction in HD. Impairment of mitochondrial transport, by depriving the axon of healthy mitochondria while failing to remove degenerated mitochondria, may play a key role in causing the cognitive and motor defects and neuropathology seen in HD.

In vivo rates of Htt transport

Using fluorescently-tagged normal and mutant Htt, we were able to visualize transport of Htt particles *in vivo* in *Drosophila*. While mutant Htt aggregates remain immobile, transport particles of both normal and mutant Htt undergo saltatory, bidirectional patterns of movement. Strikingly, transport particles of mutant Htt exhibit a dramatic increase in the duration of retrograde runs and in the length of axon traveled per retrograde run in comparison to normal Htt. Additionally, the anterograde velocity of mutant Htt particles is decreased, while duration of pauses during anterograde runs is increased. One possible explanation lies in the fact that mutant Htt binds HAP1 more tightly than normal Htt (Li et al., 1995); HAP1 directly interacts with p150glued, the largest component of the dynactin complex, which is required by dynein for retrograde transport (Li et al., 1998). The increased duration and length traveled for retrograde runs and decreased velocity and longer pauses in anterograde runs may reflect tighter binding of mutant Htt to the retrograde transport machinery. If Htt normally functions in axonal transport, changes in transport variables for Htt may impact the trafficking of all Htt-associated cargoes. Future studies will test for colocalization of movement between Htt transport particles and fluorescently-tagged transport cargoes in order to determine a subset of cargoes requiring Htt for transport, further clarifying the role of Htt in axonal trafficking. In addition, visualization of potential changes in transport of these cargoes in a mutant Htt background may elucidate cellular pathways that lead to HD pathology.

Future Directions

Our data indicates that wild-type Htt may have a role in axonal transport of cargoes including mitochondria and dense core vesicles. Future studies will focus on real-time imaging of *in vivo* axonal transport with fluorescently-tagged Htt and cargo proteins. Observation of cotransport between wild-type Htt and cargo may help to define the normal role of Htt in axonal trafficking, while effects of mutant Htt expression on transport may elucidate aggregate-dependent and –independent ways in which axonal transport defects contribute to HD pathogenesis. In addition, generation of a fluorescently-tagged version of the *Drosophila* Htt homologue may provide the most relevant information regarding normal Htt transport activity *in vivo* in flies.

Future work will also include electrophysiological analysis of mRFP-Htt-expressing lines. Interestingly, the ability to visualize Htt localization at the 3rd instar neuromuscular junction (NMJ) synapse has led to the observation that approximately one-third of these synapses contain abundant levels of Htt (Fig. 11A) while the other two-thirds exhibit no discernible Htt presence (Fig. 11B). Distribution of synapses with and without Htt appears to be random in each larva.

The ability to differentiate between synapses that physically contain Htt and those that do not enables us to perform a detailed analysis of the role of mutant Htt in synaptic pathology. Synaptic dysfunction limited to synapses containing mutant Htt would indicate that toxic effects are exerted at the synapse, whereas if synaptic defects are seen in all synapses of animals expressing mutant Htt, regardless of visible Htt presence, this may suggest mutant Htt-mediated dysfunction at the level of axonal transport or even at the level of gene transcription. Studies will include analysis of miniature excitatory junctional potentials (mEJPs), excitatory junctional potentials (EJPs), paired-pulse facilitation, and high-frequency stimulation, in order to examine the effect of mutant Htt on spontaneous vesicle fusion, basal neurotransmitter release, plasticity, and vesicle recyling, respectively. If synaptic dysfunction requires the presence of mutant Htt at synapses, it may be possible to alleviate HD pathology by preventing localization of mutant Htt to synaptic terminals.

Figure 11

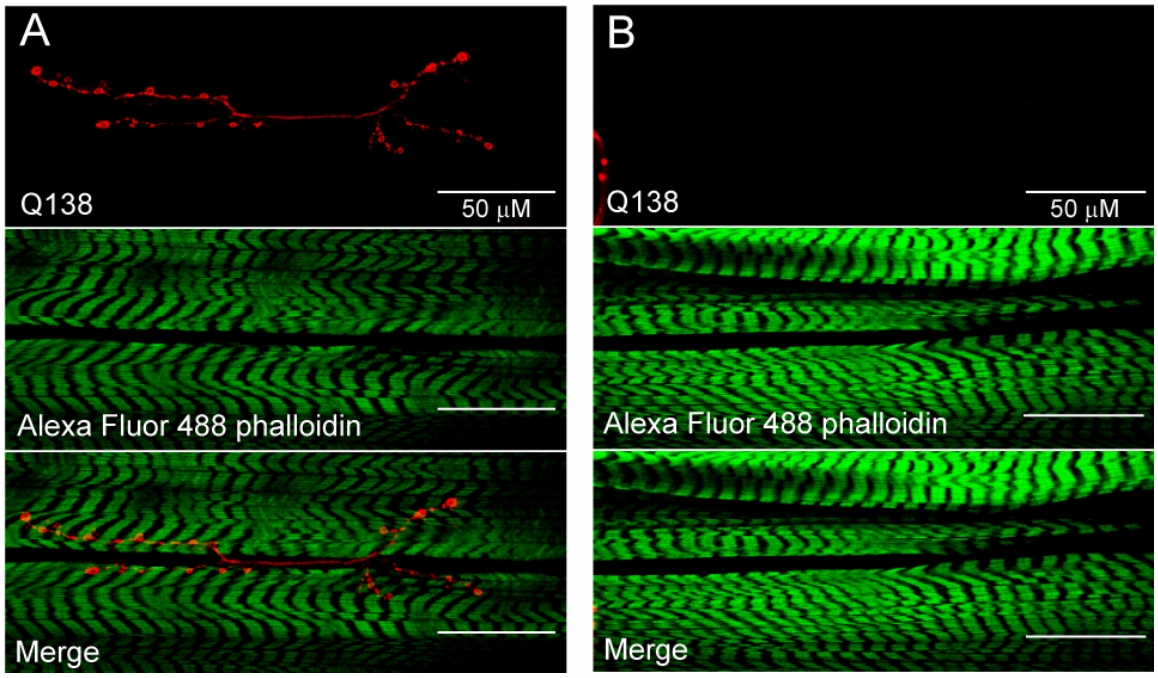
A
Q138
50 μM
Alexa Fluor 488 phalloidin
Merge
B
Q138
50 μM
Alexa Fluor 488 phalloidin
Merge

FIGURE 11. **Localization of mutant Htt to larval NMJ synapses is stochastic.** (A) Approximately one-third of muscle 6/7 NMJ synapses in 3rd instar larvae exhibit abundant levels of mRFP-HttQ138 (red). (B) mRFP-HttQ138 (red) is not observed at two-thirds of muscle 6/7 NMJ synapses. Muscles are stained with Alexa Fluor® 488 phalloidin (green).

Acknowledgments

We thank David Housman for kindly providing the HttQ96-GFP construct, Katie Lynch for help with subcloning the HttQ96-GFP construct, and Albert Su and Rupali Avasare for performing the *Drosophila* S2 cell experiments. This work was supported by grants from the NIH, the David and Lucile Packard Foundation, and the Merck/MIT Collaboration Program.

References

Albin, R. L., Reiner, A., Anderson, K. D., Penney, J. B., and Young, A. B. (1990). Striatal and nigral neuron subpopulations in rigid Huntington's disease: implications for the functional anatomy of chorea and rigidity-akinesia. *Ann Neurol,* **27**, 357-365.

Arrasate, M., Mitra, S., Schweitzer, E. S., Segal, M. R., and Finkbeiner, S. (2004). Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. *Nature,* **431**, 805-810.

Becher, M. W., Kotzuk, J. A., Sharp, A. H., Davies, S. W., Bates, G. P., Price, D. L., and Ross, C. A. (1998). Intranuclear neuronal inclusions in Huntington's disease and dentatorubral and pallidoluysian atrophy: correlation between the density of inclusions and IT15 CAG triplet repeat length. *Neurobiol Dis,* **4**, 387-397.

Block-Galarza, J., Chase, K. O., Sapp, E., Vaughn, K. T., Vallee, R. B., DiFiglia, M., and Aronin, N. (1997). Fast transport and retrograde movement of huntingtin and HAP 1 in axons. *Neuroreport,* **8**, 2247-2251.

Browne, S. E., and Beal, M. F. (2004). The energetics of Huntington's disease. *Neurochem Res,* **29**, 531-546.

Chang, D. T., Rintoul, G. L., Pandipati, S., and Reynolds, I. J. (2006). Mutant huntingtin aggregates impair mitochondrial movement and trafficking in cortical neurons. *Neurobiol Dis,* **22**, 388-400.

Chee, F., Mudher, A., Newman, T. A., Cuttle, M., Lovestone, S., and Shepherd, D. (2006). Overexpression of tau results in defective synaptic transmission in *Drosophila* neuromuscular junctions. *Biochem Soc Trans,* **34**, 88-90.

Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L., and Bates, G. P. (1997). Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell,* **90**, 537-548.

DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonsattel, J. P., Carraway, R., Reeves, S. A., and et al. (1995). Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. *Neuron,* **14**, 1075-1081.

DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P., and Aronin, N. (1997). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science,* **277**, 1990-1993.

Engelender, S., Sharp, A. H., Colomer, V., Tokito, M. K., Lanahan, A., Worley, P., Holzbaur, E. L., and Ross, C. A. (1997). Huntingtin-associated protein 1 (HAP1) interacts with the p150Glued subunit of dynactin. *Hum Mol Genet,* **6**, 2205-2212.

Feany, M. B., and Bender, W. W. (2000). A *Drosophila* model of Parkinson's disease. *Nature,* **404**, 394-398.

Fernandez-Funez, P., Nino-Rosales, M. L., de Gouyon, B., She, W. C., Luchak, J. M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P. J*., et al.* (2000). Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature,* **408**, 101-106.

Gafni, J., Hermel, E., Young, J. E., Wellington, C. L., Hayden, M. R., and Ellerby, L. M. (2004). Inhibition of calpain cleavage of huntingtin reduces toxicity: accumulation of calpain/caspase fragments in the nucleus. *J Biol Chem,* **279**, 20211-20220.

Gauthier, L. R., Charrin, B. C., Borrell-Pages, M., Dompierre, J. P., Rangone, H., Cordelieres, F. P., De Mey, J., MacDonald, M. E., Lessmann, V., Humbert, S., and Saudou, F. (2004). Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell,* **118**, 127-138.

Grunewald, T., and Beal, M. F. (1999). Bioenergetics in Huntington's disease. *Ann N Y Acad Sci,* **893**, 203-213.

Gunawardena, S., Her, L. S., Brusch, R. G., Laymon, R. A., Niesman, I. R., Gordesky-Gold, B., Sintasath, L., Bonini, N. M., and Goldstein, L. S. (2003). Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in *Drosophila*. *Neuron,* **40**, 25-40.

Hafezparast, M., Klocke, R., Ruhrberg, C., Marquardt, A., Ahmad-Annuar, A., Bowen, S., Lalli, G., Witherden, A. S., Hummerich, H., Nicholson, S*., et al.* (2003). Mutations in dynein link motor neuron degeneration to defects in retrograde transport. *Science* **300**, 808-812.

Harjes, P., and Wanker, E. E. (2003). The hunt for huntingtin function: interaction partners tell many different stories. *Trends Biochem Sci,* **28**, 425-433.

HDCRG (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell,* **72**, 971-983.

Hollenbeck, P. J., and Saxton, W. M. (2005). The axonal transport of mitochondria. *J Cell Sci* **118**, 5411-5419.

Jackson, G. R., Salecker, I., Dong, X., Yao, X., Arnheim, N., Faber, P. W., MacDonald, M. E., and Zipursky, S. L. (1998). Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron,* **21**, 633-642.

Kieran, D., Hafezparast, M., Bohnert, S., Dick, J. R., Martin, J., Schiavo, G., Fisher, E. M., and Greensmith, L. (2005). A mutation in dynein rescues axonal transport defects and extends the life span of ALS mice. *J Cell Biol,* **169**, 561-567.

Kim, Y. J., Yi, Y., Sapp, E., Wang, Y., Cuiffo, B., Kegel, K. B., Qin, Z. H., Aronin, N., and DiFiglia, M. (2001). Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains,

associate with membranes, and undergo calpain-dependent proteolysis. *Proc Natl Acad Sci U S A,* **98**, 12784-12789.

Klement, I. A., Skinner, P. J., Kaytor, M. D., Yi, H., Hersch, S. M., Clark, H. B., Zoghbi, H. Y., and Orr, H. T. (1998). Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. *Cell,* **95**, 41-53.

Krobitsch, S., and Lindquist, S. (2000). Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins. *Proc Natl Acad Sci U S A,* **97**, 1589-1594.

LaMonte, B. H., Wallace, K. E., Holloway, B. A., Shelly, S. S., Ascano, J., Tokito, M., Van Winkle, T., Howland, D. S., and Holzbaur, E. L. (2002). Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. *Neuron,* **34**, 715-727.

Lee, H. C., and Wei, Y. H. (2000). Mitochondrial role in life and death of the cell. *J Biomed Sci,* **7**, 2-15.

Lee, W. C., Yoshihara, M., and Littleton, J. T. (2004). Cytoplasmic aggregates trap polyglutamine-containing proteins and block axonal transport in a *Drosophila* model of Huntington's disease. *Proc Natl Acad Sci U S A,* **101**, 3224-3229.

Li, H., Li, S. H., Cheng, A. L., Mangiarini, L., Bates, G. P., and Li, X. J. (1999a). Ultrastructural localization and progressive formation of neuropil aggregates in Huntington's disease transgenic mice. *Hum Mol Genet,* **8**, 1227-1236.

Li, H., Li, S. H., Yu, Z. X., Shelbourne, P., and Li, X. J. (2001). Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. *J Neurosci,* **21**, 8473-8481.

Li, H., Wyman, T., Yu, Z. X., Li, S. H., and Li, X. J. (2003a). Abnormal association of mutant huntingtin with synaptic vesicles inhibits glutamate release. *Hum Mol Genet,* **12**, 2021-2030.

Li, J. Y., Plomann, M., and Brundin, P. (2003b). Huntington's disease: a synaptopathy? *Trends Mol Med,* **9**, 414-420.

Li, S. H., Gutekunst, C. A., Hersch, S. M., and Li, X. J. (1998). Interaction of huntingtin-associated protein with dynactin P150Glued. *J Neurosci,* **18**, 1261-1269.

Li, X. J., Li, S. H., Sharp, A. H., Nucifora, F. C., Jr., Schilling, G., Lanahan, A., Worley, P., Snyder, S. H., and Ross, C. A. (1995). A huntingtin-associated protein enriched in brain with implications for pathology. *Nature,* **378**, 398-402.

Li, Z., Karlovich, C. A., Fish, M. P., Scott, M. P., and Myers, R. M. (1999b). A putative *Drosophila* homolog of the Huntington's disease gene. *Hum Mol Genet,* **8**, 1807-1815.

Lunkes, A., Lindenberg, K. S., Ben-Haiem, L., Weber, C., Devys, D., Landwehrmeyer, G. B., Mandel, J. L., and Trottier, Y. (2002). Proteases acting on mutant huntingtin

generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. *Mol Cell,* **10**, 259-269.

Miller, K. E., and Sheetz, M. P. (2004). Axonal mitochondrial transport and potential are correlated. *J Cell Sci,* **117,** 2791-2804.

Persichetti, F., Trettel, F., Huang, C. C., Fraefel, C., Timmers, H. T., Gusella, J. F., and MacDonald, M. E. (1999). Mutant huntingtin forms *in vivo* complexes with distinct context-dependent conformations of the polyglutamine segment. *Neurobiol Dis,* **6**, 364-375.

Piccioni, F., Pinton, P., Simeoni, S., Pozzi, P., Fascio, U., Vismara, G., Martini, L., Rizzuto, R., and Poletti, A. (2002). Androgen receptor with elongated polyglutamine tract forms aggregates that alter axonal trafficking and mitochondrial distribution in motor neuronal processes. *Faseb J,* **16**, 1418-1420.

Praprotnik, D., Smith, M. A., Richey, P. L., Vinters, H. V., and Perry, G. (1996). Filament heterogeneity within the dystrophic neurites of senile plaques suggests blockage of fast axonal transport in Alzheimer's disease. *Acta Neuropathol (Berl),* **91**, 226-235.

Qin, Z. H., and Gu, Z. L. (2004). Huntingtin processing in pathogenesis of Huntington disease. *Acta Pharmacol Sin,* **25**, 1243-1249.

Raff, M. C., Whitmore, A. V., and Finn, J. T. (2002). Axonal self-destruction and neurodegeneration. *Science,* **296**, 868-871.

Reid, E., Kloos, M., Ashley-Koch, A., Hughes, L., Bevan, S., Svenson, I. K., Graham, F. L., Gaskell, P. C., Dearlove, A., Pericak-Vance, M. A*., et al.* (2002). A kinesin heavy chain (KIF5A) mutation in hereditary spastic paraplegia (SPG10). *Am J Hum Genet,* **71**, 1189-1194.

Rieckhof, G. E., Yoshihara, M., Guan, Z., and Littleton, J. T. (2003). Presynaptic N-type calcium channels regulate synaptic growth. *J Biol Chem,* **278**, 41099-41108.

Ross, C. A. (2002). Polyglutamine pathogenesis: emergence of unifying mechanisms for Huntington's disease and related disorders. *Neuron,* **35**, 819-822.

Saha, A. R., Hill, J., Utton, M. A., Asuni, A. A., Ackerley, S., Grierson, A. J., Miller, C. C., Davies, A. M., Buchman, V. L., Anderton, B. H., and Hanger, D. P. (2004). Parkinson's disease alpha-synuclein mutations exhibit defective axonal transport in cultured neurons. *J Cell Sci,* **117**, 1017-1024.

Sapp, E., Penney, J., Young, A., Aronin, N., Vonsattel, J. P., and DiFiglia, M. (1999). Axonal transport of N-terminal huntingtin suggests early pathology of corticostriatal projections in Huntington disease. *J Neuropathol Exp Neurol,* **58**, 165-173.

Saudou, F., Finkbeiner, S., Devys, D., and Greenberg, M. E. (1998). Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell,* **95**, 55-66.

Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Lehrach, H., and Wanker, E. E. (1997). Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates *in vitro* and *in vivo*. *Cell,* **90**, 549-558.

Sieradzan, K. A., Mechan, A. O., Jones, L., Wanker, E. E., Nukina, N., and Mann, D. M. (1999). Huntington's disease intranuclear inclusions contain truncated, ubiquitinated huntingtin protein. *Exp Neurol,* **156**, 92-99.

Steffan, J. S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B. L., Kazantsev, A., Schmidt, E., Zhu, Y. Z., Greenwald, M.*, et al.* (2001). Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature,* **413**, 739-743.

Stowers, R. S., Megeath, L. J., Gorska-Andrzejak, J., Meinertzhagen, I. A., and Schwarz, T. L. (2002). Axonal transport of mitochondria to synapses depends on milton, a novel *Drosophila* protein. *Neuron,* **36**, 1063-1077.

Szebenyi, G., Morfini, G. A., Babcock, A., Gould, M., Selkoe, K., Stenoien, D. L., Young, M., Faber, P. W., MacDonald, M. E., McPhaul, M. J., and Brady, S. T. (2003). Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport. *Neuron,* **40**, 41-52.

Tagawa, K., Hoshino, M., Okuda, T., Ueda, H., Hayashi, H., Engemann, S., Okado, H., Ichikawa, M., Wanker, E. E., and Okazawa, H. (2004). Distinct aggregation and cell death patterns among different types of primary neurons induced by mutant huntingtin protein. *J Neurochem,* **89**, 974-987.

Trushina, E., Dyer, R. B., Badger, J. D., 2nd, Ure, D., Eide, L., Tran, D. D., Vrieze, B. T., Legendre-Guillemin, V., McPherson, P. S., Mandavilli, B. S.*, et al.* (2004). Mutant huntingtin impairs axonal trafficking in mammalian neurons *in vivo* and *in vitro*. *Mol Cell Biol,* **24**, 8195-8209.

Usdin, M. T., Shelbourne, P. F., Myers, R. M., and Madison, D. V. (1999). Impaired synaptic plasticity in mice carrying the Huntington's disease mutation. *Hum Mol Genet*, ***8***, 839-846.

Vonsattel, J. P., Myers, R. H., Stevens, T. J., Ferrante, R. J., Bird, E. D., and Richardson, E. P., Jr. (1985). Neuropathological classification of Huntington's disease. *J Neuropathol Exp Neurol,* **44**, 559-577.

Warrick, J. M., Chan, H. Y., Gray-Board, G. L., Chai, Y., Paulson, H. L., and Bonini, N. M. (1999). Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat Genet,* **23**, 425-428.

Wellington, C. L., Ellerby, L. M., Gutekunst, C. A., Rogers, D., Warby, S., Graham, R. K., Loubser, O., van Raamsdonk, J., Singaraja, R., Yang, Y. Z.*, et al.* (2002). Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J Neurosci,* **22**, 7862-7872.

Williamson, T. L., and Cleveland, D. W. (1999). Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. *Nat Neurosci,* **2**, 50-56.

Wittmann, C. W., Wszolek, M. F., Shulman, J. M., Salvaterra, P. M., Lewis, J., Hutton, M., and Feany, M. B. (2001). Tauopathy in *Drosophila*: neurodegeneration without neurofibrillary tangles. *Science,* **293**, 711-714.

Zhao, C., Takita, J., Tanaka, Y., Setou, M., Nakagawa, T., Takeda, S., Yang, H. W., Terada, S., Nakata, T., Takei, Y*., et al.* (2001). Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta. *Cell,* **105**, 587-597.

CHAPTER 4

Exploring therapeutic strategies in a *Drosophila* model of Huntington's disease

Wyan-Ching Mimi Lee[1], David W. Colby[2], Vernon Martin Ingram[3], K. Dane Wittrup[2,3,4], and J. Troy Littleton[1]

[1]Picower Center for Learning and Memory, Departments of Biology and Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, Departments of [2]Chemical Engineering, [3]Biology and [4]Division of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139

The V_L12.3 intrabody was generated and subcloned into *Drosophila* vectors by David Colby. S2 cell experiments were performed by Grace Lin. All other experiments were performed by Wyan-Ching Mimi Lee.

Abstract

Conservation of cellular pathways and disease mechanisms between humans and *Drosophila* has led to the wide use of *Drosophila* disease models for the development and testing of potential therapies. Our *Drosophila* HD model provides an ideal *in vivo* environment in which to test chemical and molecular mediators of mutant Htt-induced pathology. To assay the therapeutic effect of expression of an intracellular antibody (intrabody) against Htt, we generated double transgenic lines coexpressing pathogenic Htt (mRFP-HttQ138) with the intrabody. Intrabody expression caused suppression of aggregation in both neuronal and non-neuronal cell types, but failed to rescue mutant Htt-mediated cellular dysfunction, suggesting that mutant Htt can exert toxic effects in non-aggregated form.

Introduction

Currently, no effective therapies exist for Huntington's disease. The wealth of new knowledge regarding the pathomechanisms of the disease have led to the development of numerous therapeutic strategies; some aim to inhibit the aggregation of mutant Htt, while others target different pathways affected by the disease. Because the wild-type Htt protein is known to be essential for neuronal survival and function (Dragatsis et al., 2000; O'Kusky et al., 1999), a key consideration in the design of therapies is selective targeting of the toxic effects of the mutant protein without disruption of normal protein activity. New understanding of the molecular basis of the disease will help to define effective therapeutic targets.

One obvious target in the design of HD therapies is aggregation of the mutant Htt protein. While it is still debated whether aggregates are toxic, neuroprotective, or merely a byproduct of the disease process, studies indicate that many compounds that suppress aggregation also rescue mutant Htt-mediated toxicity. Our observations of aggregate-dependent blocks in axonal transport support this approach. Drugs such as Congo Red (Heiser et al., 2000; Sanchez et al., 2003), minocycline (Chen et al., 2000; Smith et al., 2003), and the transglutaminase inhibitor cystamine (Dedeoglu et al., 2002) are able to block aggregation of mutant Htt and rescue behavioral phenotypes in R6/2 mice. Both molecular (Cummings et al., 2001; Fernandez-Funez et al., 2000; Jana et al., 2000; Vacher et al., 2005; Warrick et al., 1999) and chemical (Yoshida et al., 2002) chaperones have also been shown to diminish aggregate formation and reduce cytotoxicity in polyQ disease models. Chemical compounds (Wang et al., 2005; Zhang et al., 2005), small peptides (Kazantsev et al., 2002; Nagai et al., 2000), and intracellular antibodies (Colby et al., 2004b; Khoshnan et al., 2002; Lecerf et al., 2001; Wolfgang et al., 2005) can be engineered and/or screened for binding to mutant Htt epitopes and suppression of aggregate formation. In addition, knocking down expression of mutant Htt with RNAi inhibits aggregation and rescues motor phenotypes in mouse HD models (Harper et al., 2005; Rodriguez-Lebron et al., 2005), and offers the possibility of mutant allele-specific targeting (Rodriguez-Lebron and Paulson, 2006). Interestingly, a recent study indicates that promotion, rather than suppression, of aggregate formation may lessen HD neuropathology by rescuing proteasome function (Bodner et al., 2006).

Molecular pathways known to be involved in HD pathogenesis also provide potential targets for the development of HD therapies. Histone deacetylase inhibitors

such as suberoylanilide hydroxamic acid (SAHA) and butyrate counteract mutant Htt-mediated transcriptional dysregulation and reduce polyQ toxicity in cell culture (McCampbell et al., 2001), yeast (Hughes et al., 2001) *Drosophila* (Steffan et al., 2001), and mice (Ferrante et al., 2003; Hockly et al., 2003a). Caspase inhibitors such as cystamine (Dedeoglu et al., 2002) and minocycline (Chen et al., 2000) reduce aggregate number and behavioral abnormalities. Compounds that target the metabolic and mitochondrial defects observed in HD include dichloroacetate, creatine, and co-enzyme Q, and have been effective in treatment of transgenic HD mice (Andreassen et al., 2001; Ferrante et al., 2000) (Koroshetz et al., 1997); creatine and co-enzyme Q are currently in clinical trials. HD excitotoxicity can be targeted with NMDA receptor antagonists and metabotropic glutamate receptor agonists (Orlando et al., 1997), as well as glutamate release inhibitors such as lamotrigine (Kremer et al., 1999) and riluzole (Rosas et al., 1999; Seppi et al., 2001). Riluzole and paroxetine also increase levels of neurotrophins such as BDNF (Mizuta et al., 2001; Walker and Raymond, 2004), potentially rescuing BDNF deficiencies seen in HD.

Transgenic *Drosophila* disease models have proven invaluable for the development of therapeutic strategies. Many cellular pathways are conserved between humans and flies (Adams et al., 2000), and the ease of handling and sophisticated genetic approaches available in *Drosophila* models enable the design of unbiased genetic screens that can be used to identify new molecular targets for therapy. Screens to identify mediators of polyQ toxicity have identified several genes, including chaperones, RNA processing proteins, and chromatin-remodeling proteins (Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000), and have led to the design of therapeutic interventions that rescue toxicity in both fly and mammalian disease models (Hay et al., 2004; Hockly et al., 2003a; Steffan et al., 2001; Warrick et al., 1999).

In addition, *Drosophila* disease models can be used for high-throughput and rapid screening of candidate therapies before testing in mammals. The ability to rescue disease phenotypes in fly models with human drug treatments (Min and Benzer, 1999) suggests that the inverse may also be true – that therapies found to be effective in fly models may be beneficial in treatment of human diseases. In fly models of polyQ diseases, many characteristics of the human disorders are conserved: expression of expanded polyQ proteins in flies leads to formation of insoluble intracellular inclusions, and flies develop a late-onset, progressive disorder that ends in premature death (Fernandez-Funez et al., 2000; Jackson et al., 1998; Kazantsev et al., 2002; Marsh et

al., 2000; Takeyama et al., 2002; Warrick et al., 1999). As in humans, the length of the polyQ repeat determines the severity of disease pathogenesis, and similar thresholds of repeat length are required for disease manifestation (Fernandez-Funez et al., 2000; Jackson et al., 1998; Kazemi-Esfarjani and Benzer, 2000; Marsh et al., 2000; Takeyama et al., 2002; Warrick et al., 1999). Many therapies that have proven beneficial in mammalian HD models have also been effective in transgenic HD flies, including HDAC inhibitors (Steffan et al., 2001); (Zhao et al., 2005), Congo Red (Apostol et al., 2003), cystamine (Apostol et al., 2003), small peptides (Kazantsev et al., 2002; Nagai et al., 2003), chaperones (Iijima-Ando et al., 2005; Kazemi-Esfarjani and Benzer, 2000), and intracellular antibodies (Wolfgang et al., 2005). This evidence for conservation of disease mechanisms between mammals and flies indicates that *Drosophila* models of HD will be useful for the development and testing of potential therapies.

Our *Drosophila* HD model provides an ideal *in vivo* environment in which to test chemical and molecular mediators of mutant Htt-induced pathology, especially in the cytoplasm and neurites. This chapter focuses on use of transgenic *Drosophila* lines expressing a 588 aa N-terminal fragment of mutant Htt to test the effects of expression of an intracellular antibody against Htt on aggregation and toxicity.

Materials and Methods

S2 Cell Transfection and Analysis

cDNAs for mRFP-HttQ15 and mRFP-HttQ138 were subcloned into the BamH1 and EcoRI (blunt end ligation) sites of the pSR11 vector. cDNA for the V_L12.3 intrabody was subcloned into the pPL17 vector. Constructs were transfected with 50 μL cytofectene (BioRad) into *Drosophila* S2 cells using the BioRad Liposome Mediated Transfection Protocol. 4 μG of construct DNA were used per 5 mL S2 cell culture for single transfections while 4 μG of each construct were used for double transfections. After 72 hours, 20 μL cell suspensions were fixed with 3.7% formaldehyde in 1 x PBT, then mounted on slides with 50% glycerol in 1 x PBS. Visualization of slides was performed on a Pascal confocal microscope (Zeiss).

Morphological Analysis

Wandering 3rd instar larvae reared at 25^{o}C were dissected as described (Rieckhof et al., 2003). Visualization and quantification were performed on a Pascal confocal microscope (Zeiss).

Glue Secretion Assay

Pupae reared at 25^{o}C were isolated shortly after pupariation and adhered to slides with ventral sides facing up using double-sided tape. Visualization and quantification were performed on a Pascal confocal microscope (Zeiss).

Results

V_L12.3 is a single-domain intracellular antibody, or intrabody, against the first 20 aa of the Htt protein (Colby et al., 2004a). Most intrabodies to date have been isolated under oxidizing conditions that allow the formation of stabilizing disulfide bonds between intrabody cysteine residues; in the reducing environment of the cytoplasm, however, disulfide bonds are less readily formed, compromising the stability and effectiveness of intrabodies within the cell. V_L12.3 has been optimized for high intracellular efficacy at low expression levels by increasing its affinity for Htt in the absence of a disulfide bond. Expression of V_L12.3 in neuronal and yeast HD models inhibits aggregation and rescues toxicity. To determine whether mutant Htt-mediated aggregation and toxicity are reduced *in vivo*, we have generated double transgenic flies that express both mRFP-HttQ138 and the V_L12.3 intrabody.

Expression of the intrabody in S2 cells reduces aggregate formation

Although it has not been resolved whether mutant Htt-induced toxicity is dependent on aggregate formation, suppression of aggregation has been correlated with rescue of HD phenotypes for many prospective therapies. To determine the effect of intrabody expression on mutant Htt aggregation at the cellular level, levels of aggregation were compared between *Drosophila* S2 cells transiently transfected with mRFP-HttQ138 alone or with both mRFP-HttQ138 and the V_L12.3 intrabody. While expression of mRFP-HttQ138 alone resulted in formation of large cytoplasmic aggregates (Fig. 1A), mRFP-HttQ138 coexpressed with the intrabody exhibited diffuse localization throughout the cytoplasm (Fig. 1B). Visualization of the intrabody shows perinuclear as well as cytoplasmic localization, with increased density at areas of mRFP-HttQ138 density, indicating a direct interaction between the intrabody and Htt (Fig. 1C).

Expression of the intrabody *in vivo* reduces mutant huntingtin aggregation in neuronal and non-neuronal cells

To assess whether aggregation of mutant Htt is also inhibited by the intrabody *in vivo*, transgenic lines expressing mRFP-HttQ138 alone or mRFP-HttQ138 with the intrabody were crossed to lines expressing the pan-neuronal C155 elav-GAL4 driver. 3rd instar larval offspring of the crosses were imaged using confocal microscopy. As observed in S2 cells, in comparison to mRFP-HttQ138 expression alone (Fig. 2A),

Figure 1

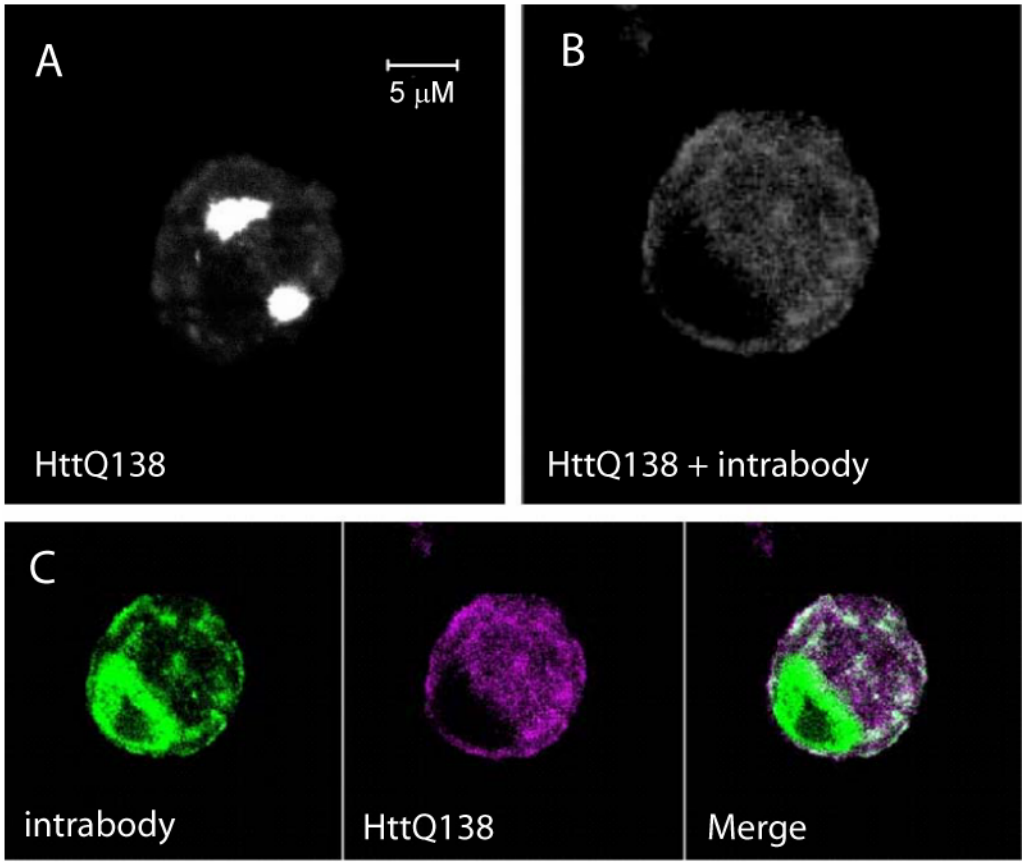
A
5 μM
HttQ138
B
HttQ138 + intrabody
C
intrabody
HttQ138
Merge

FIGURE 1. **Intrabody expression suppresses mutant Htt aggregation in *Drosophila* S2 cells.** (A) mRFP-HttQ138 forms large cytoplasmic aggregates (white). (B) Coexpression of the V_L12.3 intrabody with aggregates results in diffuse cytoplasmic localization of mRFP-HttQ138 (white). (C) Intrabody localization (green) is cytoplasmic and perinuclear, and is concentrated at sites of mRFP-HttQ138 density (magenta), indicating an interaction between the intrabody and mutant Htt.

Figure 2

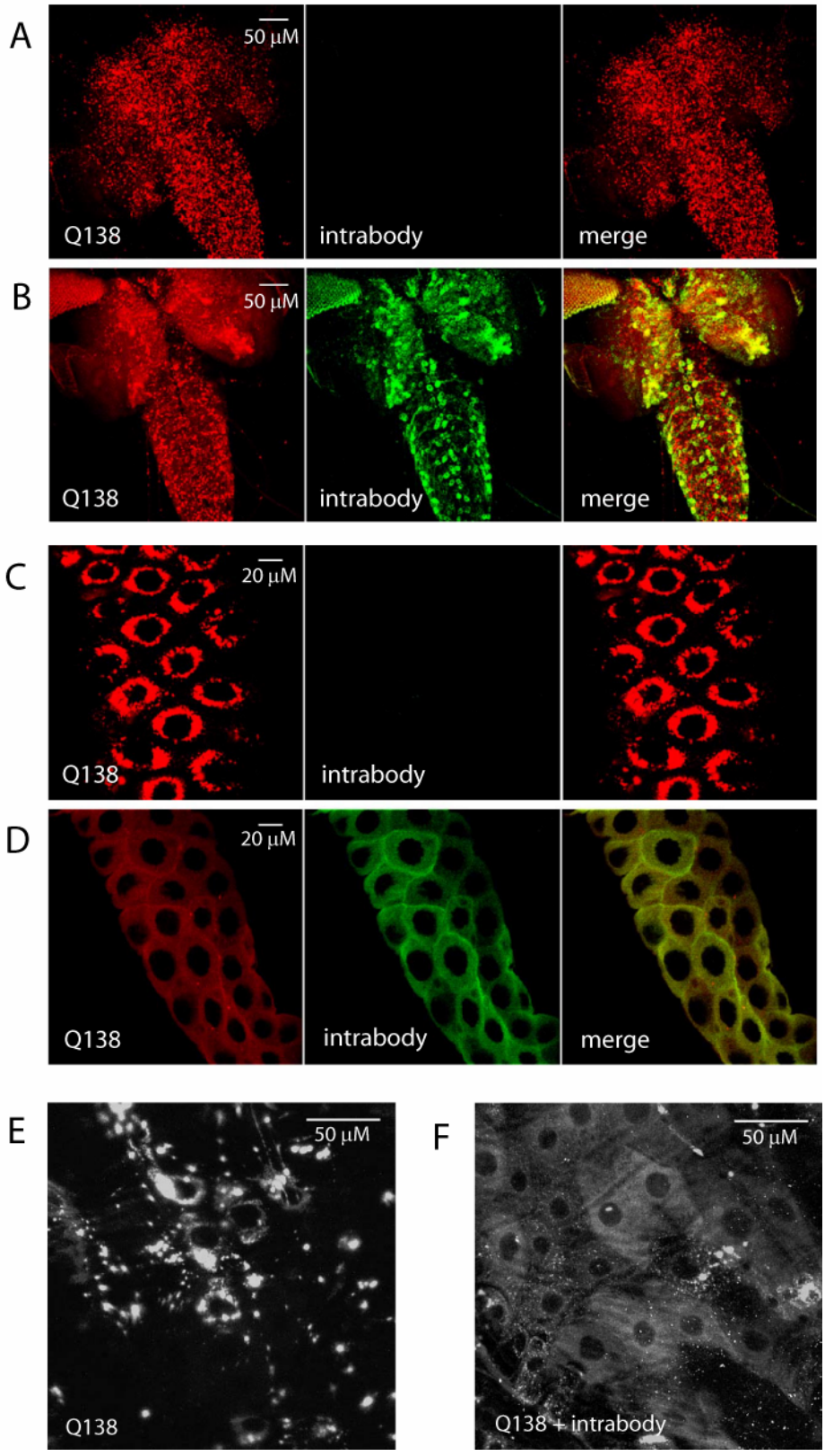
A
50 µM
Q138
intrabody
merge
B
50 µM
Q138
intrabody
merge
C
20 µM
Q138
intrabody
D
20 µM
Q138
intrabody
merge
E
50 µM
Q138
F
50 µM
Q138 + intrabody

FIGURE 2. **Intrabody expression suppresses mutant Htt aggregation *in vivo* in *Drosophila*.** Compared to expression of mRFP-HttQ138 alone (A), coexpression of the intrabody (green) reduces aggregate number and increases diffuse localization of mRFP-HttQ138 (red) in the CNS of wandering 3rd instar larvae (B). Aggregation of mRFP-HttQ138 (red) is also dramatically suppressed in salivary gland cells (C, D) and in the epidermis (E, F).

Figure 3

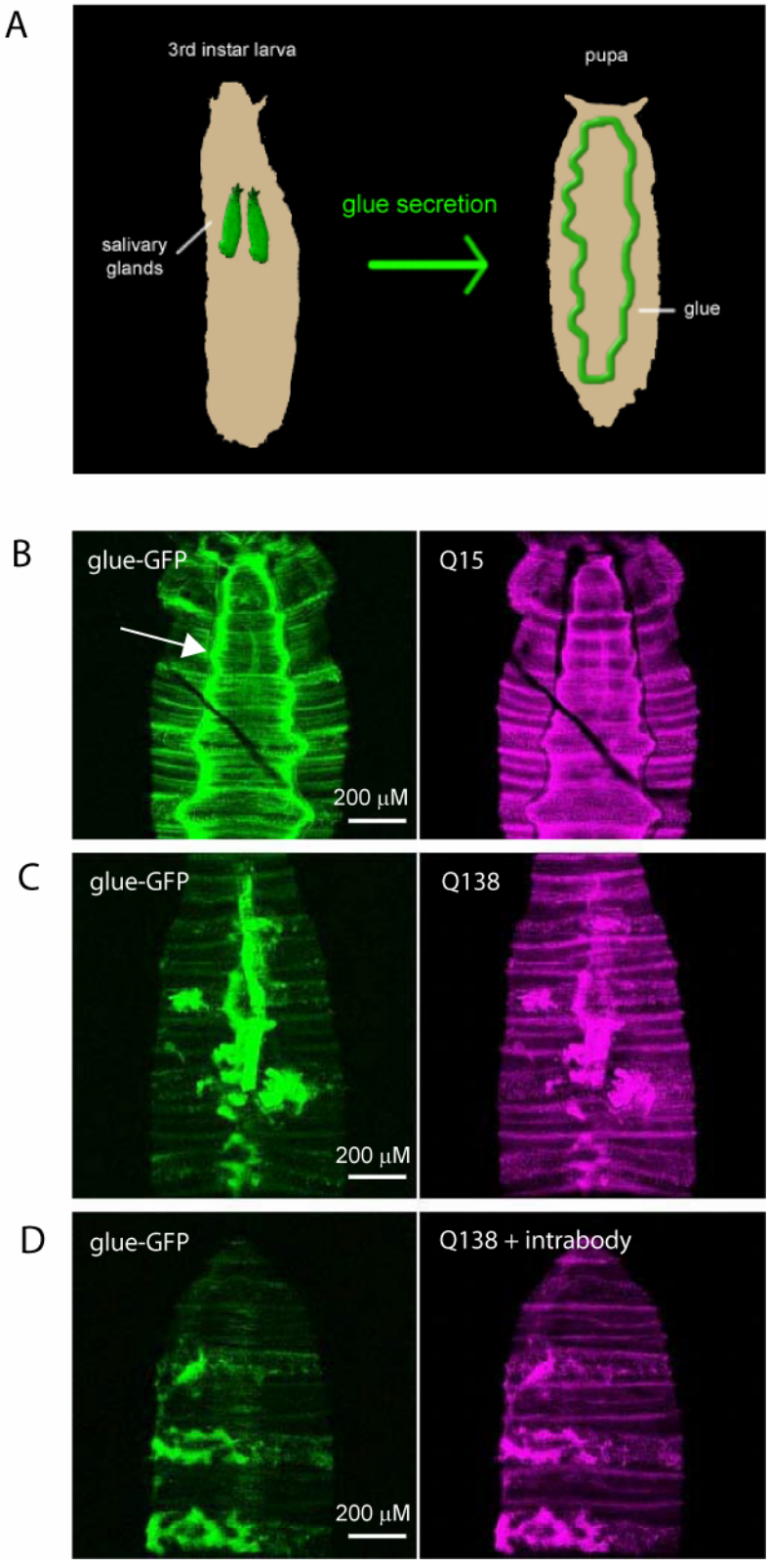
A
3rd instar larva
pupa
glue secretion
salivary glands
glue
B
glue-GFP
Q15
200 μM
C
glue-GFP
Q138
200 μM
D
glue-GFP
Q138 + intrabody
200 μM

FIGURE 3. **The intrabody does not rescue functional defects in salivary gland glue secretion in *Drosophila*.** (A) Diagram of normal glue secretion during pupariation. Expression of the glue protein fused to GFP enables *in vivo* imaging of secreted glue. (B) Secretion of glue-GFP (green) is normal in pupae expressing mRFP-HttQ15 (magenta). Arrows indicate secreted glue. (C) In pupae expressing mRFP-HttQ138 (magenta), secretion of glue-GFP (green) is strikingly decreased. (D) Coexpression of the intrabody in pupae expressing mRFP-HttQ138 (magenta) does not restore secretion of glue (green) to normal levels and does not rescue salivary gland dysfunction.

intrabody expression reduced aggregate number and increased diffuse mRFP-HttQ138 localization in the CNS (Fig. 2B). Suppression of aggregation was especially dramatic in the salivary glands; while large, distinct cytoplasmic aggregates are seen in salivary gland cells of larvae expressing mRFP-HttQ138 alone (Fig. 2C), coexpression of the intrabody resulted in diffuse, cytoplasmic localization of mRFP-HttQ138 with almost complete elimination of aggregates (Fig. 2D). Coexpression of mRFP-HttQ138 with the intrabody also reduces aggregate formation in the epidermis (Fig. 2F) in comparison to expression of mRFP-HttQ138 alone (Fig. 2E).

Intrabody expression does not rescue mutant huntingtin-induced defects in salivary gland secretion in *Drosophila*

Large cytoplasmic aggregates are abundant in the salivary glands of mRFP-HttQ138-expressing larvae (Fig. 2C), and cause a defect in the secretion of GFP-tagged glue from the salivary glands during pupariation (see Chapter 3). To determine whether this defect is rescued by intrabody expression, pupae expressing mRFP-HttQ138, glue-GFP, and the intrabody with the C155 elav-GAL4 driver were compared to pupae expressing mRFP-HttQ138 and glue-GFP alone and pupae expressing mRFP-HttQ15 and glue-GFP. While coexpression of mRFP-HttQ15 and glue-GFP leads to normal pupal glue secretion, with glue-GFP visible on the ventral side of the pupal case (Fig. 3A & B), coexpression of mRFP-HttQ138 with glue-GFP results in a lack of secreted glue (Fig. 3C). Expression of the intrabody does not increase glue-GFP secretion in mRFP-HttQ138-expressing pupae (Fig. 3D), indicating that although intrabody expression strikingly reduces aggregates in the salivary gland, the intrabody does not rescue the mutant Htt-mediated functional defect in salivary gland secretion of glue during pupariation in *Drosophila*.

Discussion

Recent studies have highlighted the potential of intracellular antibodies, or intrabodies, in the treatment of neurodegenerative diseases. Intrabodies can be isolated and affinity matured for binding to specific targets in yeast or phage display libraries, and may be especially suited for treatment of single-gene disorders such as Huntington's disease. Binding of an intrabody to a target protein, such as mutant Htt, may disrupt its interactions with other binding partners or alter protein stability, and may also be used to redirect proteins to specific subcellular compartments (Miller and Messer, 2005). Expression of intrabodies against the huntingtin protein has been shown to inhibit aggregation in various cell (Lecerf et al., 2001) and brain slice (Murphy and Messer, 2004) HD models, and has extended lifespan and delayed neurodegeneration *in vivo* in a *Drosophila* model of HD (Wolfgang et al., 2005).

One limitation of intrabody use for therapeutic purposes has been the limited intracellular efficacy of intrabodies isolated in *in vitro* environments. To optimize intracellular efficacy, the V_L12.3 intrabody was isolated and matured for binding affinity to Htt in the absence of the stabilizing disulfide bond in order to select for intrabody properties independent of the redox environment (Colby et al., 2004a). The V_L12.3 intrabody suppresses aggregate formation and rescues toxicity in neuronal and yeast HD models. To determine whether the intrabody has a similar effect *in vivo*, we generated transgenic lines expressing a 588 aa N-terminal fragment of mutant Htt with or without coexpression of the intrabody. In the presence of the intrabody, mutant Htt aggregates were greatly reduced in both neuronal and non-neuronal tissues, while levels of diffuse mutant Htt in the cytoplasm were visibly increased (Fig. 2).

Because aggregate formation has not been confirmed as a toxic step in HD pathogenesis, prospective therapies are also tested for ability to rescue mutant Htt-induced phenotypes, such as motor defects and neurodegeneration. Our *Drosophila* HD model exhibits an abundance of mutant Htt aggregates in the salivary gland, and demonstrates a clear defect in salivary gland secretion of glue during pupariation. Expression of the V_L12.3 intrabody dramatically reduced mutant Htt aggregation in the salivary gland, but did not rescue the secretion defect (Figure 3), indicating that mutant Htt-mediated toxicity may be aggregate-independent in this cell type. Future studies may reveal rescue of other functional phenotypes (see Future Directions). The conformational specificity of similar intrabodies isolated through binding affinity to Htt

may also be used for validation of sites on the mutant protein that can be used as targets for rational drug design.

Our *Drosophila* HD model recapitulates the inhibition of aggregation seen in cellular and yeast HD models expressing the V_L12.3 intrabody. While salivary gland secretion is not rescued, the effects of intrabody expression can be tested on many other mutant Htt-induced phenotypes characterized in our model, including reduced lifespan, motor defects, and neurodegeneration of photoreceptor cells. The broad characterization of cellular and functional phenotypes in our *Drosophila* HD model make it ideal for the testing of potential therapies, allowing a multimodal analysis of therapeutic effect *in vivo*.

Future Directions

Although salivary gland secretion defects are not rescued by intrabody expression, results in this non-neuronal tissue should not be used to predict the effect of the intrabody on phenotypic defects more common to HD animal models. Future studies will test the effects of intrabody expression on other defects induced by expression of a 588 aa N-terminal fragment of mutant Htt in *Drosophila*, including reduced lifespan and neurodegenerative rough-eye phenotypes.

Adult flies expressing mRFP-HttQ138 with a 2nd chromosome pan-neuronal elav-GAL4 driver undergo a dramatic, dose-dependent reduction in lifespan (see Chapter 3), with a decrease in T_{50} (time at which 50% of the culture has died) of over 70% in comparison to controls. This provides a precise assay with which to analyze intrabody effects on a mutant Htt-induced premature death phenotype. Rescue or partial rescue of reduced lifespan will indicate that intrabody expression can reduce toxicity as well as aggregation in an *in vivo* model of Huntington's disease.

Eye-specific expression of a 548 aa N-terminal fragment of mutant Htt (Htt-Q128) can be driven with the GMR-GAL4 driver, and leads to a rough-eye phenotype with loss of pigmentation, abnormal bristle pattern, and retinal photoreceptor degeneration (see Chapter 2). The rough-eye phenotype is a sensitive assay that can detect small changes in toxic insult to the eye, and should allow visualization of any rescue of eye defects by intrabody expression. A decrease in the rough-eye phenotype or in the degeneration of photoreceptors as viewed through pseudopupil analysis will provide evidence for intrabody rescue of mutant Htt-mediated toxicity.

Further analysis of intrabody effects on aggregate formation in different tissues may also be useful in assessing intrabody efficacy and function. Correlating changes in aggregate number with enhancement or suppression of functional defects may shed light on the role of mutant Htt aggregation in disease toxicity. While expression of the intrabody in cell and yeast HD models suggests that inhibition of aggregation may play a role in rescuing mutant Htt-induced toxic effects, *in vivo* analysis may show that aggregate suppression is unrelated to functional rescue, or may even intensify defective phenotypes, providing evidence that aggregates are not the toxic form of the mutant Htt protein.

A key strength of *Drosophila* disease models is the ability to perform second-site modifier screens to identify molecular pathways involved in disease pathogenesis,

leading to the discovery of new targets for therapy. Expression of the 588 aa N-terminal fragment of mutant Htt in our *Drosophila* HD model results in pharate adult lethality, enabling large-scale screens for genetic suppressors of the lethal phenotype. In addition, the mRFP tag on the mutant Htt fragment allows easy screening for suppressors and enhancers of aggregation in live 3rd instar larvae. Future screens can thus be designed to isolate genetic modifiers that affect aggregation, toxicity, or both. In summary, our *Drosophila* model of HD will be valuable both for testing of candidate therapies and for discovery of new therapeutic targets and strategies.

Acknowledgments

We thank Grace Lin for performing the S2 cell intrabody experiments. We also thank the Bloomington Stock Center for providing the GAL4 driver lines and glue-GFP lines used in this study. This work was supported by grants from the NIH, the Human Frontiers Science Program Organization, the Packard Foundation and the Searle Scholars Program.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., George, R.A., Lewis, S.E., Richards, S., Ashburner, M., Henderson, S.N., Sutton, G.G., Wortman, J.R., Yandell, M.D., Zhang, Q., Chen, L.X., Brandon, R.C., Rogers, Y.H., Blazej, R.G., Champe, M., Pfeiffer, B.D., Wan, K.H., Doyle, C., Baxter, E.G., Helt, G., Nelson, C.R., Gabor, G.L., Abril, J.F., Agbayani, A., An, H.J., Andrews-Pfannkoch, C., Baldwin, D., Ballew, R.M., Basu, A., Baxendale, J., Bayraktaroglu, L., Beasley, E.M., Beeson, K.Y., Benos, P.V., Berman, B.P., Bhandari, D., Bolshakov, S., Borkova, D., Botchan, M.R., Bouck, J., Brokstein, P., Brottier, P., Burtis, K.C., Busam, D.A., Butler, H., Cadieu, E., Center, A., Chandra, I., Cherry, J.M., Cawley, S., Dahlke, C., Davenport, L.B., Davies, P., de Pablos, B., Delcher, A., Deng, Z., Mays, A.D., Dew, I., Dietz, S.M., Dodson, K., Doup, L.E., Downes, M., Dugan-Rocha, S., Dunkov, B.C., Dunn, P., Durbin, K.J., Evangelista, C.C., Ferraz, C., Ferriera, S., Fleischmann, W., Fosler, C., Gabrielian, A.E., Garg, N.S., Gelbart, W.M., Glasser, K., Glodek, A., Gong, F., Gorrell, J.H., Gu, Z., Guan, P., Harris, M., Harris, N.L., Harvey, D., Heiman, T.J., Hernandez, J.R., Houck, J., Hostin, D., Houston, K.A., Howland, T.J., Wei, M.H., Ibegwam, C., Jalali, M., Kalush, F., Karpen, G.H., Ke, Z., Kennison, J.A., Ketchum, K.A., Kimmel, B.E., Kodira, C.D., Kraft, C., Kravitz, S., Kulp, D., Lai, Z., Lasko, P., Lei, Y., Levitsky, A.A., Li, J., Li, Z., Liang, Y., Lin, X., Liu, X., Mattei, B., McIntosh, T.C., McLeod, M.P., McPherson, D., Merkulov, G., Milshina, N.V., Mobarry, C., Morris, J., Moshrefi, A., Mount, S.M., Moy, M., Murphy, B., Murphy, L., Muzny, D.M., Nelson, D.L., Nelson, D.R., Nelson, K.A., Nixon, K., Nusskern, D.R., Pacleb, J.M., Palazzolo, M., Pittman, G.S., Pan, S., Pollard, J., Puri, V., Reese, M.G., Reinert, K., Remington, K., Saunders, R.D., Scheeler, F., Shen, H., Shue, B.C., Siden-Kiamos, I., Simpson, M., Skupski, M.P., Smith, T., Spier, E., Spradling, A.C., Stapleton, M., Strong, R., Sun, E., Svirskas, R., Tector, C., Turner, R., Venter, E., Wang, A.H., Wang, X., Wang, Z.Y., Wassarman, D.A., Weinstock, G.M., Weissenbach, J., Williams, S.M., WoodageT, Worley, K.C., Wu, D., Yang, S., Yao, Q.A., Ye, J., Yeh, R.F., Zaveri, J.S., Zhan, M., Zhang, G., Zhao, Q., Zheng, L., Zheng, X.H., Zhong, F.N., Zhong, W., Zhou, X., Zhu, S., Zhu, X., Smith, H.O., Gibbs, R.A., Myers, E.W., Rubin, G.M. and Venter, J.C. (2000) The genome sequence of *Drosophila* melanogaster. *Science*, **287**, 2185-2195.

Andreassen, O.A., Ferrante, R.J., Huang, H.M., Dedeoglu, A., Park, L., Ferrante, K.L., Kwon, J., Borchelt, D.R., Ross, C.A., Gibson, G.E. and Beal, M.F. (2001) Dichloroacetate exerts therapeutic effects in transgenic mouse models of Huntington's disease. *Ann Neurol*, **50**, 112-117.

Apostol, B.L., Kazantsev, A., Raffioni, S., Illes, K., Pallos, J., Bodai, L., Slepko, N., Bear, J.E., Gertler, F.B., Hersch, S., Housman, D.E., Marsh, J.L. and Thompson, L.M. (2003) A cell-based assay for aggregation inhibitors as therapeutics of polyglutamine-repeat disease and validation in *Drosophila*. *Proc Natl Acad Sci U S A*, **100**, 5950-5955.

Bodner, R.A., Outeiro, T.F., Altmann, S., Maxwell, M.M., Cho, S.H., Hyman, B.T., McLean, P.J., Young, A.B., Housman, D.E. and Kazantsev, A.G. (2006)

Pharmacological promotion of inclusion formation: a therapeutic approach for Huntington's and Parkinson's diseases. *Proc Natl Acad Sci U S A*, **103**, 4246-4251.

Chen, M., Ona, V.O., Li, M., Ferrante, R.J., Fink, K.B., Zhu, S., Bian, J., Guo, L., Farrell, L.A., Hersch, S.M., Hobbs, W., Vonsattel, J.P., Cha, J.H. and Friedlander, R.M. (2000) Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat Med*, **6**, 797-801.

Colby, D.W., Chu, Y., Cassady, J.P., Duennwald, M., Zazulak, H., Webster, J.M., Messer, A., Lindquist, S., Ingram, V.M. and Wittrup, K.D. (2004a) Potent inhibition of huntingtin aggregation and cytotoxicity by a disulfide bond-free single-domain intracellular antibody. *Proc Natl Acad Sci U S A*, **101**, 17616-17621.

Colby, D.W., Garg, P., Holden, T., Chao, G., Webster, J.M., Messer, A., Ingram, V.M. and Wittrup, K.D. (2004b) Development of a human light chain variable domain (V(L)) intracellular antibody specific for the amino terminus of huntingtin via yeast surface display. *J Mol Biol*, **342**, 901-912.

Cummings, C.J., Sun, Y., Opal, P., Antalffy, B., Mestril, R., Orr, H.T., Dillmann, W.H. and Zoghbi, H.Y. (2001) Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum Mol Genet*, **10**, 1511-1518.

Dedeoglu, A., Kubilus, J.K., Jeitner, T.M., Matson, S.A., Bogdanov, M., Kowall, N.W., Matson, W.R., Cooper, A.J., Ratan, R.R., Beal, M.F., Hersch, S.M. and Ferrante, R.J. (2002) Therapeutic effects of cystamine in a murine model of Huntington's disease. *J Neurosci*, **22**, 8942-8950.

Dragatsis, I., Levine, M.S. and Zeitlin, S. (2000) Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. *Nat Genet*, **26**, 300-306.

Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J., McCall, A., Canal, I., Orr, H.T., Zoghbi, H.Y. and Botas, J. (2000) Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature*, **408**, 101-106.

Ferrante, R.J., Andreassen, O.A., Jenkins, B.G., Dedeoglu, A., Kuemmerle, S., Kubilus, J.K., Kaddurah-Daouk, R., Hersch, S.M. and Beal, M.F. (2000) Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. *J Neurosci*, **20**, 4389-4397.

Ferrante, R.J., Kubilus, J.K., Lee, J., Ryu, H., Beesen, A., Zucker, B., Smith, K., Kowall, N.W., Ratan, R.R., Luthi-Carter, R. and Hersch, S.M. (2003) Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. *J Neurosci*, **23**, 9418-9427.

Harper, S.Q., Staber, P.D., He, X., Eliason, S.L., Martins, I.H., Mao, Q., Yang, L., Kotin, R.M., Paulson, H.L. and Davidson, B.L. (2005) RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. *Proc Natl Acad Sci U S A*, **102**, 5820-5825.

Hay, D.G., Sathasivam, K., Tobaben, S., Stahl, B., Marber, M., Mestril, R., Mahal, A., Smith, D.L., Woodman, B. and Bates, G.P. (2004) Progressive decrease in chaperone protein levels in a mouse model of Huntington's disease and induction of stress proteins as a therapeutic approach. *Hum Mol Genet*, **13**, 1389-1405.

Heiser, V., Scherzinger, E., Boeddrich, A., Nordhoff, E., Lurz, R., Schugardt, N., Lehrach, H. and Wanker, E.E. (2000) Inhibition of huntingtin fibrillogenesis by specific antibodies and small molecules: implications for Huntington's disease therapy. *Proc Natl Acad Sci U S A*, **97**, 6739-6744.

Hockly, E., Richon, V.M., Woodman, B., Smith, D.L., Zhou, X., Rosa, E., Sathasivam, K., Ghazi-Noori, S., Mahal, A., Lowden, P.A., Steffan, J.S., Marsh, J.L., Thompson, L.M., Lewis, C.M., Marks, P.A. and Bates, G.P. (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. *Proc Natl Acad Sci U S A*, **100**, 2041-2046.

Hughes, R.E., Lo, R.S., Davis, C., Strand, A.D., Neal, C.L., Olson, J.M. and Fields, S. (2001) Altered transcription in yeast expressing expanded polyglutamine. *Proc Natl Acad Sci U S A*, **98**, 13201-13206.

Iijima-Ando, K., Wu, P., Drier, E.A., Iijima, K. and Yin, J.C. (2005) cAMP-response element-binding protein and heat-shock protein 70 additively suppress polyglutamine-mediated toxicity in *Drosophila*. *Proc Natl Acad Sci U S A*, **102**, 10261-10266.

Jackson, G.R., Salecker, I., Dong, X., Yao, X., Arnheim, N., Faber, P.W., MacDonald, M.E. and Zipursky, S.L. (1998) Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron*, **21**, 633-642.

Jana, N.R., Tanaka, M., Wang, G. and Nukina, N. (2000) Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. *Hum Mol Genet*, **9**, 2009-2018.

Kazantsev, A., Walker, H.A., Slepko, N., Bear, J.E., Preisinger, E., Steffan, J.S., Zhu, Y.Z., Gertler, F.B., Housman, D.E., Marsh, J.L. and Thompson, L.M. (2002) A bivalent Huntingtin binding peptide suppresses polyglutamine aggregation and pathogenesis in *Drosophila*. *Nat Genet*, **30**, 367-376.

Kazemi-Esfarjani, P. and Benzer, S. (2000) Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science*, **287**, 1837-1840.

Khoshnan, A., Ko, J. and Patterson, P.H. (2002) Effects of intracellular expression of anti-huntingtin antibodies of various specificities on mutant huntingtin aggregation and toxicity. *Proc Natl Acad Sci U S A*, **99**, 1002-1007.

Koroshetz, W.J., Jenkins, B.G., Rosen, B.R. and Beal, M.F. (1997) Energy metabolism defects in Huntington's disease and effects of coenzyme Q10. *Ann Neurol*, **41**, 160-165.

Kremer, B., Clark, C.M., Almqvist, E.W., Raymond, L.A., Graf, P., Jacova, C., Mezei, M., Hardy, M.A., Snow, B., Martin, W. and Hayden, M.R. (1999) Influence of lamotrigine on progression of early Huntington disease: a randomized clinical trial. *Neurology*, **53**, 1000-1011.

Lecerf, J.M., Shirley, T.L., Zhu, Q., Kazantsev, A., Amersdorfer, P., Housman, D.E., Messer, A. and Huston, J.S. (2001) Human single-chain Fv intrabodies counteract in situ huntingtin aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci U S A*, **98**, 4764-4769.

Marsh, J.L., Walker, H., Theisen, H., Zhu, Y.Z., Fielder, T., Purcell, J. and Thompson, L.M. (2000) Expanded polyglutamine peptides alone are intrinsically cytotoxic and cause neurodegeneration in *Drosophila*. *Hum Mol Genet*, **9**, 13-25.

McCampbell, A., Taye, A.A., Whitty, L., Penney, E., Steffan, J.S. and Fischbeck, K.H. (2001) Histone deacetylase inhibitors reduce polyglutamine toxicity. *Proc Natl Acad Sci U S A*, **98**, 15179-15184.

Miller, T.W. and Messer, A. (2005) Intrabody applications in neurological disorders: progress and future prospects. *Mol Ther*, **12**, 394-401.

Min, K.T. and Benzer, S. (1999) Preventing neurodegeneration in the *Drosophila* mutant bubblegum. *Science*, **284**, 1985-1988.

Mizuta, I., Ohta, M., Ohta, K., Nishimura, M., Mizuta, E. and Kuno, S. (2001) Riluzole stimulates nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor synthesis in cultured mouse astrocytes. *Neurosci Lett*, **310**, 117-120.

Murphy, R.C. and Messer, A. (2004) A single-chain Fv intrabody provides functional protection against the effects of mutant protein in an organotypic slice culture model of Huntington's disease. *Brain Res Mol Brain Res*, **121**, 141-145.

Nagai, Y., Fujikake, N., Ohno, K., Higashiyama, H., Popiel, H.A., Rahadian, J., Yamaguchi, M., Strittmatter, W.J., Burke, J.R. and Toda, T. (2003) Prevention of polyglutamine oligomerization and neurodegeneration by the peptide inhibitor QBP1 in *Drosophila*. *Hum Mol Genet*, **12**, 1253-1259.

Nagai, Y., Tucker, T., Ren, H., Kenan, D.J., Henderson, B.S., Keene, J.D., Strittmatter, W.J. and Burke, J.R. (2000) Inhibition of polyglutamine protein aggregation and cell death by novel peptides identified by phage display screening. *J Biol Chem*, **275**, 10437-10442.

O'Kusky, J.R., Nasir, J., Cicchetti, F., Parent, A. and Hayden, M.R. (1999) Neuronal degeneration in the basal ganglia and loss of pallido-subthalamic synapses in mice with targeted disruption of the Huntington's disease gene. *Brain Res*, **818**, 468-479.

Orlando, L.R., Luthi-Carter, R., Standaert, D.G., Coyle, J.T., Penney, J.B., Jr. and Young, A.B. (1997) N-acetylaspartylglutamate (NAAG) protects against rat striatal quinolinic acid lesions *in vivo*. *Neurosci Lett*, **236**, 91-94.

Rieckhof, G.E., Yoshihara, M., Guan, Z. and Littleton, J.T. (2003) Presynaptic N-type calcium channels regulate synaptic growth. *J Biol Chem*, **278**, 41099-41108.

Rodriguez-Lebron, E., Denovan-Wright, E.M., Nash, K., Lewin, A.S. and Mandel, R.J. (2005) Intrastriatal rAAV-mediated delivery of anti-huntingtin shRNAs induces partial reversal of disease progression in R6/1 Huntington's disease transgenic mice. *Mol Ther*, **12**, 618-633.

Rodriguez-Lebron, E. and Paulson, H.L. (2006) Allele-specific RNA interference for neurological disease. *Gene Ther*, **13**, 576-581.

Rosas, H.D., Koroshetz, W.J., Jenkins, B.G., Chen, Y.I., Hayden, D.L., Beal, M.F. and Cudkowicz, M.E. (1999) Riluzole therapy in Huntington's disease (HD). *Mov Disord*, **14**, 326-330.

Sanchez, I., Mahlke, C. and Yuan, J. (2003) Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. *Nature*, **421**, 373-379.

Seppi, K., Mueller, J., Bodner, T., Brandauer, E., Benke, T., Weirich-Schwaiger, H., Poewe, W. and Wenning, G.K. (2001) Riluzole in Huntington's disease (HD): an open label study with one year follow up. *J Neurol*, **248**, 866-869.

Smith, D.L., Woodman, B., Mahal, A., Sathasivam, K., Ghazi-Noori, S., Lowden, P.A., Bates, G.P. and Hockly, E. (2003) Minocycline and doxycycline are not beneficial in a model of Huntington's disease. *Ann Neurol*, **54**, 186-196.

Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M., Kurokawa, R., Housman, D.E., Jackson, G.R., Marsh, J.L. and Thompson, L.M. (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature*, **413**, 739-743.

Stewart, B.A., Atwood, H.L., Renger, J.J., Wang, J. and Wu, C.F. (1994) Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J Comp Physiol [A]*, **175**, 179-191.

Takeyama, K., Ito, S., Yamamoto, A., Tanimoto, H., Furutani, T., Kanuka, H., Miura, M., Tabata, T. and Kato, S. (2002) Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in *Drosophila*. *Neuron*, **35**, 855-864.

Vacher, C., Garcia-Oroz, L. and Rubinsztein, D.C. (2005) Overexpression of yeast hsp104 reduces polyglutamine aggregation and prolongs survival of a transgenic mouse model of Huntington's disease. *Hum Mol Genet*, **14**, 3425-3433.

Walker, F.O. and Raymond, L.A. (2004) Targeting energy metabolism in Huntington's disease. *Lancet*, **364**, 312-313.

Wang, J., Gines, S., MacDonald, M.E. and Gusella, J.F. (2005) Reversal of a full-length mutant huntingtin neuronal cell phenotype by chemical inhibitors of polyglutamine-mediated aggregation. *BMC Neurosci*, **6**, 1.

Warrick, J.M., Chan, H.Y., Gray-Board, G.L., Chai, Y., Paulson, H.L. and Bonini, N.M. (1999) Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat Genet*, **23**, 425-428.

Wolfgang, W.J., Miller, T.W., Webster, J.M., Huston, J.S., Thompson, L.M., Marsh, J.L. and Messer, A. (2005) Suppression of Huntington's disease pathology in *Drosophila* by human single-chain Fv antibodies. *Proc Natl Acad Sci U S A*, **102**, 11563-11568.

Yoshida, H., Yoshizawa, T., Shibasaki, F., Shoji, S. and Kanazawa, I. (2002) Chemical chaperones reduce aggregate formation and cell death caused by the truncated Machado-Joseph disease gene product with an expanded polyglutamine stretch. *Neurobiol Dis*, **10**, 88-99.

Zhang, X., Smith, D.L., Meriin, A.B., Engemann, S., Russel, D.E., Roark, M., Washington, S.L., Maxwell, M.M., Marsh, J.L., Thompson, L.M., Wanker, E.E., Young, A.B., Housman, D.E., Bates, G.P., Sherman, M.Y. and Kazantsev, A.G. (2005) A potent small molecule inhibits polyglutamine aggregation in Huntington's disease neurons and suppresses neurodegeneration *in vivo*. *Proc Natl Acad Sci U S A*, **102**, 892-897.

Zhao, Y., Sun, H., Lu, J., Li, X., Chen, X., Tao, D., Huang, W. and Huang, B. (2005) Lifespan extension and elevated hsp gene expression in *Drosophila* caused by histone deacetylase inhibitors. *J Exp Biol*, **208**, 697-705.