Localization and function of the *Drosophila* huntingtin protein

by

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ABSTRACT

Huntington's Disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion of a polyglutamine tract in the huntingtin protein. This mutation leads to conformational instability, resulting in huntingtin aggregation and degeneration of neurons in the striatum and cortex. HD is characterized by motor dysfunction, personality changes, dementia, and early death. Although a number of abnormal cellular phenomena have been described in systems modeling HD, the specific events initiating pathology remain unclear. It is widely viewed that inclusions may have a toxic gain-of-function which is central to HD pathogenesis. However, evidence is accumulating that supports the loss of huntingtin function as a likely contributor to the unravelling of cellular processes early in the course of the disease.

The fruitfly *Drosophila melanogaster* has an orthologous huntingtin gene with several regions showing 40-50% similarity to mammalian huntingtin at the amino acid level. Like the mammalian huntingtin gene, the fly huntingtin lacks sequence motifs that would suggest functional correlates to other known proteins.

I have pursued a cell biological and physiological analysis of *Drosophila melanogaster* mutants with reduced huntingtin gene expression. Normal levels of huntingtin were not required for normal localization of mitochondria in neurons, synaptic transmission in the visual system, or formation of synapses.

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

Huntington’s Disease: Background

Huntington’s Disease (HD) is the most common inherited late-onset neurodegenerative disease, which afflicts approximately 1 in 10,000 individuals in North America and Europe (HDCRG, 1993). HD is a progressive disorder typically commencing in middle age (mean age of 35). However symptoms have been observed in extreme cases in infants and in adults over 80 (Myers, 2004). HD is characterized by involuntary choreiform movements, loss of motor coordination, dementia, personality changes, and ultimately premature death within 10-15 years following onset of symptoms (Vonsattel et al., 1985).

HD is caused by an expansion in the polyglutamine (polyQ)-encoding ‘CAG’ nucleotide repeat domain in the 5’ region of the gene, which resides at 4p16 in the human genome (HDCRG, 1993). The HD phenotype is exhibited when the expansion of this tract occurs beyond a threshold number (~35) (Rubinsztein et al., 1996). Age of onset and severity of symptoms are correlated with length of the CAG expansion, with longer repeats resulting in earlier age of onset and more rapid progression of symptoms, culminating in mortality (Duyao et al., 1993). Pathogenic repeat lengths exhibit the phenomenon of ‘anticipation’, where the repeat length is unstable and tends to increase in size when transmitted to subsequent generations (McInnis, et al. 1996). There is currently no cure or effective therapy for HD.
FIGURE 1. Polyglutamine length-dependence of HD age of onset. Schematic showing the relative location and variable length of the polyglutamine domain of the human huntingtin gene. Repeat number is inversely corellated to age of onset. (Gladstone Center for Translational Research, University of California at San Francisco.)
HD is among a family of late-onset, progressive neurodegenerative polyglutamine diseases showing an autosomal dominant pattern of inheritance (see Figure 2). Common to all of these diseases is polyQ expansion and subsequent conformational instability, leading to the accumulation of abnormal forms of the protein. In each condition the pathogenic protein is expressed ubiquitously in the brain, however the neuronal subtypes exhibit a selective vulnerability leading to characteristic neurodegenerative symptoms specific to each disorder. This phenomenon is observed more generally in other neurodegenerative diseases involving protein misfolding, including Alzheimer’s disease, amyotrophic lateral sclerosis (Lou Gherig’s disease), Parkinson’s disease, and the spongeiform encephalopathies (prion diseases). In general the onset of symptoms is loosely correlated with the formation of neuronal inclusions. In HD, huntingtin-immunopositive inclusions are present in neurons that degenerate, however the presence of such inclusions does not exactly correspond with neurodegeneration (Vonsattel et al., 1985; Kuemmerle et al., 1999). The role of huntingtin aggregation in HD pathogenesis continues to be controversial and specific pathways leading from protein misfolding to pathogenesis remain obscure.

HD brains show widespread abnormalities including dystrophic neurites and apoptotic neuronal death, displayed most prominently in the striatum and cortex, resulting in a loss of brain weight of up to 30% (Aylward et al., 1997). Neuropathology in HD patients is highly selective, with substantial loss of neurons in the caudate and putamen of the basal ganglia, particularly GABAergic
type II medium spiny neurons, which comprise 80% of striatal neurons (DiFiglia et al., 1991). These neurons receive glutamatergic signals from the cerebral cortex and are involved in motor control, the loss of which is consistent with HD's characteristic choreiform disorder (Albin et al., 1990).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene locus</th>
<th>Gene product</th>
<th>Normal CAG(n)</th>
<th>Expanded CAG(n)</th>
<th>Protein localization</th>
<th>Special features</th>
<th>Brain regions most affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBMA</td>
<td>Xq11-12</td>
<td>Androgen receptor</td>
<td>9-36</td>
<td>38-62</td>
<td>Nuclear and cytoplasmic</td>
<td>Intermediate alleles: 29-35</td>
<td>Anterior horn and bulbar neurons, dorsal root ganglia</td>
</tr>
<tr>
<td>HD</td>
<td>4p16.3</td>
<td>Huntingtin</td>
<td>6-34</td>
<td>36-121</td>
<td>Cytoplasmic</td>
<td></td>
<td>Striatum, cerebral cortex</td>
</tr>
<tr>
<td>SCA1</td>
<td>6p22-23</td>
<td>Ataxin-1</td>
<td>6-44</td>
<td>39-62</td>
<td>Nuclear in neurons</td>
<td>Normal alleles &gt;21 repeats interrupted with 1-4 CAT units</td>
<td>Cerebellar Purkinje cells, dentate nucleus; brainstem</td>
</tr>
<tr>
<td>SCA2</td>
<td>12p23-24</td>
<td>Ataxin-2</td>
<td>15-31</td>
<td>36-63</td>
<td>Cytoplasmic</td>
<td></td>
<td>Cerebellar Purkinje cells, brain stem, fronto-temporal lobes</td>
</tr>
<tr>
<td>SCA3</td>
<td>14q24.3-31</td>
<td>Ataxin-3</td>
<td>12-41</td>
<td>62-84</td>
<td>Cytoplasmic</td>
<td></td>
<td>Cerebellar dentate neurons, basal ganglia, brain stem, spinal cord</td>
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<td>SCA6</td>
<td>19p13</td>
<td>CACNA1A</td>
<td>4-18</td>
<td>21-33</td>
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<td></td>
<td>Cerebellar Purkinje cells, dentate nucleus, inferior olive</td>
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<td>4-35</td>
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<td>Cerebellum, brain stem, macula, visual cortex</td>
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<td>12q</td>
<td>Atrophin-1</td>
<td>6-36</td>
<td>49-84</td>
<td>Cytoplasmic</td>
<td></td>
<td>Cerebellum, cerebral cortex, basal ganglia, Luys body</td>
</tr>
</tbody>
</table>

FIGURE 2. Polyglutamine diseases. Spinobulbar muscular atrophy (SBMA), Huntington's disease (HD), and the spinocerebellar ataxias [including dentatorubropallidoluysian atrophy (DRPLA)] are the 8 neurodegenerative diseases caused by polyglutamine expansion. All are dominantly inherited, with the exception of SBMA. Although the basis of molecular dysfunction is common to all, the symptoms and regions of brain pathology are distinct. (Reproduced from Zoghbi et al., 2000)

**Huntingtin form and potential function**

The precise function of the huntingtin gene is unknown. Determining the early pathogenic events in Huntington's Disease and distinguishing them from downstream effects is essential to understanding the disease and developing essential treatment strategies. The huntingtin protein is a very large (348kD), soluble protein expressed ubiquitously and enriched in the brain and testes (DiFiglia et al., 1995). Human huntingtin comprises 3,144 amino acids and largely lacks protein domains with defined biological function, thus frustrating efforts to situate it within the family of other proteins with well-defined biological roles.
FIGURE 3. β-sheet models of polyglutamine aggregation. In this schematic representation of several proposed structural models of misfolded polyQ domains, β-sheet is represented by the red zig-zag lines. (a) The extended antiparallel β-sheet, or “polar zipper” model (Perutz et al., 1994), or alternatively a parallel β-sheet (b), anti-parallel β-hairpin (c), compact random coil composed of four anti-parallel (d) or β-strand (e) elements, and finally (f) a regular parallel β-helix with periodicity of 20 residues. (Reproduced from Ross et al., 2003)
Huntingtin is associated with various organelles including the nucleus, golgi apparatus, and endoplasmic reticulum, and localizes to synapses where huntingtin associates with clathrin-coated vesicles, endosomal vesicles, and microtubules (Velier et al., 1998; Hoffner et al., 2002; Kegel et al., 2002). This widespread subcellular localization and the inability to find structural and sequence homology with other known protein domains frustrated initial efforts to define huntingtin cell function.

The polyglutamine repeat region is often modeled to form a polar zipper structure, which may be involved in associations with Q-rich domains in transcription factors, including cAMP-responsive-element binding protein (CBP), p53 (McC Campbell et al., 2000; Steffan et al., 2000), Sp1, TAFII130 (Dunah et al., 2002), N-CoR, and Sin3A (Boutell et al., 1999). Huntingtin’s polyproline (polyP) region has been implicated in interactions with dynamin, huntingtin-interacting protein 1 (HIP1), and SH3-containing Grb2-like protein (SH3GL3) (Qin et al., 2004). Interactions through this polyP region may be direct associations with SH3 and WW protein domains, or mediated by structural stabilization of the polyglutamine domain. Other huntingtin-interacting proteins are huntingtin-associated protein 1 (HAP1), which binds with the p150glued subunit of dynactin (Li et al., 1998). Both huntingtin and HAP1 are transported bidirectionally in neurons (Block-Galarza et al., 1997). Other interacting partners are protein kinase C and casein kinase substrate in neurons 1, postsynaptic density-95, and FIP-2 (Harjes and Wanker, 2003). These proteins are involved in vesicle transport, clathrin-mediated endocytosis, apoptosis, cell-signalling,
morphogenesis, and transcriptional regulation, which suggests that huntingtin may have a role in several diverse processes.

![Diagram](image)

**FIGURE 4. Schematic of huntingtin's amino acid structure.** (Q)n and P(n) represent the polyglutamine and polyproline regions, respectively. The 37 HEAT repeat domains are clustered into 3 main groups (red boxes). The circles indicate locations of post-translational modifications (sumoylation/ubiquitination at the red circles, phosphorylation at blue). Arrowheads and triangles indicate sites of caspase and calpain cleavage, respectively. NES is the nuclear export signal sequence. (figure from Tartari et al., 2005)

Huntingtin has an active C-terminal nuclear export signal and a partially active nuclear localization sequence, which suggests huntingtin may be involved in transporting proteins between the nucleus and cytoplasm (Xia et al., 2003). This is consistent with huntingtin's nuclear and perinuclear localization. The deletion of the domain involved in associating with the nuclear protein TPR results in nuclear accumulation of huntingtin (Cornett et al., 2005).

Downstream of the polyglutamine region are the so-called 40-amino acid HEAT repeat domains, which occur repeatedly in Huntingtin, Elongation factor 3, protein phosphatase 2A, and TOR1 proteins, and are thought to be involved in protein-protein interactions (Andrade and Bork, 1995). Human huntingtin has 37 putative HEAT repeat domains in 3 clusters. *Drosophila* huntingtin has 28 HEAT repeat domains (Takano et al., 2002). The presence of these domains indicate
that huntingtin could play a role as a molecular scaffold or adaptor for a variety of different cargos undergoing cellular trafficking.

Four types of post-translational modification of huntingtin have been described. The N-terminal lysines are involved in sumoylation, which reduces the ability of N-terminal fragments to form aggregates (Steffan et al., 2004), and ubiquitination (Kalchman et al., 1996). The phosphorylation of serines at positions 421 and 434 in the human protein are associated with proteolytic cleavage. Phosphorylation of these residues is reduced in the disease form of the protein and is associated with increased cleavage and toxicity (Luo et al., 2005). Huntingtin is palmitoylated by huntingtin-interacting protein 14, which is necessary for its trafficking in axons and also consistent with its proposed role in vesicular trafficking (Difiglia et al., 1995; Yanai et al., 2006).

Protein localization studies are consistent with a role for endogenous huntingtin in axonal transport. Immunohistochemical studies in mammalian systems reveal huntingtin labeling in the neuronal cytoplasm, nerve tracks, intense punctate staining at synapses, and around secretory vesicles at the ultrastructural level (DiFiglia et al., 1995). Normal huntingtin undergoes fast axonal transport in both anterograde and retrograde directions (Block-Galarza et al., 1997). Neurodegeneration could result from interruption of trafficking of essential neurotrophic factors, e.g. BDNF, by mutant huntingtin. In accordance with this hypothesis, studies of Alzheimer's disease and other non-polyQ diseases have found that neurodegeneration is a consequence of defective axonal transport.
This variety of potential activities suggest diverse cellular roles for huntingtin. It is conceivable that the huntingtin protein is flexible in form depending on its interacting partners, lending it multi-functionality according to the timing and arrangement of its subcellular localization. This hypothesis is supported by the evidence that different huntingtin epitopes are reactive to different antibodies depending on the subcellular localization of the protein (Ko et al., 2001).

Mechanisms of pathogenesis

The causative mutation in HD is an expansion of glutamine-encoding CAG triplet repeats in exon 1 of the HD gene, huntingtin, beyond a threshold of 35-40. Huntingtin is a very large (3,144 amino acid, ~350kD) protein expressed not only in the brain of humans and mice, but also in testes, liver, heart, lungs, and pancreatic islets as well (Ferrante et al., 1997; Cattaneo et al., 2005).

A central point of debate in HD research is the role of huntingtin-immunopositive intracellular aggregates. Evidence has supported diverse claims: that they instigate neurodegeneration (Yang et al., 2002), are merely by-products of the neurodegeneration process (Szebenyi et al., 2003; Saudou et al., 1998), or are even neuroprotective in nature (Arrasate et al., 2004). Aggregate formation has been shown by several lines of evidence to be promoted by truncated polyQ-expanded N-terminal huntingtin fragments. A consensus is emerging that a cascade of cytopathic events culminate in the HD phenotype.
Caspase cleavage

Huntingtin contains 3 well-characterized protease cleavage sites, which are involved in the fragmentation of both wild-type and mutant huntingtin, although the mutant form is more susceptible to cleavage and fragments accumulate in the nucleus and cytoplasm (Goldberg et al., 1996; Wellington et al., 1998; Gafni et al., 2004). Caspase cleavage sites are conserved in vertebrates but lacking in Drosophila. The role of proteolysis in huntingtin function is not understood. However, experiments inhibiting caspase and calpain activity in cells reduces cleavage of the mutant protein and reduces toxicity (Wellington et al., 2000; Gafni et al., 2004).

Proteolytic cleavage produces short, toxic N-terminal fragments containing the polyQ expansion. Expression of huntingtin exon 1 containing a pathogenic polyQ tract is sufficient to produce HD-like symptoms in mice and flies. These truncated fragments have been demonstrated to form intracellular aggregates more easily compared to polyQ expansions in the context of longer proteins. Recently investigators have shown that specific Cdk5 phosphorylation of huntingtin in cultured human neurons reduces huntingtin cleavage, and subsequent toxicity and aggregate formation, while products of cleavage specifically inhibit the use of huntingtin by Cdk5 as a substrate. This suggests a scenario wherein toxic huntingtin fragments compromise the ability of Cdk5 to restrict huntingtin cleavage, resulting in a positive feedback loop and rapid accumulation of pathogenic huntingtin cleavage products. Soluble mutant huntingtin has also been observed to interact with mTOR kinase leading to its
loss of function, suggesting that mutant huntingtin may bind to several kinases and cause diverse physiological changes in cells. The ability of *Drosophila* huntingtin to function as a substrate for proteolysis in flies has yet to be determined.

*Transcriptional dysregulation and excitotoxicity*

Both normal and mutant forms of huntingtin have been shown to interact with a host of transcription factors, including TAFII130 and Sp1 (Dunah et al., 2002), and the cAMP response element binding protein binding protein (CBP) (McCampbell et al., 2000). Normal huntingtin stimulates levels of cortical BDNF by acting at the level of BDNF gene transcription, an effect which is lost in the presence of the mutant protein (Zuccato et al., 2001). Cleavage of full-length huntingtin has been shown to yield toxic huntingtin N-terminal fragments in mice which localize to the nucleus and sequester Q-rich transcription factors in aggregates. These reports support a general model of transcriptional dysregulation as a significant contributing factor of HD pathology. This could arise from loss of normal interactions between huntingtin and transcription factors or abnormal interactions arising from polyglutamine expansion in the pathogenic form.

Medium spiny neurons (MSNs) in the striatum are most severely affected in HD. MSNs are innervated by glutamatergic axons, glutamate being the primary excitatory neurotransmitter in the mammalian brain. This observation led to the hypothesis that mutant huntingtin exerts an excitotoxic effect on these neurons,
and has found support in experiments wherein selective loss of MSNs and HD-like symptoms are produced when glutamate receptor agonists are delivered to the striatum of test animals.

**Huntingtin loss of function phenotypes**

Huntingtin is critical for early embryonic development in mice, as homozygosity for inactivated huntingtin alleles generated by targeted disruption results in death around embryonic day 8, before the nervous system is formed. Null embryos form the three germ layers, but these become disorganized and malformed during gastrulation. Mice expressing less than 50% of normal huntingtin levels are viable, however they show abnormal brain development, neurodegeneration, and sterility (White et al., 1997; Auerbach, et al. 2001; Nasir et al., 1995). Mice that develop in the presence of huntingtin but lose normal huntingtin in post-mitotic neurons show an HD-like phenotype (Dragatsis, et al., 2000).

Reduced levels of huntingtin lead to abnormal distribution and morphology of cellular organelles in murine embryonic stem cells (Hilditch-Maguire et al., 2000). Transport of vesicles and mitochondria is diminished in *Drosophila* expressing siRNAs targeting huntingtin (Gunawardena et al., 2003) and in neurons from conditional huntingtin -/- mice (Trushina et al., 2004). Furthermore, decreased huntingtin expression in flies with only 50% of genetic complement of the motor proteins kinesin or dynein exhibited axonal swellings and organelle jams characteristic of flies with severe axonal transport deficits due to 100% loss.
of these motor proteins (Gunawardena et al., 2003; Pilling et al., 2006). This suggests that huntingtin interacts with these motor proteins at some level in the process of organelle transport, and that it is plausible that huntingtin loss-of-function may contribute to axonal swellings and blockages in HD models where the pathogenic protein is expressed. In the context of HD, a huntingtin loss of function model is supported by the observation that wild type huntingtin is sequestered among misfolded mutant huntingtin proteins within cytoplasmic aggregates.

**Axonal transport defects**

More recently, several reports have revealed a new mechanism by which mutant huntingtin disturbs brain function: disruption of axonal transport. This defect could be a manifestation of axonal blockage by huntingtin aggregates, or by a loss of function phenotype.

Evidence suggesting axonal blockages are formed from huntingtin aggregates has come from studies in cultured neurons and in *Drosophila* (Li et al. 2001; Lee et al., 2004). Huntingtin has been reported to be involved in axonal trafficking in rat sciatic nerve (Block-Galarza, J., et al. 1997), while reduced amounts of huntingtin interfere with proper axonal transport in larval neurons in *Drosophila* (Gunawardena et al., 2003). Polyglutamine-dependent inhibition of retrograde and anterograde transport has also been documented in squid giant axons (Szebenyi et al., 2003). These findings are consistent with other studies that show polyglutamine-dependent axonal pathology precedes neurodegeneration in *C. elegans* and mice (Parker et al., 2001; Li et al., 2001).
Mitochondrial toxicity

Mitochondria are normally trafficked bidirectionally in neurons. Healthy mitochondria that produce ATP and are needed to maintain membrane potential and calcium homeostasis are moved to the synapse, while damaged, aged mitochondria are moved to the cell body for repair or disposal in lysosomes (Miller and Sheetz, 2004). Deficiencies in normal mitochondrial localization may contribute to a retarded ability to respond to metabolic buffering needs, leading to neuronal dysfunction, while persistence of damaged mitochondria could lead to generation of reactive oxygen species that cause cellular damage and apoptosis (Lee and Wei, 2000). Transport of vesicles and mitochondria is diminished in Drosophila (Gunawardena et al., 2003) and in neurons from conditional huntingtin -/- mice (Trushina et al., 2004).
FIGURE 5. Potential pathways of polyglutamine pathology. (Adapted from Rudnicki and Margolis, 2003) (a) The pathogenic process (blue arrows) begins with the synthesis of a protein with an expanded polyglutamine (polyQ) tract. (b) The expanded polyglutamine tract alters the native conformation of the protein, which is reinforced by the presence of molecular chaperones. Misfolded protein undergoes two distinct proteolytic processes: (c) lysosomal-dependent proteolysis; (d) some protein is ubiquitinated (Ub) and degraded via the proteasome. (e) Cleavage produces an N-terminal fragment that is prone to aggregation. (f) The mutant proteins proceed from a monomeric random coil or β-sheet into oligomeric β-sheets and eventually into insoluble aggregates. (g) These species undergo abnormal interactions with cellular proteins, or in another model might represent a mechanism for reducing the toxicity of aggregation intermediates by sequestering toxic monomeric forms of the mutant protein. (h) Aggregation intermediates inhibit proteasomal processing. (i) The monomers or oligomers directly activate caspases or disrupt mitochondrial function. (j) Aggregates translocate into the nucleus (by an unknown mechanism) and (k) recruit specific nuclear factors, co-activators and co-repressors, inhibiting their normal activities and (l) resulting in altered gene transcription.
Chapter II: Results

Characterization of huntingtin function in Drosophila

Abstract

To observe how a loss of huntingtin function may contribute to abnormal trafficking of mitochondria in motor neurons, I examined the distribution of a GFP-tagged mitochondrial protein in motor neurons of live Drosophila under conditions of reduced huntingtin gene expression. There was no obvious difference in mitochondrial localization in the motor neurons of 3rd instar larvae expressing a transgenic dsRNA hairpin reported to significantly reduce huntingtin gene expression via RNA interference or in embryos injected with siRNAs.

In order to observe the localization of endogenous huntingtin protein in Drosophila and evaluate the extent of gene expression in flies expressing reduced levels of huntingtin protein, I pursued the generation of antibodies specific to Drosophila huntingtin. Unfortunately these antibodies were not reactive to endogenous fly huntingtin via Western or immunological approaches.

To evaluate how loss of huntingtin function affects neuronal survival and physiology in Drosophila, I pursued strategies to create a huntingtin null mutant. I examined retinal neuron function and resistance to stress via electroretinograms (ERGs) in huntingtin null flies. There was no significant difference between ERGs in huntingtin null flies and controls.
Introduction

In spite extensive efforts to understand the basis of HD pathology from the perspective of mutant protein misfolding and aggregation, the body of information resulting from these studies is often confusing. However recent evidence suggests HD pathology may be in part due to a loss of function of wild-type huntingtin. Models positing protein aggregation and wild-type loss of function are not mutually exclusive however, as wild-type huntingtin is sequestered in aggregates inside cells expressing the mutant protein.

The *Drosophila* huntingtin (Htt) gene is 11kB and encodes a large 396 kD protein that, like its vertebrate homologs, lacks domains that are conserved among known protein families. The fly ortholog lacks the continuous polyglutamine stretch that characterizes the mammalian Htt, although it retains three large regions showing approximately 25% amino acid sequence identity and 50% similarity. The dHtt locus is comprised of 29 exons and spans 43kB of genomic DNA at cytologic band 98E on the 3rd chromosome. Like the mammalian gene, the fly version lacks sequence motifs that would suggest functional correlates to other known proteins (Li et al., 1999). Vertebrate huntingtin sequences are highly conserved, with human and zebrafish sequences sharing 70% identity at the amino acid level. In contrast the *Drosophila* sequence is relatively dissimilar to those of vertebrates. The fly homolog lacks the polyglutamine repeat domain and the adjacent polyproline domain characteristic of human huntingtin (Li et al., 1999). The fly homolog is the largest of the huntingtin family, extending several hundred amino acids beyond
other vertebrate huntingtin sequences. The transcript is widely-expressed at low levels throughout the developmental cycle of *Drosophila* (Li et al., 1999).

Initial models of HD in flies were created by injecting flies with glutamine expansions and subsequently observing a neurodegenerative phenotype. At present our HD model system is comprised of transgenic flies expressing the N-terminal region of the human huntingtin gene containing pathogenic or nonpathogenic numbers of glutamines under control of the UAS/GAL4 binary expression system. Adult flies expressing pathogenic (Q128) huntingtin in the nervous system exhibit abnormal grooming behavior, defective motor coordination, and significantly abbreviated lifespan, while flies expressing non-pathogenic isoforms (Q0) are indistinguishable from wild type animals. Larvae expressing huntingtin-Q128 display cytoplasmic aggregates throughout the CNS, including axonal blockages that trap synaptic proteins. These blockages are absent in animals expressing non-pathogenic huntingtin-Q0, Q127 alone, or Q108 in the context of the non-pathogenic dishevelled gene (Gunawardena et al., 2003).
Results


Polyclonal antibodies were purified from an existing stock of antisera raised against the N-terminal 319 amino acids of the Drosophila huntingtin (d htt) protein from 3 different rats (figure 6A). Antibodies were affinity purified in batch with GST-d htt N319 bound to glutathione sepharose beads (Amersham Biosciences). From the three independently derived batches of sera (#188, 189, and 190), batch #188 showed the most reactivity and least background in probes of blots of the recombinant immunogen and endogenous protein isolated from fly heads. Unfortunately, immunostaining with these antibodies failed to reveal any reactivity to any structures in fixed 3rd instar larvae.

New polyclonal antibodies were generated in two rabbits against the same N-terminal region of the protein. This second batch of polyclonal antibodies did not show consistent reactivity in Western blots with protein from wildtype Drosophila or immunostaining of 3rd instar larvae.

To obtain samples for Western analysis of huntingtin protein expression in Drosophila, flies were frozen in liquid nitrogen and vortexed to isolate 20 heads of each genotype.
FIGURE 6. Immunoreactivity of Drosophila huntingtin antibodies. (A) Animals were injected with recombinant protein corresponding to the N-terminal 319 amino acids of the fly huntingtin gene (boxed region). (B) Huntingtin antibodies were used at several concentrations and detected using a goat anti-rabbit antibody conjugated to HRP (Jackson ImmunoResearch Laboratories). Protein extracts from 5 to 0.5 heads in sequentially decreasing amounts was loaded in each lane.

In vivo observations of mitochondrial transport in huntingtin hypomorphs

In order to see if reduced huntingtin gene expression results in mislocalization or accumulation of mitochondria in neurons, which would be consistent with a role for huntingtin in axonal trafficking of these organelles, I observed how GFP-tagged mitochondria moved in real time in individual motor neurons in 3rd instar larvae using fluorescent microscopy. I recombined the 3rd chromosome P[GawB]D42 GAL4 driver, which drives expression of UAS-tagged genes in motor neurons of 3rd instar larvae, with a 3rd chromosome UAS-mitoGFP insertion to make a homozygous line expressing fluorescently marked mitochondria in motor neurons (figure 7). Mitochondrial distribution and axonal
morphology in animals expressing dsRNAs against huntingtin RNA (Gunawardena et al., 2003) were indiscernible from control larvae with wild-type huntingtin. Injection of D42 GAL4; UAS mitoGFP embryos with two different siRNAs targeting huntingtin likewise did not show any phenotypic deviation from control animals injected with the antisense oligonucleotides.

Although preliminary observations of neuronal GFP-tagged mitochondria in two different RNAi systems failed to show any phenotype, I did not confirm that any reduction in huntingtin RNA or protein was achieved through these methods.

Approximately eighty 21-nucleotide candidate siRNA target sequences with optimal efficiency were generated using a web utility from Ambion (http://www.ambion.com/techlib/misc/siRNA_finder.html). From these eighty, two were selected based on minimal sequence overlap with non-target sequences that might complicate interpretation of any phenotype due to non-target gene knockdown. The sequences, corresponding to bases 308-328 and 1265-1285 of the Drosophila huntingtin mRNA transcript, had an E value of 3.8 for non-target sequences from a BLASTN search of the Drosophila genome map. siRNA injection of Drosophila embryos was performed by Zhou Guan. Drosophila expressing a GFP-tagged mitochondrial protein in D42 motor neurons were used in the procedure so that mitochondrial trafficking could be observed in experimental animals with decreased huntingtin expression. There was no significant phenotype in flies injected with siRNAs targeting huntingtin compared to control injected flies.
Huntingtin mutagenesis approaches in *Drosophila*.

In order to study any loss of function huntingtin phenotypes in *Drosophila*, I pursued three different approaches to generating a mutant with reduced huntingtin gene function. Initially a strategy was pursued to generate a huntingtin mutant by mobilizing the P-element P{GT1}BG01706 out of its site approximately 100kB 5' to the predicted huntingtin promoter and into the gene itself. This P-element is located approximately 200kB 5' of the huntingtin locus (figure 8a). I screened several hundred independently-derived lines, however no eye color changes (indicative of P-element transposition due to position effects of eye color marker) were observed in the F2 generations.

This approach was abandoned following our discovery of an alternative library of flies containing a *piggyBac* transposon inserted in a non-coding sequence within the huntingtin locus. Strain c07030 from the Exilixis library contains a piggyBac element less than 400bp from the predicted huntingtin promoter (Figure 8b). *piggyBac* transposons can tag and disrupt genes without the insertion biases of P-elements, however *piggyBac* elements only excise precisely, therefore mutagenesis via imprecise excision is not possible (Thibault et al., 2004). These flies were used as a template for mutagenesis of the huntingtin locus by gamma ray irradiation. Screening of over 1,000 F1 offspring for loss of red eye color due to lesioning of genomic DNA in the vicinity of the *piggyBac* produced no potential candidates.
The third and final strategy for pursuing a huntingtin mutant involved precise excision of DNA between two transposable elements flanking huntingtin genomic sequence. Within the Exelixis collection of PBac transgenic flies, we found three strains of *Drosophila* with *piggyBac* transposons inserted into non-coding regions near or within the huntingtin locus (Figure 7A)(Thibault et al. 2004). The sequences of these transposon constructs include short (48-bp) FRT recombination sites from the yeast 2μ plasmid. In the presence of FLP recombinase expressed via an exogenous Hsp70 promoter on the X chromosome, the intervening huntingtin coding sequence flanked by the PBac FRT sites is excised precisely and with relatively high efficiency.

FLP recombinase-expressing females bearing each of the dHtt PBac alleles were mated to white-eyed males with the third chromosome balancer TM3 GFP. I created ~200 lines derived from single flies coming from this crossing, the non-balancer third chromosome in each line being uniquely derived from an individual transposition event. The mutagenesis plan called for screening for putative dHtt recombinant F1 progeny via a PCR-based strategy to confirm deletion of the targeted Htt sequence in the excision event. However at this time we discovered that a huntingtin mutant had been generated using this strategy by Sheng Zhang from the Perrimon lab at Harvard Medical School. Subsequently I pursued phenotypic analysis of these flies.
FIGURE 7. Genomic maps of Drosophila strains containing transposons used in mutagenesis of huntingtin gene. (A) Schematic showing position of the P element used in the initial transposition and imprecise excision strategy, and the three piggyBac elements used in final mutagenesis attempt. (B) Composition of piggyBac elements in WH strains from Exilixis collection (Thibault et al. 2004). (C) Recombination resulting in deficiencies is efficient (~10%) when proximal (<100kB) FRT sites are recombined in trans in the presence of FLP transposase (Parks et al. 2004).
Analysis of Huntingtin mutant flies

To detect any abnormal synaptic transmission at a time when behavioral motor defects were evident, I measured electroretinograms (ERGs) in the visual system of adult flies whose huntingtin gene was deleted. ERGs are extracellular recordings of photoreceptor depolarization that indicate synaptic transmission to second order neurons in response to light. Synaptic events occur at the onset and termination of a light pulse and are represented by the on- and off-transients of the ERG. Mutants that disrupt synaptic transmission lose on/off transients (Littleton et al., 1998). Young mutant flies (adults 1-2 days old) show a normal ERG response at 20 degrees and when stressed at 37 degrees (figure 9A). Both old (40-45 days) and young flies show inconsistent loss of ERGs: the mutants show a higher percentage loss of ERGs under these conditions (~60%), however the control animals show the same loss albeit at a lesser penetrance (20%) (figure 9B). There was no consistent defect in spiking or seizure activity in the DLM when assayed at either temperature.
FIGURE 8. Electrophysiological analysis of Htt mutants by Sudipta Saraswati and J. Troy Littleton. (A) Electroretinograms (ERGs) recorded from control and Htt mutants aged 40-45 days at 25°C, or from Htt mutants aged 1-3 days. Flies were rapidly heated from 20°C to 37°C, with test light pulses (black bar below trace) given at regular intervals. Htt mutants showed a more severe temperature-induced loss of phototransduction than controls, suggesting Htt mutant photoreceptors were stress-sensitive compared to controls. (B) Percent of adult animals aged 40-45 days with a loss of phototransduction at 37°C. The number of preparations analyzed was: control (10); Htt (16).
Materials and methods

Antibody generation

Recombinant immunogen was expressed in BL21 E.coli cells as an N-terminal GST fusion in E. coli from the pGEX2T plasmid encoding the N-terminal 319 amino acids of the Drosophila huntingtin gene. Two rabbits were immunized as per standard protocol (Invitrogen: http://www.invitrogen.com/content.cfm?pageid=3987).

Huntingtin mutagenesis in Drosophila

To mutate the huntingtin gene with gamma rays, male piggyBac{PB}huntingtin^{07030} Drosophila containing a w’ eye color marker approximately 125 bp from the 5’ end of the huntingtin locus were dosed with 4,000 RADs at 662 kEV from a ^{137}Cs source and mated with w^- virgins.

Electrophysiology in Huntingtin mutants

Electrophysiological analysis of wandering stage 3rd instar larva was performed in Drosophila HL3.1 saline (NaCl, 70 mM; KCl, 5; MgCl₂, 4; CaCl₂, 0.2; NaHCO₃, 10; Trehalose, 5; Sucrose, 115; HEPES-NaOH, 5; pH 7.2) using an Axoclamp 2B amplifier (Axon Instrument). Electroretinograms were performed as previously described (Rieckhof et al., 2003). Temperature shifts were performed by heating mounting clay encompassing the fly to the desired temperature with a peltier heating device.
Chapter III: Concluding remarks

In conclusion, the huntingtin loss of function phenotype appears to be very subtle in *Drosophila* and may not be a contributing factor in the gross defects observed in axonal transport in existing *Drosophila* HD models. However the physiological basis of mild movement defects observed in these huntingtin mutants remains to be determined. An investigation into genetic interactions between huntingtin and other axonal motor protein genes, such as kinesin, dynactin, and p150Glued may help to elucidate the mechanism by which huntingtin performs any role at synapses and facilitates vesicular and organelle transport along axons.

It remains to be reconciled how the null *Huntingtin* mutant we studied fails to demonstrate a phenotype consistent with UAS-GAL4 dsRNA loss of function huntingtin flies reported by Gunawardena et al., 2003. A more basic question is how *Drosophila* manage to develop and largely achieve full neuronal functions, which are severely disturbed in mammalian *Huntingtin* mutants. It would be interesting to see if *Huntingtin* null flies have a more exaggerated disease phenotype when the mutant form of the protein is expressed, in experiments akin to those showing that wildtype huntingtin mitigates the effects of the pathogenic form (Ho et al., 2001; Leavitt et al., 2001).

In spite of its subtle null phenotype, *Drosophila* retain abundant potential to reveal how huntingtin does its job in neurons, particularly with respect to axonal transport. The facility associated with observation and measurement of in vivo trafficking of fluorescently labeled axonal cargos via fluorescent microscopy...
is a powerful tool for functional screening of therapeutic compounds emerging as candidates from cell-based assays, such as Geldanamycin (Herbst, M. and Wanker, E.E. 2007). The behavioral phenotype observed in Huntingtin mutants and fly HD models is a valuable marker for identifying relevant genetic factors in large-scale screens of expanding RNAi and transposon insertion libraries (Dietzl et al., 2007; Thibault et al., 2004).
APPENDIX A: Genetic schemes

huntingtin gamma ray allele generation

\[
P \xrightarrow{w, PBac^{\text{w+}}\text{PBac}^{\text{w+}}} X \xrightarrow{w, Tm3 \text{ Tm6}}
\]

\[
F1 \xrightarrow{1 w, PBac^* \text{ PBac}^* \text{ white eyes}} X \xrightarrow{w, Sb \text{ Tm3-GFP}}
\]

\[
F2 \xrightarrow{w, PBac^* \text{ Tm3-GFP}} X \xrightarrow{w, PBac^* \text{ Tm3-GFP}}
\]

\[
F3 \xrightarrow{w, PBac^* \text{ Tm3-GFP}} \rightarrow \text{viability, PCR}
\]

FLP-FRT excision of huntingtin

\[
\text{Chr. II or III}
\]

\[
\text{isow} \quad \text{Y} \quad \text{Bal} \quad \times \quad \text{hs-FLP} \quad \text{Dom} \quad \text{Bal}
\]

\[
\text{hs-FLP} \quad \text{Y} \quad \text{Bal} \quad \times \quad \text{isow} \quad \text{Bal} \quad \triangle
\]

Heat shock progeny larvae to activate FLP expression

\[
\text{hs-FLP} \quad \text{Y} \quad \triangle \quad \times \quad \text{isow} \quad \text{Bal} \quad \text{Bal}
\]

\[
isow \quad \text{Y} \quad \text{Bal}
\]

Initial PCR done in next generation on males with putative deficiency
References


disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. Cell 81, 811–823


