Huntington disease (HD) is an inherited neurodegenerative disorder caused by a polyglutamine (polyQ) expansion in the huntingtin (Htt) gene. Despite years of research, there is no treatment that extends life for patients with the disorder. Similarly, little is known about which cellular pathways that are altered by pathogenic Huntingtin (Htt) protein expression are correlated with neuronal loss. As part of a longstanding effort to gain insights into HD pathology, we have been studying the protein in the context of the fruitfly Drosophila melanogaster. We generated transgenic HD models in Drosophila by engineering flies that carry a 12-exon fragment of the human Htt gene with or without the toxic trinucleotide repeat expansion. We also created variants with a monomeric red fluorescent protein (mRFP) tag fused to Htt that allows in vivo imaging of Htt protein localization and aggregation. While wild-type Htt remains diffuse throughout the cytoplasm of cells, pathogenic Htt forms insoluble aggregates that accumulate in neuronal soma and axons. Aggregates can physically block transport of numerous organelles along the axon. We have also observed that aggregates are formed quickly, within just a few hours of mutant Htt expression. To explore mechanisms of neurodegeneration in our HD model, we performed in vivo and in vitro screens to search for modifiers of viability and pathogenic Htt aggregation. Our results identified several novel candidates for HD therapeutics that can now be tested in mammalian models of HD. Furthermore, these experiments have highlighted the complex relationship between aggregates and toxicity that exists in HD.

Introduction

Huntington disease (HD) is a neurodegenerative disorder characterized by progressive motor disturbances, cognitive decline, and psychiatric symptoms. The disease usually appears between the ages of 30 and 40, and causes death 10–20 y later. HD affects 1 in 10,000 individuals of European descent. Despite decades of research, there are no targeted therapeutics capable of curing HD or retarding disease onset. HD is caused by an autosomal dominant mutation in a single gene, and manifests with 100% penetrance. In 1993, the Huntington’s Disease Collaborative Research Group cloned the huntingtin (Htt) gene, and discovered that the disease results from an unstable trinucleotide repeat expansion. The CAG repeat in exon 1 of Htt remains diffuse throughout the cytoplasm of cells, pathogenic Htt forms insoluble aggregates that accumulate in neuronal soma and axons. Aggregates can physically block transport of numerous organelles along the axon. The other polyQ diseases are: spinal and bulbar muscular atrophy, dentatorubral and pallidoluysian atrophy, and the spinocerebellar ataxias 1, 2, 3, 6, 7, and 17. Together, the polyQ diseases are the most common class of inherited neurodegenerative diseases. Understanding the mechanisms of mutant Htt toxicity may shed light on cellular dysfunction in other polyQ expansion disorders.
The Advantages of Studying HD Pathology in Drosophila

Drosophila is an excellent system for studying HD pathology and disease mechanisms. First, the *Htt* gene is conserved across evolution. All *Htt* homologs contain regions known as HEAT repeats, which form an α-helical scaffold structure important for Htt's protein-protein interactions.6,7 The *Drosophila* homolog of *Htt* contains 5 regions of strong homology, including HEAT repeats. *Drosophila*
and vertebrate Htt proteins are also similar in size: 3583 and 3144 amino acids, respectively.6

Studying mutant Htt in Drosophila allows use of the powerful molecular tools available in this model organism. One can modulate gene expression with spatial and temporal specificity, permitting inquiry into molecular interactions within specific cellular subpopulations.8 The short lifecycle and ease of genetic manipulation allows for large-scale, in vivo genetic screens that are not available in vertebrate models. Drosophila larval preparations are another strength of the model system, allowing live imaging of fluorescent-tagged proteins in neurons. This extra view will highlight how our lab and others have used screens and live imaging in Drosophila to gain insights into HD pathogenesis.

Strategy for Modeling HD in Drosophila

A key consideration in engineering a transgenic Drosophila HD model was determining which fragment of human Htt to use. The importance of protein context in disease has been well-documented.9 PolyQ expansions within different proteins are the root pathology of 9 different neurodegenerative diseases— each of which affects a distinct subpopulation of neurons and presents unique symptoms. The polyQ proteins also localize to distinct areas of the neuron.10 These differences are likely accounted for by protein context or differential protein interactions. Additional evidence for the importance of protein context comes from studies showing that expression of a polyQ stretch alone can be more toxic than a polyQ stretch embedded in a disease-causing protein.11

Expressing the full-length human Htt gene has been a challenge because of its large size: the Htt gene contains 67 exons and generates a protein product of 3,144 amino acids. The polyQ region, however, resides in exon 1 (Fig. 1A). To capture the polyQ region without the burden of the full-length transcript, one approach is to use only the first exon of Htt. These exon 1 constructs lose many key sites of known protein-protein interactions. For example, exon 1 models do not include the region of well-conserved HEAT repeats, which are thought to be critical for Htt’s interactions with several binding partners, including HIP1, HAP1, and HIP14.12 Another important portion of the Htt protein is a caspase-6 cleavage site at amino acid 586; a mouse model resistant to cleavage by caspase-6 at this locus showed reduced neurotoxicity.13 Several post-translational modification sites near the N terminus of the protein have also been implicated in disease progression. These include phosphorylation at serine residues 13, 16, and 421, and acetylation of lysine 444.14-17 Many of these sites are also missing in exon 1 models.

To try and capture a more physiologically relevant Htt fragment, we engineered constructs that included the first 12 exons of the human Htt gene. The resulting protein, 588 amino acids in addition to the polyQ stretch, contain the sites for post-translational modification, caspase cleavage, and many of the HEAT repeats (Fig. 1A). The flies carry an upstream activating sequence (UAS) in front of the gene to allow temporal and spatial expression via the UAS-GAL4 system. Two variations were engineered. One contained 15 polyQ repeats, representing the nonpathogenic form of Htt. The other contained a stretch of 138 polyQ repeats, representing a severe form of the disease observed in juvenile onset HD. We also generated constructs with a monomeric red fluorescent protein (mRFP) tag fused to the N-terminal of the protein. The fluorescent tag can be used for live imaging, allowing one to study the location, trafficking, and aggregation kinetics of Htt in vivo.

Aggregates: Beneficial, Toxic, or Byproduct?

The most salient feature of HD models is aggregation of the polyQ expanded Htt protein. Yet, it is unclear how aggregates contribute to toxicity in HD and other polyQ diseases. Drosophila and mouse studies suggest aggregates are not necessarily linked to pathology. Expression of a human HttQ128 in the Drosophila CNS has been shown to increase neurotransmitter release, trigger neuronal degeneration, impair motor function, and reduce lifespan, but no Htt aggregates were visible in soma or axons.18 Conversely, a mouse model that expressed an N-terminal fragment of HttQ128 resulted in extensive aggregation, but no behavioral dysfunction or neuronal loss.19 A separate mouse model that expressed mutant Htt only in cortical pyramidal neurons yielded the same results.20 These studies suggest that aggregates are not required or sufficient for neuronal pathology.

An alternative model has suggested that aggregates may be beneficial for neurons. A study in N2a cell cultures showed that soluble oligomeric mutant Htt—and not aggregated mutant Htt—caused toxicity.21 Similarly, tracking mutant Htt aggregation in cultured primary mouse neurons showed aggregates were inversely proportional to cell death.22 This data suggests aggregates may be neuroprotective by sequestering a toxic soluble Htt species.

However, evidence suggesting aggregates are toxic has also been reported. Overexpressing cellular chaperones known as heat shock proteins eliminates aggregates and rescues cell death induced by mutant polyQ proteins in yeast, mammalian cell culture, Drosophila, and mouse models.23-26 In a conditional mouse model of HD, one observes the same link between aggregates and survival. At 18 weeks of age, when pathological symptoms began to appear, turning off HttQ94 gene expression caused aggregates to disappear, and motor and behavioral deficits were dramatically improved.27 Pharmacological studies also suggest aggregates are toxic in HD. Small molecule screens to identify inhibitors of polyQ aggregation demonstrated that C2–8, a potent inhibitor of aggregation, also suppressed neurodegeneration in Drosophila and mouse HD models.28,29 In short, a variety of therapeutic approaches have implicated aggregates as a noxious species in HD.

Axonal Transport Defects in Drosophila Models of Huntington Disease

To examine the link between mutant Htt aggregation and neurodegeneration, we began investigating deficits in our Htt polyQ expressing Drosophila. A previous HD model in the lab suggested
axonal transport defects were likely to be a contributing factor to pathology. This model contained the first 548 amino acids of Htt, with a polyQ stretch of 128 repeats, representing the pathogenic form of the protein, or Q0, a non-pathogenic form (Fig. 1A). Driving expression of UAS-HttQ128 in the eye with GMR-GAL4 caused photoreceptor degeneration and aberrant photoreceptor function. Weak, pan-neuronal expression resulted in decreased lifespan, while stronger pan-neuronal expression caused 100% pharate adult lethality. Expressing HttQ0 did not cause any obvious deficits.

Unlike exon 1 models, where pathogenic Htt can accumulate in the nucleus, pathogenic polyQ Htt aggregates from the larger fragment localized exclusively to the cytoplasm. This was observed for both neuronal and non-neuronal cell types. Interestingly, HttQ128 aggregates were differentially distributed in polarized cell types, including epidermal and neuronal cells. Non-pathogenic Htt remained diffusely localized throughout the cytoplasm, while pathogenic Htt formed aggregates in cell bodies and along axons and dendrites.

Whether the primary toxicity induced by mutant Htt is the result of its interactions in the nucleus or in the cytoplasm is widely debated. Other animal models of Htt-polyQ have shown both nuclear and cytoplasmic localization of Htt. Previously, the presence of exon1 Htt-polyQ in the nucleus led to the belief that Htt exerts toxicity through nuclear dysfunction, including impaired transcription. But, the Drosophila HD model demonstrates that HttQ128 is also pathogenic in the absence of nuclear localization, providing evidence for cytoplasmic dysfunction. The exclusion of nuclear Htt

Figure 2. FRAP analysis of HttQ138 aggregates in vivo shows aggregates continue to grow in size. (A) Averaged traces comparing the FRAP recovery signal in regions of the axon without aggregates, with small aggregates, or with large aggregates that exceed the diameter of the axon. Regions without aggregates recover quickly, due to fast axonal transport. Large and small aggregates recover more slowly, but to a higher level. Large aggregates recover more rapidly than small ones. (B) FRAP recovery traces of aggregates in the cell body and axons shows that both regions of the motor neuron recover fluorescence. (C) Time-lapse images of a FRAP experiment showing recovery of a large HttQ138 aggregate in the motor neuron of a live, anesthetized 3rd instar larvae. Modified from Weiss et al., 2012.
offered the unique opportunity to focus solely on cytoplasmic mechanisms of toxicity in the fly system.

The discovery that HtrQ128 was differentially distributed in polarized cells suggested that Htr aggregates may associate with cytoskeletal machinery to undergo directed transport, a process highly relevant for axonal function. We began further investigating Htr’s role in axonal transport. Using the eye-specific driver GMR-GAL4, HtrQ128 aggregates were transported along axons to the CNS and accumulated in photoreceptor growth cones of the developing visual system. Larval motor axons similarly transported HtrQ128 along axons, leading to accumulations in axon terminals at neuromuscular junctions. Visualizing HtrQ128 in motor neurons revealed that large aggregates sometimes caused axons to swell beyond their normal diameter, indicating they might be physically blocking axonal transport. Immunohistochemistry for synaptotagmin I, a synaptic vesicle protein, revealed that synaptic vesicles accumulated in large clumps at sites of Htt aggregates. In HttQ0 animals, synaptic vesicles maintained their normal diffuse distribution. These results suggested a role for cytoplasmic polyQ-Htt in HD pathogenesis that might be mediated in part through alterations in axonal transport.

Axonal transport defects have been observed in other Drosophila and mouse HD models. An exon 1 Drosophila model, HtrQ93 animals accumulated organelles along axons, indicative of impaired transport. TUNEL staining showed that HtrQ93 expression also caused neuronal apoptosis. Both axonal transport defects and neurodegeneration could be rescued with the overexpression of the protein chaperone HSC70. Another exon 1 Drosophila HD model yielded similar results. Expressing mutant Htt with motor neuron driver D42-GAL4 produced aggregates of various sizes. When mutant Htt was co-expressed with fluorescent axonal transport proteins, altered trafficking of organelles and proteins, including synaptic vesicles, could be observed. Axonal transport defects have also been found in mouse models of HD. The R6/2 exon 1 model has Htt inclusions blocking neurites, along with axonal degeneration. In another model, generated with a full-length Htt construct, mutant Htt aggregates can be seen in the axons of striatal projection neurons. Since striatal neurons are the neuronal population most vulnerable to mutant Htt toxicity, this suggests a significant role for axonal transport defects in HD. Researchers also observed degenerated axons, in which Htt aggregates accumulated organelles that appeared to be damaged mitochondria. Another transgenic mouse model expressing full-length mutant Htt found impaired trafficking of synaptic vesicles and mitochondria in vivo and in cultured striatal neurons. By measuring the uptake and transport of a neurotracer in vivo, slower axonal transport, even in pre-symptomatic animals, could be observed. In vitro trafficking studies showed mitochondria were transported more slowly and stopped more frequently in HttQ72 primary neurons, compared with controls. Hence, the role of axonal transport defects in HD appears to be a common phenotype across many model systems.

With the development of mRFP-tagged HtrQ138 transgenics, we also investigated the kinetics of Htt aggregation in live larvae. As with HtrQ128, expression of HttQ138-mRFP causes pharate adult lethality when strongly expressed throughout the nervous system. Weaker, pan-neuronal expression is viable, but animals show progressive motor dysfunction and reduced lifespan. HttQ138-mRFP also formed large, strictly cytoplasmic aggregates (Fig. 1C, E, and G). HtrQ15mRFP did not aggregate (Fig. 1B, D, and F), nor did it impair motor function or decrease lifespan. For axonal transport experiments, we expressed HttQ138-mRFP in a single neuron per hemisegment using CCAP-GAL4 and imaged aggregates in anesthetized, live larvae using a spinning disk confocal microscope. Time-lapse imaging showed aggregates were immobile over 2 h. Fluorescence recovery after photobleaching (FRAP) was used to determine if HttQ138-mRFP aggregates remained constant in size or grew with the addition of soluble HtrQ138 monomers. HttQ138-mRFP axonal aggregates recovered up to 40% of original fluorescence over 50 min imaging sessions (Fig. 2A and C), indicative of the constant addition of new Htt monomers. Larger aggregates tended to recover more quickly (Fig. 2A). Furthermore, aggregates in both axons and cell bodies were capable of recovering fluorescence, indicating that aggregation kinetics were not strictly dependent on fast axonal transport (Fig. 2B).

Next, we conditionally expressed HttQ138-mRFP using a temperature sensitive GAL4 to investigate how aggregates were developed and degraded. Under the control of GAL80, a temperature sensitive GAL4, gene expression is restricted at 18°C, and permitted at 30 °C. Animals were raised at 18 °C to inhibit expression of HttQ138 until larvae reached the 3rd instar larval stage. Animals were then shifted to 30 °C. After 4–8 h, Htt could be seen in neuronal cell bodies of the ventral nerve cord (VNC) and proximal axons (Fig. 3). Aggregates appeared in VNC cell bodies and axons after 12 h of expression. Beyond 12 h, aggregates grew mostly in size, rather than in number (Fig. 3). To determine if cells can remove aggregates, HttQ138-mRFP was expressed for 24 h and then expression was turned off. After 72 h of recovery with no further HttQ138 expression, a reduction in size and number of aggregates was observed. These experiments indicate that HttQ138 aggregates can form rapidly in multiple neuronal compartments, including the cell body and neurites. Likewise, blocking further HttQ138 expression can allow neurons to reduce aggregate load.

**Screening for Suppressors of Mutant Htt Toxicity**

To further investigate mutant Htt pathology, we also conducted an in vivo forward genetic screen for suppressors of HttQ138-mRFP induced aggregation or lethality, using a deficiency collection covering ~80% of the *Drosophila* genome. Our rationale was that finding a dominant suppressor that could rescue pathogenesis through haploinsufficiency might represent a good target for new therapeutics, since HD symptoms could be improved by reducing protein function just 50%. The deficiency collection was first used to identify in vivo suppressors of HttQ138-mRFP aggregation.
Four large deletions that reduced mutant Htt aggregates were identified. Next, we used the deficiency collection to screen for suppressors of pharate adult lethality caused by strong pan-neuronal expression of HttQ138-mRFP. If introducing a deficiency into this background allowed adult flies to eclose, it would suggest a deleted gene might reduce mutant Htt pathogenesis. The screen identified 11 deficiencies that suppressed HttQ138-mRFP induced lethality. Ten of the 11 were partial rescues, improving viability 20–30%, while one deficiency showed a near complete rescue of lethality. Western blot analysis showed that the deficiencies had varying impact on HttQ138-mRFP protein levels: some reduced levels of soluble HttQ138-mRFP, while others had no change. Despite different effects on Htt protein production, all deficiencies were tested for aggregation in the ventral nerve cord and axons, and found to have a varying effect on HttQ138-mRFP lethality.

The screen illustrated the complex relationship between aggregation and lethality. In general, we observed that decreasing mutant Htt expression caused a decrease in aggregation and an increase in viability. We did not find a single suppressor of aggregation that did not also improve viability. Interestingly, all 4 deficiency lines that reduced Htt aggregation were independently identified in the lethality suppressor screen. This highlights the probable toxicity of aggregates in HD pathogenesis. But 4 deficiency lines that decreased HttQ138-mRFP lethality did not yield any change in axonal aggregates. The increased viability in these lines is likely correlated with a decrease in soluble Htt. Thus, we surmised that both aggregated and soluble forms of Htt are likely to represent pathogenic species.

To expand the search for suppressors of mutant Htt aggregation or lethality, a large-scale in vitro screen using Drosophila primary neuronal cultures was conducted in the lab.37 Primary neurons were prepared from animals expressing HttQ138-mRFP with a neuronal driver. These animals also expressed membrane-associated GFP (CD8-GFP) to visualize neurite morphology. HttQ138-mRFP expression in primary neurons resulted in cytoplasmic aggregates and dystrophic neurites, providing phenotypes that could be assayed in 2 distinct suppressor screens. The first was an RNA interference (RNAi) screen designed to identify new therapeutic targets for HD drugs. The RNAi screen targeted genes in a kinase/phosphatase collection (468 genes). Additionally, we conducted a small molecule screen using libraries enriched for FDA-approved drugs (~2600 compounds). Cultured Htt neurons were visually scored independently for phenotype suppressors, and automated microscopy was used to score aggregation and morphology with custom algorithms. The RNAi screen identified 1 new target, lkb1, which has roles in mTOR signaling, autophagy, and nutrient sensing. We examined the role of lkb1 in vivo by using 2 genetic loss-of-function mutants. In vivo tests with heterozygous lkb1 mutant flies showed that reducing lkb1 activity partially rescued...
pharate adult lethality in HttQ138mRFP adults. In a different HttQ138-mRFP line with lower expression, lkb1 mutations significantly improved motor function. Interestingly, while lkb1 knockdown restored neuronal viability, it did not have any effect on aggregation. Next, from the small molecule screen, 62 novel aggregation inhibitors were identified. Only 8 of these 62 compounds improved neurite morphology in primary cultures. We then tested a subset of the small molecules in vivo using Drosophila larvae reared in liquid culture. Larvae expressing HttQ138-mRFP die during the 2nd instar stage under these conditions. The addition of the small molecules Camptothecin or 10Hydroxycamptothecin (inhibitors of topoisomerase 1) significantly increased survival, suggesting suppressive effects in vivo. 37

The results of this screen point to new directions for HD therapeutics, and again underscore the complex relationship between aggregation and toxicity. We found that inhibiting lkb1 dramatically improved neuronal viability, but had no effect on aggregates. Conversely, we found over 50 compounds that decreased Htt aggregation, but did not improve neurite morphology in vitro. Lastly, we discovered that the class of small molecules known as Camptothecins suppressed HttQ138-mRFP aggregation, improved neurite morphology in vitro, and increased larval survival. 37

The primary interest of the lab’s HD research program is how mutant Htt exerts toxicity, and what can be done to ameliorate it. Our results illustrate the danger of relying on secondary characteristics (especially changes in aggregation) to indirectly address viability. Screening only for genes or compounds that change Htt aggregation may yield numerous false negatives and false positives when it comes to cellular toxicity. Using metrics of neuronal health and the survival of HttQ138-mRFP animals allows one to focus on genes and compounds that are likely to be more related to neurodegeneration in HD.

Conclusions

The cause of pathogenesis in HD remains an open question. Since the Htt gene was first identified in 1993, many research groups have reported mechanisms by which expanded Htt may exert toxicity. The relative contribution from diffuse vs. aggregated Htt is still a topic of debate. Furthermore, it is unclear which cellular changes are primary to Htt-polyQ pathogenesis vs. secondary consequences of expanded Htt, or cellular programs induced by stress and apoptosis. The ease of creating transgenic flies has allowed the lab to engineer Drosophila models expressing fragments of the human Htt gene. Recognizing the importance of protein context in disease, the model uses a 588-amino acid protein fragment that includes many key sites of post-translational modification, caspase cleavage, and protein-protein interaction domains. Adding an N-terminal mRFP tag allows study of Htt dynamics in real time in vivo.

Using this model, we have addressed questions about HttQ138 distribution, its role in axonal transport, and the kinetics of aggregate formation. The lab has also taken advantage of the unique opportunity Drosophila present to conduct large-scale screens. The results highlighted the complex relationship between mutant Htt aggregation and toxicity. One model consistent with our data are that aggregates in the cell body may be neuroprotective by sequestering toxic soluble forms of pathogenic Htt, while aggregates formed within neurites are detrimental by disrupting axonal transport. Through in vitro suppressor screens for dystrophic neurites induced by pathogenic Htt, several new candidates for the development of HD therapeutics were also identified. We hope continued dissection of HD pathogenesis in the Drosophila model will ultimately provide clues into the human disorder.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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