CHAPTER SIX

Neurotoxicity Pathways in *Drosophila* Models of the Polyglutamine Disorders

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Abstract

Although polyglutamine expansion diseases are the most common genetically inherited neurodegenerative disorders, the key pathogenic mechanisms that lead to neuronal cell death are unclear. The expansion of a polyglutamine tract in specific proteins is the defining molecular insult, leading to cell-type and region-specific neuronal death. Intraneuronal aggregates of the affected protein can be found in the nucleus and/or cytoplasm and are a hallmark of these disorders. Whether and how aggregation leads to pathology, however, is under debate. In this chapter, we will review some of the key observations using *Drosophila* models of polyglutamine disorders that have highlighted a host of potential contributing pathologies, including defects in transcription, autophagy, and mitochondrial biology. We will also examine how genetic screening approaches have been used in *Drosophila* to provide insights into potential therapeutic approaches for polyglutamine disorders.

1. INTRODUCTION

The trinucleotide repeat disorders arise from genetically inherited expansions of unstable repetitive elements within specific loci. While some

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variability in repeat length is observed in normal alleles, pathology results when repeat length exceeds a specific threshold. Disease-causing expansions can occur in both coding and noncoding regions of genes depending upon the disorder (Orr & Zoghbi, 2007). The polyglutamine (polyQ) class of trinucleotide repeat disorders occur as a result of a CAG tract expansion in the coding region, leading to a protein product with an extended polyQ tract that forms intracellular aggregates. The nine well-known polyQ diseases include Huntington disease (HD), spinobulbar muscular atrophy (SBMA), dentatorubropallidoluysian atrophy (DRPLA), and spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7, and 17 (Orr & Zoghbi, 2007). Together, the polyQ diseases comprise the most common class of inherited neurodegenerative disorders.

A defining feature of the polyQ disorders is that different neuronal populations are uniquely vulnerable in each disease, despite the fact that the pathogenic proteins are widely expressed throughout the nervous system. For instance, D2-expressing GABAergic medium spiny neurons of the striatum are the first population to degenerate in HD (Zuccato, Valenza, & Cattaneo, 2010). Similarly, basal ganglia degeneration is also a hallmark of DRPLA, with neuronal loss occurring in the globus pallidus, the red nuclei of brainstem and dentate nuclei of cerebellum (Yamada, Sato, Tsuji, & Takahashi, 2008). Cerebellar degeneration is the primary neuropathology of the SCA diseases (Yamada et al., 2008). In contrast to the other polyQ diseases, SBMA affects lower motor neurons in the brainstem and spinal cord (Suzuki, Kastuno, Banno, & Sobue, 2009). How these cell-type-specific pathologies arise is a fundamental question in the field.

The specific protein disrupted in each of the polyQ disorders is unique. It is generally accepted that the disorders result in a dominant gain-of-function phenotype caused by the expanded repeat. However, there is also evidence that loss of function from disruption of the normal properties of the protein may contribute to aspects of toxicity (Cortes et al., 2014; Lam et al., 2006; Orr, 2012; Schulte & Littleton, 2011). Each of these disorders is highlighted by the presence of aggregated polyQ proteins within affected neurons. The proteins affected in the polyQ disorders are normally present in a variety of cellular compartments, from the plasma membrane to the cytosol to the nucleus. In the case of HD, the repeat expansion occurs within the first coding exon of huntingtin (HTT), a large HEAT repeat-containing cytosolic protein of unknown biological function (The Huntington's Disease Collaborative Research Group, 1993). The SCA family of disorders result from expansions in an unrelated subset of proteins. The nuclear

transcriptional and splicing regulator ataxin-1 is disrupted in SCA1 (Orr et al., 1993). The cytoplasmic ataxin-2 protein is altered in SCA2 (Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996), while the nuclear ataxin-3 protein has an expanded repeat in SCA3 (Kawaguchi et al., 1994). SCA6 results from polyQ expansion in the plasma membrane-resident alpha 1 subunit of the P/Q-type calcium channel (Zhuchenko et al., 1997), a key regulator of neurotransmitter release. The nuclear and cytoplasmic ataxin-7 protein is part of an acetyltransferase complex and is mutated in SCA7 (David et al., 1997). SCA17 results from a polyQ expansion in the nuclear RNA polymerase component TATA-binding protein (Nakamura et al., 2001). SBMA results from expansion in the nuclear androgen receptor (La Spada, Wilson, Lubahn, Harding, & Fischbeck, 1991), while DRPLA results from expansion in atrophin-1 (Koide et al., 1994; Nagafuchi et al., 1994). Many of the polyQ proteins have also been shown to undergo proteolytic cleavage, which can lead to changes in their normal subcellular localization with nuclear accumulation a common feature. Beyond cell-type specific pathology, another key question in the polyQ field is determining whether the aggregated version of the protein is the pathogenic form. Similarly, determining how the disease-relevant form of the protein generates toxicity in a specific cellular compartment has also been challenging, with evidence suggesting both nuclear and cytoplasmic dysfunction in some cases.

This review highlights how the *Drosophila* model has contributed to the characterization of several toxic pathways implicated in the polyQ disorders. *Drosophila* is particularly amenable to dissecting pathogenic mechanisms in this class of neurodegenerative diseases (Perrimon, Bonini, & Dhillon, 2016). Since causative mutations have been characterized for the polyQ disorders, one can generate transgenic *Drosophila* expressing the human gene of interest or characterize *Drosophila* homologs to study loss-of-function phenotypes. The rapid generation time and ease of genetic manipulation make it possible to conduct large-scale forward genetic screens to identify disease modifiers (Ugur, Chen, & Bellen, 2016). There are also a wide variety of tools available to record neuronal activity in vivo, including physiology and live imaging (Harris & Littleton, 2015). A wide variety of *Drosophila* stocks can be used to examine the effects of loss-of-function or overexpression genetic interactions (Venken & Bellen, 2014). Where null and hypomorph mutants are not readily accessible, genetically encoded RNAi lines are available to knockdown nearly every gene in *Drosophila* (Mohr, Smith, Shamu, & Neumüller, 2014). The advent of CRISPR gene editing is making it increasingly easy to generate mutants or tag endogenous loci

with fluorescent markers (Gratz, Harrison, Wildonger, & O'Connor-Giles, 2015). *Drosophila* also has the GAL4-inducible system that allows modulation of gene expression with temporal and spatial specificity (Elliott & Brand, 2008; Venken & Bellen, 2012). In cases where global expression of a transgene would prove lethal, one can also confine expression to non-essential cell populations such as the eye. A popular approach in the field takes advantage of suppressor and enhancer screens for morphological phenotypes induced by polyQ expression in the *Drosophila* eye.

The polyQ field is a large one with extensive data, suggesting that a host of cellular pathways are likely to be altered in dying neurons (Orr & Zoghbi, 2007; Weber, Sowa, Binder, & Hubener, 2014). One can find evidence for dysfunction of almost any molecular pathway of interest, creating a difficult arena for parsing out the most pathogenic insults created by polyQ expansion. To cope with this large and expansive literature, we have restricted our coverage of the polyQ field into four subsections. First, we survey evidence from the mammalian and *Drosophila* fields that transcriptional dysregulation in the polyQ models is likely to be a key factor in cell toxicity. We next highlight some key findings from modifier screens performed in *Drosophila* polyQ models that highlight several cellular pathways that are relevant for neurodegeneration in fly neurons. We also discuss issues surrounding mitochondrial function and mobility that have been linked to toxicity. Finally, we examine how *Drosophila* mutants and RNAi lines have been used to characterize the role of autophagy in the polyQ disorders. Although these pathways are likely to be only a subset of the toxicity mechanisms at play, they highlight how *Drosophila* can be used to model polyQ pathogenesis (Fig. 1).

2. TRANSCRIPTIONAL AND NUCLEAR DYSFUNCTION

Transcriptional aberrations were noted in early studies of polyQ patient brain tissue and supported by findings in mouse and *Drosophila* disease models. Transcriptional dysregulation has been studied most extensively in the context of HD (Sugars & Rubinsztein, 2003). Given that many of the pathogenic polyQ proteins accumulate in the nucleus, either as aggregates or as more soluble forms, there has been a heavy focus on whether and how these aggregates might alter nuclear biology. A common finding has been the ability of such nuclear aggregates to sequester various transcription factors or nuclear proteins, setting the stage for a loss-of-function phenotype in transcriptional regulation.

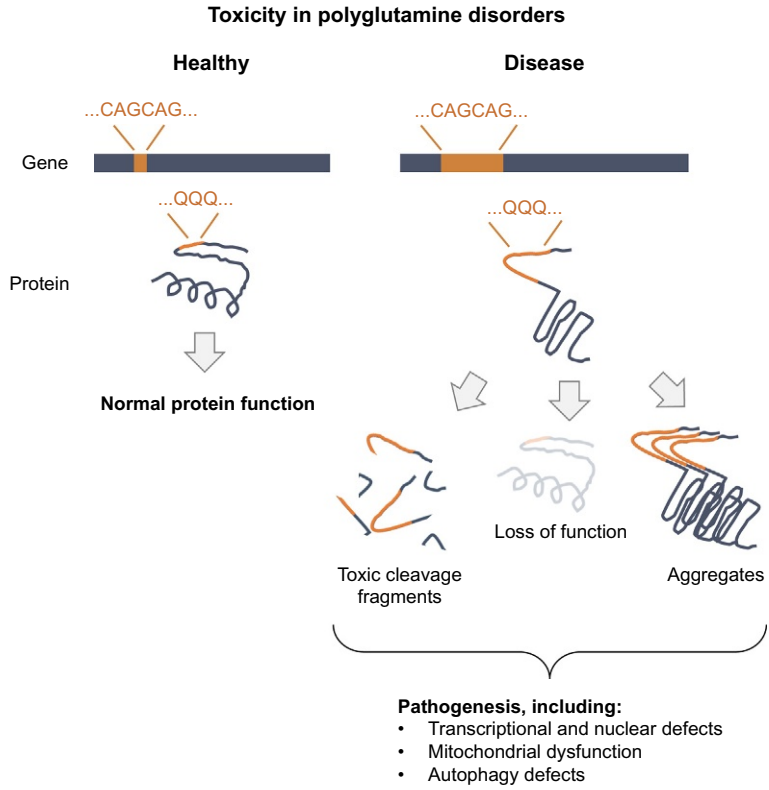

Fig. 1 Toxicity pathways in the polyQ disorders. CAG repeat expansions beyond a critical length (typically >40) result in an expanded polyQ tract within the affected protein and the formation of intracellular aggregates. Numerous downstream molecular pathways have been implicated in subsequent pathology, including defects in transcription, autophagy, and mitochondrial function. The expanded polyQ tract may also disrupt the normal function of the affected protein, leading to loss-of-function pathology as well. In addition, proteins containing the expanded polyQ tract may form novel protein–protein interactions that lead to aberrant signaling. Affected proteins can also undergo cleavage that may lead to altered cellular localization and enhance aggregate formation. It is still unclear which form of the polyQ protein (monomers, small aggregates, or larger inclusions) is the most toxic to neurons, and why only selected neuronal populations are highly sensitive to the expanded polyQ protein.

Microarray analysis has provided a powerful approach to measure gene expression changes in HD models. The first microarrays from R6/2 mouse striatum showed changes in less than 2% of 6000 genes analyzed. Most genes showed decreased expression, but a handful of genes, mostly related to inflammation, were increased. Genes central to neuronal signaling (neurotransmitter receptors, neurotransmitters, and neuropeptides), retinoid

signaling, and calcium homeostasis were among those showing decreased expression (Luthi-Carter et al., 2000). In 2002, a consortium published a series of microarray experiments from various HD and polyQ disease models (Orr, 2002). Decreases in transcription were more commonly observed, with changes seen in genes associated with neurotransmission, intracellular signaling, calcium homeostasis, and transcriptional processes (Luthi-Carter, Hanson, et al., 2002; Luthi-Carter, Strand, et al., 2002). Interestingly, initial results indicated that expressing a short N-terminal fragment of HTT resulted in more dramatic changes than those observed in animals expressing longer fragments or full-length HTT transgenes. Short N-terminal fragments are expressed in R6/2 (67 amino acids) and N171-82Q (171 amino acids) models and were compared to longer fragments found in HD46 and HD100 mice (964 amino acids), and YAC72 mice (full-length, 3144 amino acids) (Chan et al., 2002; Pouladi, Morton, & Hayden, 2013). While R6/2 and N171-82Q mice have mRNA profiles similar to those observed in HD patients, the HD46, HD100, and YAC72 mice profiles were more subtle and qualitatively different (Chan et al., 2002). However, more recent analysis indicates that the longer full-length HD models have some similarities in transcriptional dysregulation to N-terminal fragment models and HD patients if older animals (1½ to 2 years old) are analyzed (Kuhn et al., 2007). As such, transcriptional dysregulation is commonly observed in all HD models, suggesting that a complex genomic response is occurring in HTT-expressing cells.

Several mechanisms may account for transcriptional changes in HD. One model proposes a physical interaction between expanded polyQ proteins and certain transcription factors. For example, pathogenic HTT and other mutant polyQ proteins have been shown to interact with and sequester CREB-binding protein (CBP), impairing regulation of CBP transcriptional targets (McCampbell et al., 2000; Nucifora et al., 2001; Steffan et al., 2000). The mechanism of transcription factor sequestration and transcriptional interference fits with the observation that nuclear localization of the polyQ protein is central to toxicity in many polyQ diseases, including HD. However, aberrant gene regulation must result from mechanisms other than just transcription factor sequestration, given that cellular dysfunction in polyQ disease models can occur before the appearance of nuclear inclusions, and some studies demonstrate a neuroprotective role for aggregates. Besides physical sequestration of transcription factors, HTT has been suggested to physically bind to DNA to directly repress transcription (Benn et al., 2008; Zhai, Jeong, Cui, Krainc, & Tjian, 2005). It has also been proposed

that alterations in mRNA levels may be the result of physical changes to the nucleus (invagination of nuclear membrane) and nuclear pores (nuclear pore density) (Cha, 2000; Davies et al., 1997). Lastly, transcriptional abnormalities may be the result of chromatin modifications in polyQ diseases (Mohan, Abmayr, & Workman, 2014). HTT has been shown to interact with methyl-CpG-binding protein 2 (MeCP2), and expression of mutant HTT decreases CBP-dependent acetylation and increases methylation of H3K9 (McFarland et al., 2014; Nucifora et al., 2001; Ryu et al., 2006).

Early in vitro studies revealed that immunoprecipitation of HTT can pull down CBP, a cofactor in CREB-mediated transcriptional activation. Interactions with CBP were enhanced when the HTT fragment was expanded to include the polyproline domain, a proline-rich stretch of residues adjacent to the polyQ stretch near the N-terminus of HTT (Steffan et al., 2001). Additional in vitro experiments demonstrated that HTT also binds other acetyltransferases, including P/CAF. Furthermore, HTT binding impairs transcriptional activators' acetyltransferase activity, as measured by acetylation of histone H4 peptides (Steffan et al., 2001). In PC12 cell cultures transfected with a fragment of HTT-Q103 plus the polyproline region, H3 and H4 acetylation levels were restored after treatment with histone deacetylase (HDAC) inhibitors (Steffan et al., 2001).

In vivo experiments in *Drosophila* have supported a role for HDAC biology in polyQ pathology. Expression of a pathogenic HTT exon 1 fragment in *Drosophila* eyes caused progressive rhabdomere degeneration, along with ~70% lethality and reduced lifespan (Steffan et al., 2001). Rearing larvae on medium that contained HDAC inhibitors ameliorated degeneration in a concentration-dependent manner. HDAC inhibitors also increased viability and extended lifespan. Similar results were obtained with the expression of an isolated polyQ tract (Q48) (Steffan et al., 2001). HDAC inhibitors have also proven effective in models of SCA7 and SCA3 (Jung & Bonini, 2007; Latouche et al., 2007), suggesting a potential convergence on HDAC pathology.

Drosophila mutants have been used to further examine the role of histone acetylation state in polyQ diseases, specifically CBP-regulated acetylation. Using live-cell imaging, coexpression of GFP-tagged CBP and expanded HTT or ataxin-3 in cell culture resulted in CBP sequestration and subsequent immobilization after its incorporation into polyQ-containing aggregates (Chai, Shao, Miller, Williams, & Paulson, 2002). This adds to a body of evidence, suggesting that polyQ proteins impair transcription via CBP loss of function (Chai et al., 2002; McCampbell et al., 2000; Nucifora et al.,

2001; Steffan et al., 2000). Indeed, overexpression of *dCBP*, which encodes the *Drosophila* CBP ortholog, fully rescued Q127-induced eye neurodegeneration in *Drosophila* models (Marek et al., 2000). In addition to improvements in eye morphology, a visual challenge demonstrated that eye function was also restored by upregulation of *dCBP* (Taylor et al., 2003). How does dCBP overexpression rescue polyQ toxicity? Analysis of *Drosophila* homogenates showed that acetylation of histones H3 and H4 was reduced in flies expressing Q127 (Taylor et al., 2003). Acetylation levels were restored by the overexpression of dCBP. Given that reduced acetylation would promote a chromatin state that is less accessible to transcription factors, it was hypothesized that changes in H3 and H4 acetylation would be accompanied by altered gene expression.

Expanding upon this model, a set of *Drosophila* mutants and shRNA lines have been used to test whether loss of function or overexpression of *Drosophila* HDACs can modify HTT-induced pathology (Fernandez-Funez et al., 2000). *Drosophila* have 10 HDACs: 5 are considered classic zinc-dependent HDACs (Rpd3, HDAC3, HDAC4, HDAC6, and HDAC11) and the other 5 are NAD-1-dependent deacetylases (Sir2, Sirt2, Sirt4, Sirt6, and Sirt7). A combination of *Drosophila* mutants and shRNA silencing revealed that reduced levels of Rpd3, but not the other zinc-dependent HDACs, significantly improved eye degeneration and survival following pan-neuronal expression of an HTT-Q93 exon 1 fragment. Reducing levels of Sir2 (the *Drosophila* homolog of mammalian SIRT1) also improved eye degeneration and survival. Furthermore, the modifier effects were additive (Pallos et al., 2008), indicating that the two types of HDACs may act in parallel pathways to moderate transcription. The findings are also interesting in light of the fact that *Rpd3* and *Sir2* were identified in an SCA1 suppressor screen, suggesting that some toxic pathways may be shared among different polyQ diseases (Fernandez-Funez et al., 2000).

Modification of acetylation levels by genetic intervention in HD model flies has also been performed. *Drosophila Sin3A* encodes a corepressor protein that plays an essential role in HDAC complexes. Partial loss of function of Sin3A reduces histone deacetylation and also reduces HTT-induced eye degeneration and increases viability (Steffan et al., 2001). *Sin3A* was also identified in a forward genetics screen for modifiers of phenotypes in an SCA1 *Drosophila* model (Fernandez-Funez et al., 2000).

Forward genetic screening in *Drosophila* indicates that pathogenic polyQ proteins physically entering the nucleus may be detrimental (Doumanis, Wada, Kino, Moore, & Nukina, 2009). Using a combination of in vitro

and in vivo model systems expressing an N-terminal fragment of expanded HTT, Doumanis et al. identified 21 high-confidence modifiers of aggregation and toxicity. In addition to genes with functions in general transcription and RNA processing, a set of hits were identified with critical roles in nuclear pore transport. In vivo knockdown of *CG4738*, the *Drosophila* homolog of human *NUP160,* suppressed aggregation and pathology in the brains of *Drosophila* expressing HTT-Q93, HTT-Q152, or nuclear-targeted HTT-Q48-NLS (Doumanis et al., 2009). Doumanis et al. hypothesized that this rescue may be due to reduced formation of HTT inclusions in the nucleus, which would match the evidence that nuclear localization is central to toxicity in several polyQ diseases, including SCA1, SCA7, DRPLA, and SBMA (Walsh, Storey, Stefani, Kelly, & Turnbull, 2005). Early observations from HD patient brain tissue showed that nonexpanded HTT is consistently localized to the cytoplasm (DiFiglia et al., 1995). However, pathogenic mutant HTT forms dense inclusions in the nuclei, as well as aggregates in the cytoplasm (DiFiglia et al., 1997). The appearance of nuclear inclusions coincides with disease onset in human patient tissue and mouse models of the disease (Davies et al., 1997; DiFiglia et al., 1997).

Experimental manipulations to prevent N-terminal HTT from entering the nucleus further supported a role for nuclear toxicity in HD. Cell culture experiments revealed that truncating the pathogenic HTT protein enhances its localization to the nucleus and exacerbates toxicity (Cooper et al., 1998; Hackam et al., 1998; Lunkes & Mandel, 1998; Martindale et al., 1998). Toxicity can also be suppressed by preventing cleavage and nuclear translocation of the truncated N-terminal fragment (Gafni et al., 2004). In another set of experiments, a nuclear localization signal (NLS) or nuclear export signal (NES) was added to truncated mutant HTT. The NES tag rescued toxicity, as measured by cell loss, while the NLS exacerbated the toxic effects of HTT (Peters et al., 1999). Although nuclear localization of mutant HTT is detrimental to neurons, both nuclear and cytoplasmic events likely contribute to pathogenesis (Benn et al., 2005; Lee, Yoshihara, & Littleton, 2004).

In addition to HD models, transcriptional dysregulation has also been analyzed in the context of other polyQ disorders, including SCA1. Overexpression of the *Drosophila* ataxin-1 homolog, dAtx-1, induces phenotypes similar to those observed from overexpression of an expanded human ataxin-1, including patterning defects and bristle loss in the wing margin (Tsuda et al., 2005). The bristle phenotype suggested that Sens, a zinc-finger transcription factor required for sensory organ development, may have abnormal activity following overexpression. Both dAtx-1 and human

Atx-1 physically interact with Sens. In vivo, dAtx-1 and Atx-1-Q82 decrease levels of endogenous Sens protein, and overexpression of Sens can suppress the loss-of-bristle phenotype caused by expression of *Drosophila* or human ataxin-1. The vertebrate homolog of Sens is Gfi-1. In mice, coimmunoprecipitation assays revealed that Atx-1 interacts with Gfi-1 in vivo. In SCA1 transgenic mice, which overexpress human Atx-1 in Purkinje cells, there was a marked decrease in Gfi-1 protein levels prior to Purkinje cell death. Indeed, human ataxin-1 enhanced proteosomal degradation of Gfi-1, and loss of Gfi-1 leads to Purkinje cell degeneration (Tsuda et al., 2005).

Ataxin-1 has also been shown to interact with the transcriptional repressor Capicua. Regulation of this interaction through mutation of a specific residue (S776A) in ataxin-1 suppresses SCA1 neuropathology (Lam et al., 2006), implicating another route for ataxin-1 to drive transcriptional alterations. Genetic approaches in both *Drosophila* and mouse SCA1 models revealed that alterations in the RAS–MAKP pathway could reduce SCA1 pathology (Park et al., 2013). The authors demonstrated that specific MAPK kinases phosphorylate residue S776 in ataxin-1 to regulate the stability of the protein. Inhibitors of the MAPK pathway reduced ataxin-1 levels, leading to less toxic protein in the nucleus, and providing a potential therapeutic approach for targeting SCA1 toxicity.

3. GENETIC AND PHARMACOLOGICAL SCREENS FOR SUPPRESSORS OF PolyQ PATHOLOGY

There are a variety of tools available to screen for modifiers of polyQ toxicity in *Drosophila*, including RNAi, transposable elements, chemical mutagenesis, and small-molecule assays. Kazemi-Esfarjani and Benzer (2000) performed one of the first screens searching for modifiers of polyQ toxicity in *Drosophila*. Using P-element mutagenesis, 7000 mutant strains were tested for their role in dominantly modifying the eye degeneration phenotypes observed in *Drosophila* following expression of a simple polyQ tract (Q127) using the *GMR-GAL4* eye driver. The screen identified 30 suppressors and 29 enhancers, including suppressor hits in *dHJD1*, the homolog of human *HSP40/HDJ1*, and *dTPR2*, the homolog of human *TPR2*. Both insertions increased transcription of the J (Hsp40) class of cochaperones that stimulate activity of Hsp70. Kazemi-Esfarjani and Benzer generated transgenic flies overexpressing *dHDJ1* or *dTPR2* and confirmed that upregulation of either gene was protective against eye degeneration caused

by Q127 expression. This screen set the stage for future studies that explored the mechanisms by which cellular chaperones suppress toxicity in polyQ disorders and other neurodegenerative diseases (for reviews, see Muchowski & Wacker, 2005; Opal & Zoghbi, 2002; Paul & Mahanta, 2014).

In addition to in vivo screening in *Drosophila*, primary neurons can also be generated for in vitro screening assays of RNAi suppressors or candidate small-molecule therapeutics. Using *Drosophila* engineered to express a membrane-bound GFP (CD8-GFP) and a 12-exon fragment of HTT (HTT-Q138), primary neuronal cultures have been shown to accumulate cytoplasmic aggregates and result in altered neuronal morphology, including dystrophic neurites (Schulte, Sepp, Wu, Hong, & Littleton, 2011). These cultures were screened with RNAi libraries to identify suppressors of either aggregation or defects in neurite morphology. The screen revealed a novel target, lkb1, a kinase with roles in mTOR signaling and autophagy, as a potential suppressor of polyQ pathology in this neuronal population. Heterozygous loss of *lkb1* was sufficient to partially suppress lethality in adult HD model flies, suggesting that lkb1 may be linked to pathology in this model. Cultured neurons expressing pathogenic HTT were also screened using a collection of 2600 small molecules enriched for FDA-approved drugs. Sixty-two novel aggregation inhibitors, of which eight also improved the morphology of dystrophic neurites, were identified in this screen (Schulte et al., 2011). The topoisomerase inhibitors camptothecin or 10-hydroxycamptothecin extended the lifespan of HTT-expressing animals, indicating that the compounds are neuroprotective in vivo (Schulte et al., 2011).

In another screen designed to identify new FDA-approved modifiers of toxicity, Jimenz-Sanchez et al. used mammalian cell culture and *Drosophila* to search for targets that suppressed HTT-induced degeneration. Like the screens performed by Doumanis et al. and Schulte et al., Jimenez-Sanchez et al. began with an RNAi screen in vitro for suppressors of HTT-Q138 toxicity. Of the 257 RNAi screened, a subset with *Drosophila* homologs were selected for validation in vivo using neurodegeneration observed in the *Drosophila* eye following expression of a polyQ tract (Jimenez-Sanchez et al., 2015). In mammalian cell lines and primary neurons, siRNA against the gene encoding QPCT modified HTT-induced toxicity. RNAi against the *Drosophila* homologs, *Glutaminyl cyclase* (*QC*) and *iso Glutaminyl cyclase* (*isoQC*), partially rescued photoreceptor loss induced by expression of Q48 or an exon 1 fragment of HTT-Q120. Three lead compounds designed to inhibit QPCT activity demonstrated dose-dependent decreases in HTT

aggregation and cellular toxicity in vitro. QPCT localizes to the endoplasmic reticulum and exhibits glutaminyl cyclase activity. It is thought to exert neuroprotective effects through increasing levels of the chaperone alpha-B-crystallin, a small heat-shock protein.

In vivo screens to search for modifiers of HTT aggregation and toxicity have also been performed in *Drosophila* expressing a larger 12-exon fragment of the human *HTT* gene with 138 CAG repeats (HTT-Q138). This longer fragment, which is tagged with mRFP to allow visualization of aggregation in live animals, maintains many key binding sites for HTT-interacting proteins, along with critical residues for posttranslational modification and caspase cleavage. Weiss, Kimura, Lee, and Littleton (2012) used a deficiency collection kit covering 80% of the *Drosophila* genome to screen for suppressors of HTT toxicity in a haploinsufficiency screen where 50% loss of the deleted gene product uncovered by the deficiency would modify toxicity. These experiments identified four gene regions that reduced HTT-Q138 aggregation when deleted. The deficiency collection was also used to find new suppressors of lethality induced by neuronal expression of HTT-Q138 following expression with the *elav-GAL4* pan-neuronal driver. On its own, *elav>HTT-Q138* is pharate lethal. In 11 deficiency backgrounds, viability improved 20–30%, and in another line, lethality was almost fully suppressed. These findings provided promising targets for future HD research (Weiss et al., 2012).

The connection between aggregation and toxicity is a perplexing relationship. On one hand, *Drosophila* screens performed by Doumanis et al., Desai et al., 2006, Schulte et al. and Weiss et al. all found correlation between aggregates and toxicity. In the in vivo screen by Weiss et al. the four deficiency lines identified in the screen for suppressors of aggregation were each identified in the lethality screen as well. However, four other lines that suppressed lethality did not have any observable changes in HTT-Q138 aggregate load (Weiss et al., 2012). Similarly, Schulte et al. found that only a subset of aggregation inhibitors were effective at suppressing cellular toxicity (Schulte et al., 2011). In the case of the screen performed by Doumanis et al., the top suppressor of aggregation in vitro (*CG1109*) strongly suppressed HTT-induced toxicity in vivo (Doumanis et al., 2009). More support for the connection between aggregation and toxicity comes from additional small-molecule screens that found strong inhibitors of aggregation suppressed neurodegeneration in *Drosophila* and mouse models of HD (Chopra et al., 2007; Zhang et al., 2005). However, other studies suggest that aggregates are not directly related to toxicity. For example, two

in vitro studies show that HTT-induced toxicity is most strongly correlated with soluble HTT, not aggregates, and that aggregates may play a protective role (Arrasate, Mitra, Schweitzer, Segal, & Finkbeiner, 2004; Lajoie & Snapp, 2010). It remains unclear if aggregates are toxic, beneficial, or merely a by-product in polyQ diseases (Krench & Littleton, 2013).

4. MITOCHONDRIAL DYSFUNCTION

Many studies have demonstrated a role for mitochondrial dysfunction in HD pathogenesis (Damiano, Galvan, Déglon, & Brouillet, 2010). Research has shown that HTT can bind directly to mitochondria, impairing metabolic function and motility (Orr et al., 2008; Panov et al., 2002; Trushina et al., 2004). HTT also causes energy defects and neurodegeneration via its interference with CREB-Taf4-dependent transcription of PGC-1α, a regulator of mitochondrial biogenesis (Cui et al., 2006). Several models describe other mechanisms by which expanded HTT may affect mitochondria, including impairing trafficking or reducing their ability to buffer cytosolic calcium. One theory hypothesizes increased production of reactive oxygen species (ROS) and oxidative damage in HD. The vast majority of ROS in cells are produced by mitochondria (Balaban, Nemoto, & Finkel, 2005). In support of this theory, samples from postmortem tissue and blood samples from HD patients show increased oxidative damage and reduced mitochondrial function (Browne, Ferrante, & Beal, 1999; Chen et al., 2007). Further support comes from studies in yeast, which shows that expression of polyQ proteins leads to mitochondrial transport chain impairment and significantly higher levels of ROS (Solans, Zambrano, Rodríguez, & Barrientos, 2006). In vitro studies also show increased ROS generation induced by mutant HTT (Fukui & Moraes, 2007; Lim et al., 2008; Solans et al., 2006).

Defects in metabolism may be exacerbated by mislocalized mitochondria in HD. Mitochondria are generally distributed throughout the cell in response to local energy demands. However, there is ample evidence from *Drosophila* demonstrating that expression of pathogenic HTT impairs axonal transport (Lee et al., 2004). In a *Drosophila* HD model expressing an exon 1 fragment of HTT, neuronal expression of HTT-Q93 caused organelle accumulations along axons (Gunawardena et al., 2003). Live imaging of fluorescent transport proteins in flies expressing HTT-Q93 showed that trafficking of organelles and proteins along axons was impaired by mutant HTT (Sinadinos et al., 2009).

Animal models and postmortem brain tissue from HD patients show mitochondrial components and trafficking motors are also sequestered in mutant HTT aggregates. In vitro experiments suggest that aggregates impair the movement of mitochondria down the axon (Chang, Rintoul, Pandipati, & Reynolds, 2006). Mutant HTT has also been shown to disrupt the association of microtubule-based transport of mitochondria. As a consequence of reduced mitochondrial motility, distal axon terminals are starved of ATP (Orr et al., 2008). By impairing mitochondrial energy production and distribution, mutant HTT results in lower levels of cellular ATP, which may contribute to increased vulnerability (Zuccato et al., 2010). In addition to the toxic gain-of-function effects of mutant HTT, loss of function of normal HTT may also contribute to aberrant mitochondrial localization. Studies in both *Drosophila* and mammalian neurons demonstrate that reducing levels of endogenous HTT results in defective mitochondrial trafficking (Trushina et al., 2004).

5. AUTOPHAGY DEFECTS

Another emerging pathway that has been linked to cellular dysfunction in polyQ diseases is the bulk cellular degradation process of autophagy. In macroautophagy (hereafter referred to as autophagy), cytoplasmic cargo is sequestered and enveloped by a double-membrane vesicle termed an autophagosome. The autophagosome fuses with the lysosome, creating an autolysosome, where the membrane and contents of the autophagosome are degraded by lysosomal enzymes. As new proteins and organelles are synthesized, autophagic processes degrade old or damaged organelles, along with misfolded or aggregated proteins (Levine & Kroemer, 2008). Dysfunctions in autophagy have been implicated in a variety of diseases, ranging from neurodegenerative diseases to metabolic disorders (Cuervo, 2011; Levine & Kroemer, 2008). Some disease states may induce an "autophagic traffic jam," where autophagy induction is highly active, but there are not enough lysosomes to receive all of the cargo for degradation (Cuervo, 2011). Neurons with polyQ aggregates rely heavily on autophagy to clear these cellular obstructions. In doing so, expanded HTT may overwhelm the autophagy system, impairing routine housekeeping activities (Tooze & Schiavo, 2008). Furthermore, it has been suggested that the other main degradative pathway, the ubiquitin–proteasome system, is also impaired in HD (Tydlacka, Wang, Wang, Li, & Li, 2008; Wang et al., 2008), placing an additional burden on autophagy. The increased levels of autophagosomes found

in HD brains may represent hyperactive autophagy that is attempting to degrade damaged components of the cell or intracellular aggregates (Levine & Kroemer, 2008). Another link to autophagy is the recent observation that one normal function for HTT is to scaffold autophagy cargo receptors and autophagosome components to initiate the autophagy process (Rui et al., 2015). PolyQ expansion within HTT could potentially disrupt this process, putting additional stress on the autophagic pathway.

In support of a neuroprotective theory of autophagy, increasing the process via inhibition of mammalian TOR (mTOR) is neuroprotective in *Drosophila* and mouse HD models. In *Drosophila* expressing a fragment of HTT-Q120, rhabdomere degeneration was reduced following treatment with rapamycin, which inhibits TOR function (Ravikumar et al., 2004). mTOR inhibition was also assayed in mammalian models. The rapamycin derivative CCI-779 enhanced clearance of expanded HTT in HD cell culture models and in the Ross/Borchelt HD mouse model (Huang & Houghton, 2001). Treatment with CCI-779 also reduced striatal aggregates and improved behavior and motor function (Ravikumar et al., 2004). These findings in *Drosophila* and mouse models of HD suggest a neuroprotective benefit when activating autophagy pathways.

Experiments in *Drosophila* also demonstrate that mutant HTT can be cleared when autophagy is stimulated through mTOR-independent pathways. Several autophagy-stimulating compounds were validated in vivo in *Drosophila* expressing a fragment of HTT-Q120 in the eye: verapamil (L-type calcium channel antagonist), clonidine (binds to alpha-2 adrenergic receptors and type I imidazoline receptors to activate G_i signaling pathways), and valproic acid (reduces inositol and IP_3 levels). Treatment with all three compounds suppressed rhabdomere degeneration (Williams et al., 2008). In addition to pharmacological efforts to increase autophagy, Pandey et al. used RNAi knockdown of *Drosophila* autophagy genes to study the interplay between autophagy and the UPS system in an SBMA model (AR-Q52). Knockdown of the autophagy genes *atg6* or *atg12* exacerbated eye degeneration in this SBMA model, indicating that the autophagy pathway was critical to clearance (Pandey et al., 2007).

Multiple studies have highlighted an interplay between autophagy and the UPS system (Pandey et al., 2007; Saitoh et al., 2015). The p62/sequestosome 1 protein (p62) allows for select ubiquitinated proteins to be targeted for autophagic degradation, bridging together the two main cellular degradation pathways. Previous *Drosophila* screens for modifiers of SCA3 toxicity have shown that truncated MJD-Q78 can undergo either

autophagic degradation or degradation via the UPS system (Bilen & Bonini, 2007). In agreement with that finding, Saitoh et al. found that knockdown of either autophagy (*Atg12* or *alfy*) or proteasome (*Prosβ2*) genes exacerbated the rough eye phenotype induced by expression of truncated MJD-Q78. RNAi knockdown of p62 also enhanced the MJD-Q78 rough eye and increased aggregation of cytoplasmic MJD-Q78. These data support the model that p62 triggers the clearance of polyQ proteins via autophagic degradation (Saitoh et al., 2015).

In a *Drosophila* DRPLA model, transcriptional profiling offered new perspective into how autophagy is involved in pathogenic pathways triggered by expanded Atrophin. *Atrophin* (*Atro*), the *Drosophila* ortholog to human *Atrophin-1*, contains two polyQ stretches. DRPLA models have been generated by expanding the polyQ region near either the N-terminus (Atro-75QN) or the C-terminus (Atro-66QC). Atro-75QN contains a polyQ stretch in the middle of a polyproline domain, similar to where the polyQ stretch is found in human Atrophin-1 (Nisoli et al., 2010). Overexpression of expanded (and to a lesser extent wild-type) *Atro* in the eye caused a downregulation of *ft*, a tumor suppressor gene that encodes a cadherin and regulates planar polarity through interactions with *Atro*. Loss-of-function *ft* mutants exacerbate Atro-75QN-induced retinal degeneration. Introducing additional mutants supported the idea that the Hippo tumor suppressor pathway was implicated in *ft* neurodegeneration, and the mechanism of toxicity is linked to defective autophagy (Napoletano et al., 2011).

Nisoli et al. also examined the role of autophagy in *Drosophila* DRPLA models. In contrast to HD and SBMA models, enhancing autophagy did not suppress toxicity in the DRPLA model. Photoreceptors overexpressing polyQ atrophins show an increase in autophagosomes and autophagic markers. How autophagy contributed to toxicity was assayed by using the autophagy mutant, $atg1^{\Delta 3D}$, a putative null allele of *atg1*. Blocking the induction of autophagy with the $atg1^{\Delta 3D}$ mutant enhanced retinal neurodegeneration by Atro-Q75N. Similarly, RNAi knockdown of *atg5* also enhanced Atro-Q75N neurodegeneration. Further study indicated that expanded atrophins block clearance at the lysosomal level after fusion between autophagosomes and lysosomes. These data indicated that increasing autophagy is insufficient to rescue neurodegeneration because downstream lysosomal functions remain impaired in the DRPLA model (Nisoli et al., 2010). Thus, while defective autophagy appears to be a common dysfunction among many polyQ diseases, enhancing autophagy may fail to rescue toxicity if defects exist downstream of the early autophagy steps.

6. CONCLUSION

The rapid generation time and the ease of genetic manipulations have made *Drosophila* an ideal model system to screen for modifiers of toxicity in the polyQ disorders. By using the *Drosophila* eye, suppressor or enhancers can be identified in a nonlethal cell population. In addition to screening for novel modifiers, *Drosophila* provides a complimentary model organism for testing hypotheses generated from in vitro experiments or candidates derived from other disease models. Research in *Drosophila* has provided a window into neurotoxic pathways in the polyQ disorders. As described in this review, toxicity in polyQ disorders may stem from a variety of mechanisms, including transcriptional and nuclear dysregulation, mitochondrial dysfunction, autophagy, and more. A number of additional mechanisms are also likely to contribute to the pathogenesis of these diseases. For example, it has been shown that overexpression of molecular chaperones can suppress aggregation and toxicity in polyQ disease models (reviewed in Muchowski & Wacker, 2005; Sakahira, Breuer, Hayer-Hartl, & Hartl, 2002; Xu, Tito, Rui, & Zhang, 2015). As the field of polyQ research progresses, it will be important to determine whether similar pathogenic mechanisms that occur downstream of polyQ protein expression in *Drosophila* are found in human HD tissue as well.

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