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REVIEW

Experimental Models for Identifying Modifiers of Polyglutamine-Induced Aggregation and Neurodegeneration

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Abstract Huntington's disease (HD) typifies a class of inherited neurodegenerative disorders in which a CAG expansion in a single gene leads to an extended polyglutamine tract and misfolding of the expressed protein, driving cumulative neural dysfunction and degeneration. HD is invariably fatal with symptoms that include progressive neuropsychiatric and cognitive impairments, and eventual motor disability. No curative therapies yet exist for HD and related polyglutamine diseases; therefore, substantial efforts have been made in the drug discovery field to identify potential drug and drug target candidates for disease-modifying treatment. In this context, we review here a range of early-stage screening approaches based in *in vitro*, cellular, and invertebrate models to identify pharmacological and genetic modifiers of polyglutamine aggregation and induced neurodegeneration. In addition, emerging technologies, including high-content analysis, three-dimensional culture models, and induced pluripotent stem cells are increasingly being incorporated into drug discovery screening pipelines for protein misfolding disorders. Together, these diverse screening strategies are generating novel and exciting new probes for understanding the disease process and for furthering development of therapeutic candidates for eventual testing in the clinical setting.

Keywords Polyglutamine diseases · High-throughput screening · Drug discovery · Model organism

Introduction

At least nine dominantly inherited human neurodegenerative diseases, including Huntington's disease (HD), spinal-bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy, and spinocerebellar ataxias (SCAs) are caused by CAG-repeat expansions in the coding region of the respective disease gene [1]. The *huntingtin* gene (*HTT*) mutation was identified in 1993 [2] as a trinucleotide CAG expansion encoding polyglutamine (polyQ) near the amino terminus (N-terminus) of the large Huntingtin (HTT) protein. Similarly, spinal-bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy, and ataxias were found to be caused by CAG-repeat expansions in the genes encoding the androgen receptor, atrophin, and ataxin proteins, respectively. In each case, an abnormally expanded polyQ tract causes misfolding and accumulation of the mutant protein in cellular inclusions, which are associated with progressive neuronal degeneration and cell loss in different regions of the brain. These neurodegenerative diseases are characterized by progressive motor, cognitive, and neuropsychiatric deficits, with only modest symptomatic management achievable with medication and physical and occupational therapy [3, 4]. As no curative therapy exists for any of these polyQ-driven diseases, there is an urgent need to develop and improve drug discovery screening technologies targeting these devastating disorders.

The discovery that the proximal causes of these diseases emanate from CAG expansions within single genes has enabled the generation of genetic models with high relevance and specificity to each disease. For example, expression of all, or part of the *HTT* gene containing the CAG expansion in mice recapitulates key aspects of HD, including the development of cellular inclusions, motor and cognitive symptoms, and neuronal pathology [5, 6]. Analogous genetic models for HD and other polyQ diseases have also been developed in the budding yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*, as well as in neuronal cell lines and most recently in induced pluripotent stem cells (iPSCs) derived from patients (Fig. 1). While any such model will have intrinsic advantages, as well as limitations,

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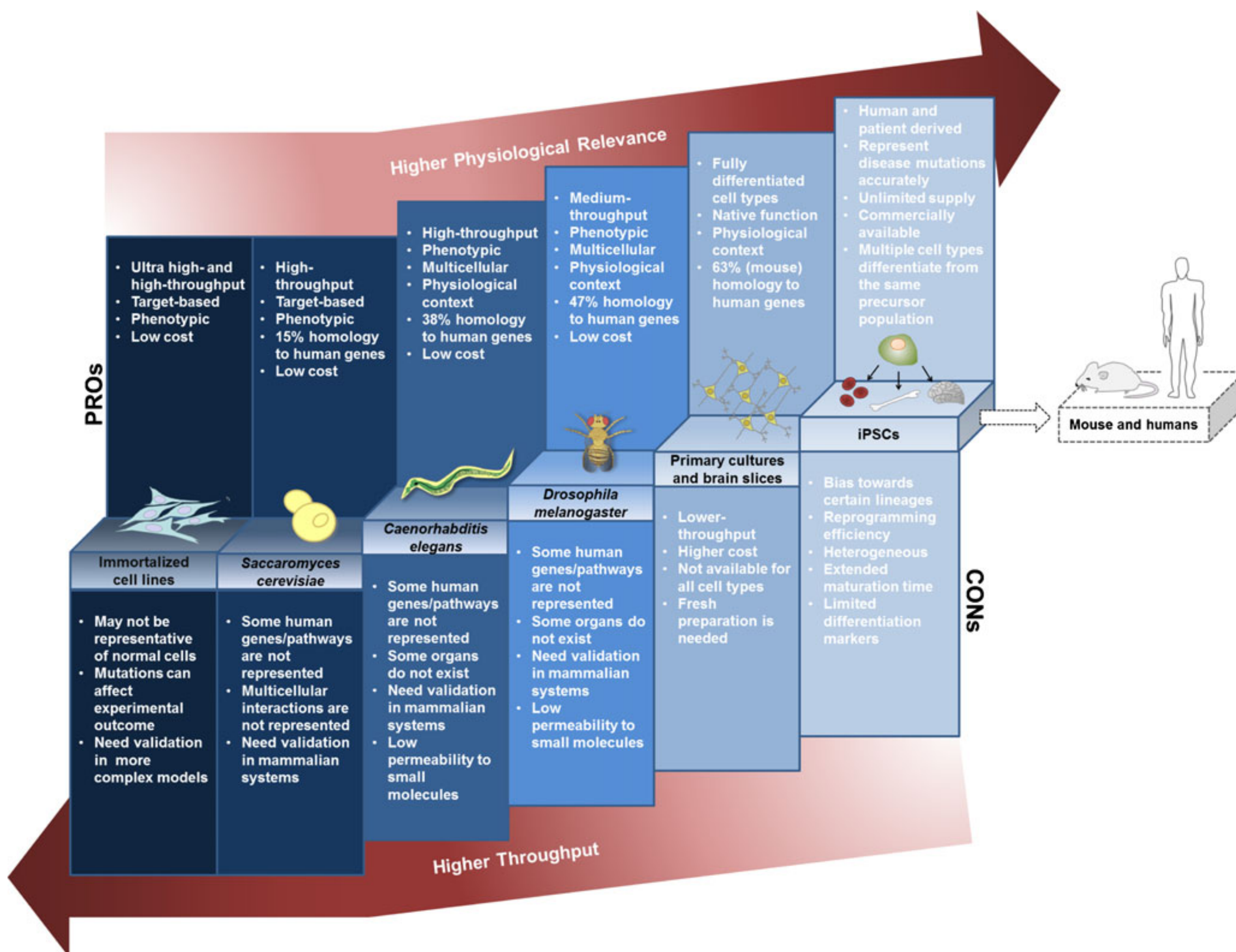

Fig. 1 Model systems for drug and target discovery in polyQ disorders balance physiological relevance against cost and throughput. The simplest models have high throughput, but limited biological complexity, whereas tissue and organismal models retain high biological complexity, but have more modest throughput capacities. Note that the categories shown are not intended to represent hard distinctions, but rather a continuum of screening solutions balancing throughput against biological complexity

in the context of drug discovery—generally weighing physiological/clinical relevance against throughput and cost—collectively these screening models provide a powerful critical path for the discovery, optimization, and validation of new candidate drugs and drug targets.

Here we will review some recently developed models of polyQ disease and how these models have been implemented in high-throughput screening (HTS) campaigns to identify new therapeutic targets and compounds for disease modification. Rather than describing each type of molecular or cell/organism-based model in turn, we will discuss how these models have been employed to achieve different goals in the drug discovery development process, including *compound screening* with disease gene-specific and non-specific assay readouts; *genetic screening* using RNA interference (RNAi), chemical mutagenesis, or transposon insertions; and *proteomic screening*. The latter two approaches have been applied primarily for target identification and validation, while compound screens have been done in the full range of such disease models, from yeast to cell- and tissue-based models to whole animal model organisms. While we will provide examples of new drug and drug target candidates emerging from such studies, we will regrettably not be able to review the field in a comprehensive manner owing to space limitations. We thus refer the reader to recent and excellent reviews focusing on other aspects of polyQ drug discovery and disease models [7–14].

Molecular Screening to Target PolyQ Protein Levels and Aggregation

The monogenic nature of HD, together with the likelihood that a major portion of disease pathogenesis arises from

gain-of-function mechanisms [15, 16], suggest that molecular screening assays based on mutant HTT (mHTT) proteins could be used to identify proximal events in disease initiation. In fact, one of the first observable molecular phenotypes of polyQ diseases was the presence of large visible protein inclusions, comprised of the mutant misfolded protein and associated cellular proteins [17–19]. Thus, molecular screening assays to inhibit mutant polyQ protein aggregation were among the first high-throughput screens developed for these diseases (see [8] and [20] for review). A number of such campaigns done *in vitro* and in cellular models identified small molecules and peptides that reduced aggregation (Table 1) [21–25].

Molecular assays directed against polyQ disease proteins have been designed in diverse ways. Examples of pure *in vitro* assays for aggregation of recombinant proteins include quantification of aggregate formation using the filter-trap aggregation assay [21, 24, 26] and the sedimentation assay [27]. Both assays have been used for the identification of compounds with inhibitory properties against mHTT aggregation. More recently, *in vitro* assays have been used to define more clearly the toxic protein species [28, 29]. For example, methylene blue (MB) reduced aggregation as measured by the filter trap method when added to monomers or preformed fibrils *in vitro* [30]. MB was then shown to diminish different mHTT species isolated from primary cortical neurons using an agarose gel method that distinguishes aggregates and oligomers [31]. MB is currently in late-stage clinical trials as a tau aggregation inhibitor for mild-to-moderate Alzheimer's disease (AD) [32].

To incorporate cellular context, a number of laboratories have designed assay platforms in which aggregation can be monitored within living cells. For example, aggregation-prone proteins fused to fluorescent tags that emit a Förster/fluorescence resonance energy transfer (FRET) signal were used to screen a ~2,800 compound library and identified the Rho kinase (ROCK) inhibitor Y-27632 as a hit compound [25]. Y-27632 reduced the FRET signal by nearly half and reduced the loss of photoreceptor neurons expressing mHTT exon 1 in a *Drosophila* model. Although Y-27632 has limited potency [33], the discovery of ROCK inhibitors as aggregation inhibitors enabled the characterization of a ROCK–profilin signaling pathway [34, 35]. Profilin binding to mHTT appears to maintain mHTT solubility, and ROCK phosphorylation of profilin reduces the protective profilin–mHTT interaction. Recently, administration of the clinically-approved ROCK inhibitor HA-1077 (Fasudil) was shown to rescue retinal degeneration in the R6/2 mouse model expressing human *HTT* exon 1 with >150 CAGs [36, 37]. Thus, ROCK inhibition has been strongly implicated as a mechanistic pathway for intervention in HD.

Automated time-lapse microscopy has been used to analyze live primary neuronal cultures for aggregation of GFP-tagged polyQ proteins [38, 39]. By following individual cells over time, visible aggregate morphology could be anticorrelated with the risk of cell death. Another aggregation model that used restoration of luciferase activity from a polyQ-luciferase reporter identified MB and several other compounds as aggregation inhibitors [40]. Terflunomide reduced incorporation of polyQ-containing proteins into aggregates resulting in smaller aggregates, but did not disaggregate polyQ protein or reduce polyQ-associated toxicity in human embryonic kidney (HEK) 293 cells [40]. Such complex screening methodologies highlight the value of distinguishing mechanisms of action for aggregation inhibitors, even at the primary screening stage.

Aggregation-based screens can also be done in the context of genetic model organisms, such as a yeast-based primary screen of 16,000 small molecules for suppressors of HTT103Q-mediated aggregation [41]. A subsequent secondary screen of a focused compound library in mammalian cell models of polyQ disease identified compounds that increased soluble HTT103Q levels. The potency of these compounds in inhibiting mHTT aggregation was then tested in organotypic hippocampal slices obtained from R6/2 mice. Among the 7 compounds tested in this model, only the sulfobenzoic acid derivative C2-8 showed a marked effect in reducing mHTT aggregate load in neurons, despite having no significant effect in reducing the total number of aggregates. Compound C2-8 was also shown to have neuroprotective activity *in vivo* by rescuing photoreceptor degeneration in a *Drosophila* eye model of HD. Although the precise mechanism by which C2-8 reduced aggregation remains unknown, the authors provided evidence that the compound could be targeting the polymerization step of the polyQ aggregation process. Of interest, compound C2-8 was recently shown to cross the blood–brain barrier, improve motor function, and reduce striatal neuronal atrophy in R6/2 mice, although overall survival was not extended [42]. The discovery of C2-8 and other sulfobenzoic acid derivatives also uncovered a hitherto unknown connection between SIRT2 inhibition, cholesterol homeostasis, and mHTT aggregation [43, 44]. Compounds with SIRT2 inhibitory activity were also neuroprotective against mutated alpha-synuclein in cell culture models [45].

Finally, methods that employ time-resolved FRET (TR-FRET) have also been designed to quantify soluble and aggregated mHTT levels from samples derived from screening campaigns, such as those described above, from whole-animal models, and from patient samples as potential progression and drug response biomarkers in the clinical context [46, 47]. Detection of mHTT in this system depends on the use of two labeled antibodies, including one that recognizes the polyQ expansion; thus, the TR-FRET signal is

Table 1 Chemical modifier screens

Phenotype	Expressed protein	Assay system	Hits	Secondary assays	Ref.
HSF-1 activation		~1×10^6 compound screen in HeLa cells	A1, A3, C1, D1, F1	Mammalian cellular and *Caenorhabditis elegans* models of HD	[51]
HSF-1 activation		10,000 compound library screen in yeast	HSF1A (TRiC/CCT complex)	Mammalian cellular and *Drosophila* models of HD	[52]
Fly motor defect	HTT Q128	521 quinazoline-derived compound screen in *Drosophila* neurons	EVP4593	Medium spiny neurons	[81]
Toxicity	HTT480 Q68	40,000 compounds in primary striatal neuron culture			[56]
Toxicity	HTTN90 Q8 and Q73	~400 selected small molecule screen in primary cultures of cortical and striatal neurons	Inhibitors of Rho kinase, phosphodiesterase, adenosine 2A receptor, and IKKβ		[57]
Toxicity	HTT588 Q138 and Q15	~2600 small molecule screen in *Drosophila* primary neuronal culture	Camptothecin, OH-camptothecin, 18β-glycyrrhetinic acid, and carbenoxolone	*Drosophila* HD model	[66]
Toxicity	HTT exon 1 Q25- and Q103-EGFP	68,887 compound screen in PC12 cells	16F16, thiomuscimol, cystamine	Cortico-striatal brain slice models of HD and AD	[61]
Toxicity	HTTN90 Q73	74 drug-like compound screen in rat brain slices	Inhibitors of IKK complex, CXCR3 chemokine receptor, c-Jun N-terminal kinase, and adenosine 2A receptor		[60]
Toxicity	HTT exon 1 Q150	9 selected compound screen in *C. elegans* ASH neurons	LiCl, TSA, SAHA, and mithramycin		[80]
Aggregation and toxicity	HTT Q103-EGFP	16,000 compound library screen in yeast	C2-8	Mammalian cells, brain-slice, and *Drosophila* models of HD; R6/2 transgenic mouse	[41]
Aggregation	GST-HTT exon 1 Q51	*In vitro* screen of ~184,000 small molecule library screen	Benzothiazoles	293 Tet-Off cells	[21]
Aggregation	HTT171 Q58	*In vitro* screen of 1040 FDA-approved drugs and bioactive compounds	Gossypol, gambogic acid, juglone, celastrol, sanguinarine, and anthralin	HdhQ111/Q111 striatal cells	[22]
Aggregation	GST-Q62	*In vitro* screen of peptide phage display library	Six tryptophan-rich peptides (polyQ-binding peptide 1)	COS-7 cell model of HD	[23]
Aggregation	GST-HTT exon 1 Q51	*In vitro* screen of ~5000 natural substances	EGCG and related polyphenols	Yeast and *Drosophila* models of HD	[24]
Aggregation	AR127 Q65	*In vitro* screen of 2800 biologically active compounds	Y-27632	*Drosophila* model of HD	[25]
Aggregation	GST-HTT exon 1 Q51	*In vitro* screen of 11 small molecules	Congo red, thioflavine S, chrysamine G, and Direct fast yellow	COS-7 cells transfected with HTT 52Q	[26]
Aggregation	HTT^{NT} fragment	*In vitro* screen of 15 peptides	HTT^{NT}-based peptides	SH-SY5Y cells	[27]
Aggregation	HTTQ72-Luciferase	2687 small molecule screen in HEK-293 cells	Leflunomide and teriflunomide		[40]
Degradation (clearance)	HTT573 Q72	10,000 natural compound library screen in HN10 neuronal cell line	TSA analogue, staurosporine, anisomycin, cycloheximide, borrelidin, BAY 61-3606		[46]
Degradation (clearance)	HTT573 Q72 and Q25	~2×10^6 compound screen in HN10 neuronal cell line	Heat shock protein 90 inhibitors	HdhQ150 embryonic stem cells and in embryonic stem cell-derived neurons	[48]

Blank entry indicates not included in the study.

HSF = heat shock factor; HTT = huntingtin; TriC/CCT = chaperonin containing TCP-1; EGFP = enhanced green fluorescent protein; GST = glutathione-S-transferase; FDA = Food and Drug Administration; HEK = human embryonic kidney; IKKβ = IκB kinase; CXCR3 = chemokine (C-X-C motif) receptor 3; LiCl = lithium chloride; TSA = trichostatin A; SAHA = suberoylanilide hydroxamic acid; EGCG = (−)-epigallocatechin-3-gallate; AR = androgen receptor; HD = Huntington's disease; AD = Alzheimer's disease.

specific for mHTT, with little or no crossover to the wild type (WT) protein. Importantly, this method has the benefit of being homogeneous, robust and scalable for HTS [46]. Although, to date, TR-FRET screens have primarily identified previously known target classes [46, 48], they have strong potential to identify novel targets as well. A quantification method for aggregated mHTT TR-FRET has also been developed based on dual labeling of a single antibody that recognizes mHTT [49].

Target-Based and Phenotypic Screening Using High-Content Analysis

In addition to reducing soluble and/or aggregated mHTT, screening assays can also be based on defined molecular targets (target-based) or specific cellular states, and/or features believed to be associated with the disease state (phenotypic screening; including high-content analysis). Such assay endpoints are often linked within a screening campaign to associate target engagement directly with a desired phenotypic outcome, such as protection against cell death. A limitation in the field is that no truly validated molecular targets for HD are known in the strictest sense, as there are, currently no approved drugs for HD with disease-modifying ability. However, numerous candidate molecular targets have emerged from basic science studies, as well as target screening campaigns (see below), and many of these have been employed in screening assays to generate tool compounds and/or biologics for subsequent hypothesis testing and potential clinical development. Alternatively, many laboratories have generated phenotypic screening assays that are hypothesis-neutral with respect to molecular target(s), and instead focus on rescue of particular cellular phenotypic/pathogenic features induced by the introduction of mutant polyQ proteins.

HTT is intrinsically the most relevant molecular target in HD, and itself can be the focus of a targeted molecular screen. For example, a high-throughput Western blot-based screen done in cell-based models demonstrated the involvement of matrix metalloproteinases in proteolytic cleavage of mHTT—a key event in the pathogenesis of HD—and in associated cellular toxicity [50]. Another example of a target-based screen was one in which the primary endpoint was the activation of a heat shock protein HSP70 promoter-luciferase reporter construct responsive to the activity of heat shock factor-1 (HSF-1), the master regulator of the heat shock response [51]. Large-scale screening was designed to identify compounds that could increase HSF-1 activity and thus drive the luciferase reporter. Hit compounds were then advanced to show suppression of aggregate formation and reduction of toxicity in a *C. elegans* polyQ model (see following section on model organisms). Importantly, the hit compounds did not seem to act by inducing protein misfolding, or by inhibiting HSP90 or the proteasome.

A similar approach was taken in a yeast strain that depended on human HSF-1 activation for growth in which a library of 10,000 non-biased small molecules was screened for activators of HSF-1 [52]. Two benzyl-pyrazole compounds (HSF1A and HSF1C) were identified as effective HSF-1 activators and subsequently shown to reduce polyQ aggregation and cell death in a cell culture model; they were also shown to ameliorate disease-like phenotype in a *Drosophila* model of SCA3. The compound HSF1A was shown to bind to the TRiC/CCT cytosolic chaperone complex, which had been previously identified as a mHTT binding partner in a proteomic screen [53], and had also been shown to reduce polyQ aggregation/toxicity in mammalian and yeast models [54, 55]. Thus, CCT might play an essential role in modulating the folding of polyQ-expanded proteins and could be a prime target for further therapeutic development.

Cell-based phenotypic screening for polyQ disorders has frequently used cell degeneration and/or death as primary endpoints. For example, expressing a 480-amino acid mHTT fragment with 68Q in primary striatal neuronal cultures led to 50 % cell death in a 6-day assay window [56]. This assay was used to screen 40,000 compounds and generated candidate compounds undergoing further characterization. Similarly, a high-content, composite cortical neuron, striatal neuron, and astroglial co-culture screening platform was described [57]. Striatal and cortical cell populations could be visualized separately through electroporation of different fluorescent proteins before co-culturing, with loss of fluorescence as a quantitative readout for cell survival. In this platform, expression of m*HTT* exon 1 fragment (73 CAG) reduced striatal and cortical neuron survival compared with normal Q-length controls. Screening in 96-well plate format led to identification of several compound classes with known and novel neuroprotective actions, including compounds that had been previously identified in screens using different endpoints (e.g., the ROCK inhibitor Y-27632) [25] or acted on gene targets implicated in other HD models (e.g., inhibitor of kappa B (IκB) kinase and the adenosine 2A receptor) [58–60].

Induction of apoptosis by a mHTT fragment in the pheochromocytoma cell line PC12 was used to screen nearly 70,000 compounds [61]—several hits from which appeared to bind to a common protein target. The cellular target was subsequently identified as the molecular chaperone protein disulfide isomerase by using a novel adaptation of the "click" chemistry approach [62]. These compound hits were subsequently shown to be neuroprotective in *ex vivo* HD and AD brain slice models, supporting the predictive value of phenotypic screens based in PC12 cells. Moreover, the authors presented evidence that protein disulfide isomerase

may also be a molecular target for cystamine, which had previously been shown to provide benefit in a whole-animal HD model [63, 64].

A large-scale screen using nearly 8,000 small interfering RNA pools representing the "druggable genome" was carried out in a HEK293 cell line that underwent caspase activation when transfected with an N-terminal mHTT fragment (558 amino acids with 141Q) [65]. Interestingly, although this initial screen was done in a non-neuronal cell line, mHTT modifier gene hits were enriched in neurologic disease functions. Moreover, connecting nodes in a suppressor gene network included genes such as *nuclear factor-kappa B* [58, 60] with established roles in HD pathology. A novel pathway was also identified, as small interfering RNAs to multiple components of the related rat sarcoma (RRAS) viral oncogene homolog pathway emerged as mHTT toxicity suppressors. Subsequently, aberrant RRAS signaling was confirmed in the striatum of R6/2 mice, and RRAS inhibition was protective in a knock-in cell line (HdhQ111), as well as a *Drosophila* HD model [65].

For phenotypic screening in neurons, the neuronal dendritic arbor can be a very sensitive and quantitative indicator of cell state and vitality. An interesting example of such a screen was done in primary neuronal cultures generated from *Drosophila* transgenic lines expressing a 588-amino acid fragment of HTT with either 15Q or 138Q, and tagged with monomeric red fluorescent protein (mRFP) to monitor aggregation [66]. The strain was also transgenic for a membrane-targeted green fluorescent protein (GFP), which allowed for visualization of dendrites in the primary neurons. mHTT-expressing primary neurons showed altered dendritic and axonal morphologies that could be distinguished from the normal Q-length control. Using these assay endpoints for mHTT aggregation, neuromere size, and dendritic length, 2,600 compounds and a 468-gene kinase/phosphatase RNAi sub-library were screened in 384-well plates with the mammalian target of rapamycin/insulin pathway negative regulator lkb1, as well as camptothecins, emerging as hits.

Similarly, synapses and synaptic function can be very sensitive surrogate endpoints for neuronal phenotype and function, and for states of neural circuits. In this context, a HTS was established to measure synaptic activity using a pH-sensitive GFP fused to synaptophysin [67]. Fluorescence develops only upon synaptic vesicle fusion and exposure of the GFP tag to the normal pH of the synaptic cleft. Using a fully automated 96-well plate based system, neurons could be stimulated and the rate of exocytosis of the synaptic vesicle label measured. Adenosine 1 receptor agonists were found to suppress synaptic vesicle movement using this technique. Alternatively, synaptic connections can be directed through microchannels (“synapse microarray”) in a more stereotyped fashion to facilitate automated analysis [68]. Using this system, 22 compounds were evaluated at 3 doses and several histone deacetylase (HDAC) inhibitors that enhanced the synaptic signal were identified.

High-content analysis methods have also been used in primary cultured cortical neurons to detect compounds that specifically influenced synaptogenesis and dendritic outgrowth [69]. Using immunocytochemistry against microtubule-associated protein 2 to label dendrites and against synapsin to label synapses, several compounds were tested and found to reduce dendritic lengths and/or synapse numbers in a concentration-dependent manner. Interestingly, the compound mevastatin appeared to suppress synapse number specifically without shortening dendrites.

Three-dimensional (3D) culturing systems represent a way to build biological complexity while maintaining the scalability of cell culture. For example, 3D hydrogel tissue constructs formed through extracellular matrix remodeling can measure contractile force, which does not occur in two-dimensional culture [70]. Similarly, 3D spheroid cultures derived from SH-SY5Y neuroblastoma cells expressing tau variants have been used as a model of tauopathy [71].

To mimic the native 3D tissue context of neurons even more closely, brain slice explants can be used as a screening platform [72, 73]. For example, biolistic transfection has been used to introduce mHTT constructs together with fluorescent visual reporters to develop phenotypic assays for mHTT-induced neuronal degeneration [74]. The brain slice preparation allows a variety of neuronal features to be assayed, including dendritic arborization, and allows direct quantitative readouts of neuronal loss. Moreover, intact brain tissue also retains cell-based neuroinflammatory responses, and a number of neuroprotective compounds identified in brain slice screens were likely to have neuroinflammatory pathways as their primary targets [60]. The application of novel methodologies such as these to HD cell- and tissue-based models are supporting the development of valuable new screening platforms for phenotypic features that may not be expressed in conventional monolayer cell cultures (Fig. 1).

Drug and Drug Target Screening in Invertebrate Animal Models

Combining biological complexity with amenability to high-throughput technologies, invertebrate model organisms, such as yeast, flies, and worms, can help to bridge the gap between *in vitro* and mammalian *in vivo* assays (Fig. 1). Such model organisms can provide powerful genetic screening platforms for drug and especially drug target discovery in polyQ diseases. In the following sections we will describe examples of small molecule, genetic, and RNAi screens that have been performed in model organisms, both at the small

and large scale (Table 2). We will also discuss recent proteomic studies and associated follow-on validation studies in model organisms. As more data emerge from such screens and can be harmonized and analyzed, key concepts and driving pathways for disease pathogenesis should increasingly emerge.

Model organisms commonly used in polyQ HTS drug and target discovery include *S. cerevisiae*, *C. elegans*, and *D. melanogaster*. Despite being a simple unicellular organism *S. cerevisiae* has made fundamental contributions to the elucidation of essential eukaryotic pathways and cellular mechanisms (reviewed in [75]). In fact, about one third of the yeast genome has direct human orthologs; in addition, nearly 500 human disease genes are orthologous to yeast genes, making this organism an apt tool for the study of human diseases [76]. While yeast cannot model the complexity of mammalian neural networks, the presence of conserved eukaryotic pathways allows extrapolation of findings obtained in this organism to humans. For example, conservation of protein-control quality machinery between yeast and higher eukaryotes makes *S. cerevisiae* an extremely useful model organism to study neurodegenerative diseases associated with protein misfolding and aggregation [75]. Finally, yeast is highly amenable to HTS, allowing for the identification of compounds with inhibitory properties against disease protein-mediated cellular phenotypes.

C. elegans also offers an effective model in which to study neurodegenerative diseases, including diseases associated with polyQ expansions (reviewed in [77]). Although like yeast *C. elegans* does not contain its own *HTT* ortholog, expression of expanded CAG constructs induces cellular and behavioral phenotypes reminiscent of cellular dysfunction and degeneration in mammalian systems. Worm polyQ models have thus been used to test the effects of known drugs or to identify novel drugs and gene candidates that modify aggregation/toxicity of polyQ-disease proteins.

Finally, *D. melanogaster* has proven to be a powerful tool for studying the pathology of human diseases both at the molecular and cellular levels (reviewed in [78]). *Drosophila* genes share about 50 % homology with the human genome, and important biological pathways are highly conserved. In addition, *Drosophila* has a relatively complex nervous system and a brain, providing relevance for the study of neurodegenerative diseases. Together with the extensive genetic tools available, these features have enabled *Drosophila* to be incorporated very effectively into large-scale genetic and pharmacologic screens for the identification of human disease genes and potential therapeutic leads [78].

Small Molecule Screening With Model Organisms

Several groups have taken advantage of the amenability of model organisms to high-throughput technologies to screen libraries of chemical compounds for the identification of inhibitors of polyQ-induced phenotypes (Table 2). For example, a *C. elegans* model of HD in which neuronal expression of the N-terminal fragment of human HTT carrying a tract of 150Q (HTT exon 1 Q150) induced progressive neurodegeneration of ASH sensory neurons was used to screen a collection of compounds previously reported to ameliorate death in cellular and animal models of polyQ diseases [79]. A subset of the compounds tested was able to decrease polyQ-mediated death of ASH sensory neurons [80].

Analogously, a *Drosophila* HD model in which pan-neuronal expression of mHTT resulted in a motor phenotypic impairment (limb tremors and climbing) was used in a screen of 521 quinazoline-derived compounds that identified compound EVP4593 as a potent suppressor of the climbing defect [81]. In secondary assays, EVP4593 was also shown able to protect cultured primary medium spiny neurons (MSNs) isolated from transgenic HD mice (YAC128) from glutamate-induced toxicity. Follow-up studies suggested that one or more subunits of the transient receptor potential (TRP) channels could be the molecular targets of the compound, adding to other reports supporting a role of TRP channel family members [82] in neurodegeneration [83].

Genetic and RNAi Screens

In addition to allowing the identification of lead compounds, model organisms provide an excellent tool for the discovery of genes (this section) and proteins (see following section on proteomic screening approaches) as potential drug target candidates. Genome-wide genetic screens for modifiers of mHTT-mediated toxicity in yeast have identified several genetic pathways and molecular mechanisms underlying neurodegeneration in HD that could be exploited for therapeutic purposes. For example, genes that enhance mHTT-mediated toxicity have been found to be involved in the response to cellular stress, protein folding, and degradation [84], whereas genes with a predicted role in vesicle transport, vacuolar degradation, transcriptional regulation, and prion-like aggregation have been shown to be suppressors of mHTT toxicity [85].

A recent phenotypic yeast-based screen was used to assay for transposon insertion mutants that could restore the activity of the Ade-2 protein containing 97Q [86]. An interesting hit from this screen was *SPT4*, a transcription elongation factor that reduces RNA polymerase II dissociation from its template, thereby increasing polymerase processing [86]. Accordingly, a deletion in *SPT4* led to a general decrease in transcription elongation, which, surprisingly, was more pronounced in genes encoding long stretches of CAG or other repetitive sequences (e.g., CAA

Table 2 Genetic, RNA interference (RNAi) and proteomic modifier screens

Phenotype	Expressed protein	Tissue	Assay system	Hits	Secondary assays	Ref.
Toxicity	HTT exon 1 Q20 and Q53		Genome-wide loss-of-function enhancer screen in yeast	Genes involved in stress responses, protein folding, and ubiquitin-dependent protein degradation		[84]
Toxicity	HTT exon 1 Q103		Genome-wide loss-of-function suppressor screen in yeast	Genes involved in vacuolar transport, transcriptional regulation/maintenance of chromatin structure, other processes (*bna4*) and prion genes		[85]
Aggregation	Ade-2 Q25 and Q97		Transposon insertion mutants in yeast	Genes involved in vacuolar transport, transcriptional regulation *(Spt4)*	Murine striatal neuron cell models of HD	[86]
Toxicity	HTT exon 1 Q2 and Q150	ASH sensory neurons	Mutation screen in *Caenorhabditis elegans*	polyQ enhancer-1 *(pqe-1)* gene		[95]
Toxicity	HTT exon 1 Q128	Touch receptor neurons	6034 candidate RNAi screen in *C. elegans*	Genes involved in cell death, protein folding, intracellular transport, metabolic processes, response to stress, stress-activated pathways	Striatum of HD mouse models	[101]
Aggregation	Q35-YFP	BWM	Genome-wide RNAi in *C. elegans*	Genes involved in RNA synthesis/processing, protein synthesis, transport, folding, and degradation		[102]
Aggregation	Q35-YFP	BWM	Genome-wide RNAi in *C. elegans*	Genes involved in cell cycle, DNA and RNA synthesis/processing, energy and metabolism, and protein synthesis, transport and folding	*C. elegans* expressing Q37-YFP, SOD^{G93A}, and temperature sensitive proteins	[105]
Toxicity	Q127	Retina	7000 P-element insertions in *Drosophila*	DNAJ domain-containing proteins dHDJ1 and dTPR2	Transgenic *Drosophila* lines	[90]
Toxicity and aggregation	Ataxin-1 Q30 and Q82	Retina, central nervous system	Transposon insertion in *Drosophila*	Genes involved in protein folding, degradation, transcriptional regulation, RNA binding and stress response		[91]
Toxicity	Ataxin-1 Q82, ataxin-3 Q78 and Q127	Retina	55 modifier strains in *Drosophila*	Genes involved in protein folding and apoptosis	*Drosophila* brain	[145]
Toxicity and aggregation	Ataxin-3tr Q78	Retina	2300 enhancer/promoter element insertion library in *Drosophila*	Chaperones and ubiquitin-pathway components, and genes involved in transcription, translation, and RNA binding	*Drosophila* model of tau-induced degeneration	[92]
Toxicity and aggregation	HTT588 Q138	Neuronal and non-neuronal tissues	Autosomal deficiency (Df) kit for chromosome II and III (160 Df lines)	Small C-terminal domain phosphatases and Antennapedia complex	*Drosophila* HD model	[93]
Toxicity	HTT588 Q138 and Q15		Whole genome (468 genes) kinase/phosphatase RNAi sub-library in *Drosophila* neuronal culture	*lkb1*	*Drosophila* HD model	[66]
Toxicity	HTT exon 1 Q97	Mouse brain, mouse muscle	Y2H, MS	Proteins involved in folding, synaptic transmission, cytoskeletal organization, signal transduction, and transcription	*Drosophila*	[53]
	HTT Q144	Mouse brain	Affinity purification/MS	Proteins involved in translation		[113]
Toxicity	HTT Q97	Mouse brain	Affinity purification/MS	Proteins involved in protein folding, 14-3-3 signaling, microtubule-based intracellular transport, and mitochondrial function	*Drosophila*	[114]
Toxicity	HTT Q72	iPSC lines	2-DE/MS	Genes involved in oxidative stress and apoptosis		[136]

Blank entry indicates not included in the study.

HTT = huntingtin; HD = Huntington's disease; YFP = yellow fluorescent protein; BWM = body wall muscle; SOD = superoxide dismutase; TRP2 = tetratricopeptide repeat protein 2; Y2H = yeast 2-hybrid; MS = mass spectrometry; iPSC = inducible pluripotent stem cell; 2-DE = two-dimensional electrophoresis.

and AAA repeats). In turn, reduced transcription of expanded polyCAG stretches was shown to reduce aggregation of the mutant Ade-2 protein, and also to diminish its co-aggregation with Ade-2-containing short polyQ stretches. The beneficial effects of deleting *SPT4* in yeast were reproduced in a mouse model of HD, where knockdown of the ortholog *Supt4h* led to reduction of HTTQ111 aggregation, with no effect on HTTQ7 expression.

Transgenic *Drosophila* models of human polyQ-mediated diseases have been also used in high-throughput genetic screens for modifiers of mutant protein-induced aggregate/toxicity (reviewed in [87–89]). Using the fly eye as a model to study neurodegeneration, two groups ran genetic screens to identify modifiers of either pure polyQ tracts (127Q repeats) or ataxin-1 82Q-mediated toxicity [90, 91]. A screen for modifiers of polyQ-induced toxicity identified two suppressor genes encoding the dHDJ1 and dTPR2 proteins, which contain a J domain known to stimulate HSP70 activity and prevent protein aggregation [90]. Similarly, a screen for modifiers of mutant ataxin-1 toxicity identified components of the protein folding (dHDJ1) and clearance pathways (ubiquitin conjugases), as well as novel modifiers involved in RNA processing, transcriptional regulation, and cellular detoxification [91]. A genetic screen for modifiers of *Drosophila* eye degeneration induced by mutant ataxin-3 (ataxin-3tr Q78) identified 14 suppressors and one enhancer [92]. Analogous to the two studies described above [90, 91], some suppressors belonged to the chaperone and ubiquitin-pathway component classes in addition to suppressor genes with roles in transcription, translation, and nuclear export. Interestingly, some of the suppressor genes, such as the co-chaperone TPR2 and polyubiquitin, were also able to reduce tau-mediated degeneration, indicating that chaperones and the ubiquitin-proteasome system may be general modifiers for different protein conformation diseases [92].

More recently, a haplo-insufficiency screen for suppressors of aggregation and toxicity mediated by expression of a 588-amino acid N-terminal fragment of HTT with a 138Q repeats (HTT588 138Q) was done in *Drosophila* [93]. Although these toxicity and aggregation screens were done in neuronal and non-neuronal tissues, respectively, they both identified the same chromosomal regions as hits. Two major classes of suppressors were identified: one class that rescued fly viability by decreasing HTT expression and aggregation, and a second class that rescued viability without reducing HTT aggregation, suggesting that aggregation and toxicity are separable, and that mutant HTT-associated toxicity depends on both the soluble and aggregation-prone forms of the mutant protein. Two single genes were mapped: one was a *piggyBac* insertion that reduced the expression of CG5830, which shares homology with small nuclear protein phosphatases known to silence neuronal gene expression. As disruption of transcriptional regulation (e.g., of brain-derived neurotrophic factor) has been implicated in HD [14, 94], it is possible that loss of CG5830 rescues mutant HTT-induced toxicity by restoring transcription. The other mapped gene was *Labial*, a member of the *Antennapedia* complex that has been linked to neural stem cell survival in *Drosophila*; the rescue in *Drosophila* viability elicited by this mutation was attributed to its ability to reduce mutant HTT levels. Interestingly, other members of the *Antennapedia* complex were also able to suppress HTTQ138-induced toxicity.

Caenorhabditis elegans models of HD and other polyQ-associated diseases have also been widely used to screen for genetic modifiers of polyQ aggregation and toxicity. The *C. elegans* model in which mHTT exon 1 Q150 expression caused degeneration of ASH neurons (see above) was also used in a genetic screen for mutations that increase mHTT neurotoxicity, leading to the identification of the *polyQ enhancer-1* (*pqe-1*) gene [95]. A loss of function mutation in *pqe-1* was shown to increase mHTT toxicity, whereas its overexpression rescued the toxic phenotype. The authors suggested that *pqe-1* provides protection against polyQ toxicity in *C. elegans* neurons through its glutamine/proline-rich domain by competing with other proteins for binding to mHTT.

This mHTT exon 1 Q150 model has also been used to investigate the roles of specific histone deacetylases in regulating mHTT toxicity [96]. As several studies indicated that mHTT can sequester cyclic adenosine monophosphate response element-binding protein-binding protein (CBP), reducing its histone acetyltransferase activity [97, 98]; inhibition of histone deacetylase activity could counteract this effect and subsequently reduce mHTT toxicity. In fact, using RNAi, Bates et al. [96] reported that lowering *hda-3* in *C. elegans* reduced polyQ toxicity. Analogously, chemical manipulation of HDAC function by histone deacetylase inhibitors such as trichostatin A also led to a decrease in polyQ-mediated neurodegeneration in this study.

C. elegans expressing an N-terminal fragment of the HTT exon 1 protein containing expanded (Q88 and Q128) or unexpanded (Q19) polyQ stretches in touch receptor neurons showed polyQ-dependent neuronal dysfunction [99]. This system was used to investigate the effect of increased *sir2.1* deacetylase activity in rescuing nematode neurons from polyQ-mediated toxicity [100]. The Q128 nematode model was also used in a large-scale RNAi screen to identify genes that would either reduce or exacerbate the loss of response to light touch [101]. An intriguing observation was that some of the RNAi hits from this *C. elegans* screen overlapped with striatal gene expression data derived from the CHL2 (Q150/Q150 knock-in) and the transgenic R6/2 mouse models of HD, underscoring the relevance of using invertebrate models for the identification of potential therapeutic targets for human neurodegenerative diseases.

In particular, the druggable *pha* gene, which encodes the enzyme phenylalanine-4-hydroxylase, was of interest because of its connection to the tyrosine–dopamine biosynthesis pathway, which is known to be associated with early HD phenotypes [101]

In a broader approach, a genome-wide RNAi screen was done in a *C. elegans* transgenic strain expressing stretches of 35 Q (polyQ35) fused with yellow fluorescent protein specifically in muscle cells [102]. This study yielded 186 genes that, when knocked down, caused premature aggregation of polyQ35 aggregates, including the TCP-1 chaperonin ortholog later confirmed to have a role in aggregation of mHTT in mammalian cells [54, 55, 103]. In addition, a subsequent RNAi screen of human orthologs of these *C. elegans* modifiers showed a subset to also be suppressors of mHTT aggregation in human cells (HEK293) expressing HTT-Q74–GFP [104].

A complementary approach was undertaken recently [105] in which the polyQ35 *C. elegans* muscle model was used to screen for genes that, when down-regulated, increased cellular protein folding capacity and thereby suppressed polyQ35-mediated toxicity [105]. Gene hits were subsequently tested for suppression of aggregation in a mutant superoxide dismutase 1 (SOD1G93A) model of amyotrophic lateral sclerosis. This strategy led to the identification of 9 genes that work as core proteostasis network modulators and not only reduce protein aggregation but also enhance the protein-folding environment [106, 107].

An interesting observation from these *C. elegans* genetic screens was the minimal overlap between the hits derived from aggregation screens [102, 105] and those from the toxicity screen described previously [101]: only 15 modifiers were identified in common. This emphasizes the critical roles of assay readouts (e.g., aggregation *vs* toxicity), and of molecular and cellular context in such genetic screens. For example, whereas the two aggregation screens used a polyQ-only expansion expressed in muscle cells, the toxicity screen used HTT exon 1 Q128 expressed in neurons.

Proteomic Screening

A parallel and complementary systems biology approach to the identification of genetic modifiers is the direct identification of mHTT interacting proteins that may play mechanistic roles in polyQ-related disease pathogenesis. Initial yeast two-hybrid screens identified interactors of mHTT with widespread roles in protein trafficking, turnover, mRNA biogenesis and metabolism, and transcription and signaling pathways [108–111], underscoring the broad range of proteins and core cellular pathways that interact with mHTT.

More recently, an extensive proteomic study was done using a combination of yeast two-hybrid and pull-down assay/mass spectrometry [53]. A total of 234 novel HTT-fragment-interacting proteins were identified that belonged to different functional classes, such as protein folding and degradation, signal transduction, synaptic transmission, and metabolism. Sixty of these protein hits were subsequently tested in a *Drosophila* model of HD, with 48 of the 60 shown to be either enhancers or suppressors of mHTT-induced neurodegeneration. An interesting observation was that some of the modifiers were involved in synaptic vesicle fusion, suggesting that WT HTT could have a role in modulating neurotransmitter secretion, which could be impaired by mHTT. In addition, two components of the cytosolic CCT (TriC) were identified, and these proteins were also confirmed to alter the course of polyQ-induced toxicity in mammalian cells [54, 55].

Recently, proteomic studies to identify HTT-associated proteins have also been done in the context of full-length HTT expressed in mouse brain, using affinity purification-mass spectrometry [112, 113]. Using subcellular fractionation of brain homogenates, one study found molecular chaperones as mutant HTT interactors, in support of previous findings and highlighting the central role of these folding machineries as HTT modifiers [112]. Interestingly, components of the translational machinery were also identified as mutant HTT interacting proteins, providing a novel molecular pathway for HTT-mediated toxicity. In another study distinct regions of WT and BACHD mouse brains of different ages were used to identify 747 HTT interacting proteins [113], of which several had previously been found as HTT interactors or as modifiers of mHTT toxicity [53, 114]. A subset of these protein hits was also shown to act as genetic modifiers of mHTT-induced neurodegeneration in a *Drosophila* model. Of importance, several of these proteins are targets of compounds that have been effective in mouse HD models, such as geldanamycin targeting HSP90. Interestingly, although the majority of HTT interactors were relatively conserved across different brain tissue and age, brain region- and developmental stage-specific interactors were also found, suggesting that some of these proteins might contribute to the selective neuronal susceptibility and age-dependent degeneration observed in HD patients. A comparison to other proteomic datasets reported previously [53, 111, 115] showed greater overlap among HTT datasets than between HTT and ataxia datasets, suggesting that unique protein–protein interactions may contribute to specific pathogenic mechanisms in different, but related, polyQ-mediated diseases [113].

Chemical, Genetic and Proteomic Screens: What Have We Learned?

With an increasing number of studies completed we can begin to compare the hits emerging from a range of diverse

screening strategies (Tables 1 and 2). Perhaps not surprisingly, there have been relatively few common genes [89] and compounds found across screens. Numerous factors are likely to contribute to such low hit overlap. For example, different technical methodologies may intrinsically bias towards or limit against certain subsets of targets and mechanisms: proteomic interaction methods tend to identify abundant proteins and may miss rare interacting partners, while genetic screens may fail to detect essential genes owing to lethality or gene families with redundant functions. In addition, not all models share all genetic pathways. Differences in constructs used to drive disease models (e.g., fragment *vs* full-length HD models) may also emphasize different hit pathways and categories.

However, such high-throughput genetic and proteomic screens in the context of different polyQ-driven neurodegenerative diseases are also identifying functional gene classes represented across disease states, suggesting conserved roles for protein classes in common and, perhaps, core disease mechanisms. For example, molecular chaperones and components of the proteolytic machineries involved in refolding and degradation of misfolded proteins, respectively, have commonly been found as hits in screens for suppression of polyQ aggregation and toxicity [106, 107]. Overexpression of selected or multiple chaperones by genetic and pharmacologic strategies has, indeed, proven effective in rescuing aggregation-mediated toxicity of mHTT and other polyQ-related proteins in multiple models. Analogously, induction of autophagy by rapamycin, an inhibitor of mammalian target of rapamycin, was shown to ameliorate neurodegenerative phenotypes both in fly and mouse models of HD [116] in support of these screening results, as described in previous sections.

In addition many genes involved in transcriptional regulation and RNA processing are consistently found as modifiers of polyQ-mediated aggregation and toxicity in yeast, *Drosophila*, and *C. elegans* [2]. Indeed, accumulating evidence suggests that chromatin structure and epigenetic regulation are involved in polyQ-mediated pathology. It has been shown in several models that mutant polyQ proteins localize in the nucleus, often as inclusions, and cause the redistribution of key transcription factors, such as the nuclear receptor corepressor, CBP, and p300/CBP-associated factor [117]. Sequestration of such factors could compromise the acetylation level of the chromatin and lead to altered transcription [110].

Remarkably, in both flies and mice loss in histone deacetylation and subsequent HD-induced neurodegeneration could be rescued not only by CBP overexpression, but also by pharmacological means, using HDAC inhibitors [117]. HDAC inhibitors show a therapeutic potential for the treatment of several disorders, including HD [118]. For example, the HDAC inhibitors suberoylanilide hydroxamic acid [119], sodium butyrate [120], phenylbutyrate [121], and 4b [122] have all been shown to ameliorate mutant HTT-mediated degeneration in different HD mouse models, and some (e.g., sodium butyrate) have reached clinical trials for the treatment of HD. These encouraging studies suggest that clinical development of HDAC inhibitors for HD therapy may be possible.

That certain targets and mechanisms could be common to all polyQ diseases, such as the involvement of molecular chaperones and the ubiquitin-proteasome system [123], may not be surprising. It might be expected, however, that some pathways involved in polyQ-mediated disease progression will be disease-specific. In fact, a comparative study of known ataxin-1 Q82 modifiers in *Drosophila* found that only certain genes were able to modify mutant ataxin-1- and HTT-induced phenotypes in a similar manner, whereas other genes were disease-specific or even modified the two disease models in opposite ways [124]. In addition, post-transcriptional/translational modifications outside of the polyQ tract have been found to have profound effects on the pathogenicity and mechanisms of action of polyQ proteins, including HTT [125, 126], and potentially driving disease-specific or "disease-emphasized" pathways.

The Promise of iPSC

Recent advances in iPSC technology represent a tremendous opportunity to apply drug discovery assays and approaches such as those described in the previous sections to patient-derived cells that can be differentiated into neurons. Monogenic diseases such as HD were among the first to take advantage of iPSC technology to generate neurons and other cells types from patient fibroblasts (reviewed in [127–131]). The benefits of using *bona fide* human and patient-derived cells that can be differentiated into relevant neuronal and/or glial species are inestimable for new drug discovery and development. Moreover, the pluripotency of iPSCs can theoretically provide a nearly unlimited and consistent supply of such cellular reagents. In addition, embryonic and neural stem cells from HD mouse models have also been characterized and could serve as critical bridging tools for supporting preclinical translational studies [132, 133]. Initial studies showed no differences in the ability of HD iPSCs to differentiate into neurons [123, 134, 135].

The first iPSC-based proteomic analysis for HD has recently been reported. This study compared normal with HD patient-derived iPSC lines expressing HTT Q25 and identified 26 differentially regulated genes involved in a range of processes [136]. In particular, genes involved in programmed cell death and oxidative stress were enriched in HD iPSC lines compared with normal iPSC lines, indicating that these cell lines could be highly vulnerable to oxidative stress and apoptotic stimuli. In fact, levels of antioxidant enzymes, such as SOD1 and glutathione-S-

transferase, were found to be lower in HD iPSC lines, which were also more susceptible to apoptotic cell death than control lines. Critically, it has been recently shown that correction of the polyQ expansion in HD patient iPSCs (i.e., in the identical genetic backgrounds) normalizes pathogenic HD signaling pathways and phenotypes such as susceptibility to cell death and altered mitochondrial bioenergetics [137].

For use in phenotypic studies and screening, a set of HD iPSCs consisting of normal (28- and 33-CAG) and long CAG repeats characteristic of juvenile onset HD (60-, 109-, and 180-CAG) was recently generated, with HD neurons found to have increased caspase activity and susceptibility to stressors such as glutamate and brain-derived neurotrophic factor withdrawal [138]. Interestingly, iPSC-derived neurons with shorter polyQ repeat lengths may require more extended times in culture to mature and develop HD-associated cellular phenotypes. For example, iPSC derived neurons with adult onset CAG length (42, 44) showed little phenotype in culture [139], whereas the longest (109, 180) CAG repeat length had more pronounced deficits [138]. However, significant challenges remain in implementing iPSC in HTS protocols [129], including definition, consistency, and yields of cellular differentiation and phenotypes.

Conclusions and Perspectives

While current clinical options remain limited for HD and other polyQ diseases, rapid progress in developing focused preclinical models and drug screening technologies for these disorders promises that new generations of drug candidates will be entering clinical evaluation in coming years, including biologic molecules targeting the mutant genes and proteins themselves [10, 140–142]. Moreover, emerging robotic and automated imaging technologies, together with powerful new analytic tools, are offering new possibilities for the higher throughput use of complex models in drug discovery, ranging from iPSCs to brain slices to *C. elegans* and *D. melanogaster*. For example, the Complex Object Parametric Analyzer and Sorter Biosort [143], which is able to automatically sort and dispense an accurate number of worms and *Drosophila* eggs and embryos into multiwell plates, and the WormToolbox [144], an automated image analysis tool for HTS of *C. elegans* phenotypes, are contributing considerably to increase the throughput of screens using model organisms. Together with recent and rapid progress in novel therapeutic modalities; disease and response biomarker development, including image-based biomarkers and target engagement assays; blood barrier penetrant antibodies; and inhibitory RNAs, peptides, and antisense oligonucleotides for protein misfolding and aggregation, there is much optimism that the polyQ diseases may soon make the transition from "fatal" to manageable.

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