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*Fifteen years after discovery of **huntingtin**, knowledge of disease pathophysiology is limited, and no effective therapy exists. Recent advances in assay systems have increased the number of potential drug molecules.*

Small molecule drug discovery for Huntington's Disease

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Huntington's Disease (HD) is a rare neurodegenerative disease caused by mutation of the *huntingtin* gene that results in a protein with an expanded stretch of glutamine repeats (polyQ). Knowledge of validated targets is in its infancy, and thus, traditional target-based drug discovery strategies are of limited use. Alternative approaches are needed, and early attempts were aimed at identifying molecules that inhibited the aggregation of polyQ huntingtin fragments. More recently, phenotypic assays were used to find molecules able to reverse some of the pathogenic mechanisms of HD. Such discovery strategies have an impact on the configuration of screening cascades for effective translation of drug candidates toward clinical trials.

Introduction

Huntington's Disease (HD) is a fatal, progressively degenerative brain disorder for which only symptomatic treatments but no efficacious anti-neurodegenerative therapy exists at present [1]. The disease is caused by an expansion of glutamine repeats (polyQ, >35 glutamine residues) at the N-terminal part of a large protein called huntingtin (Htt), which is ubiquitously expressed. Mutant Htt is cleaved by proteolytic enzymes, which results in the release of N-terminal fragments containing the expanded polyQ sequence. These fragments are able to aggregate with themselves and other proteins, and form large nuclear and cytoplasmatic inclusions [2]. There are conflicting reports on whether these large inclusions mediate subsequent cell death [3] or if they are rather cytoprotective, being the result of a mechanism by which the cell protects itself against the production of soluble toxic monomers or oligomers [4].

In any case, the production of these polyQ-containing fragments has several pathophysiological consequences for the affected neurons. They suffer from mitochondrial dysfunction, resulting in reduced ATP levels, decreased Ca^{2+} uptake and oxidative stress [5]. Mitochondrial impairment leads to excitotoxicity, that is, hypersensitivity to excitatory amino acids, in particular glutamate [6]. Several genes from key signaling pathways, such as the ones induced by cAMP and retinoic acid, were found to be downregulated in different rodent HD models [7],

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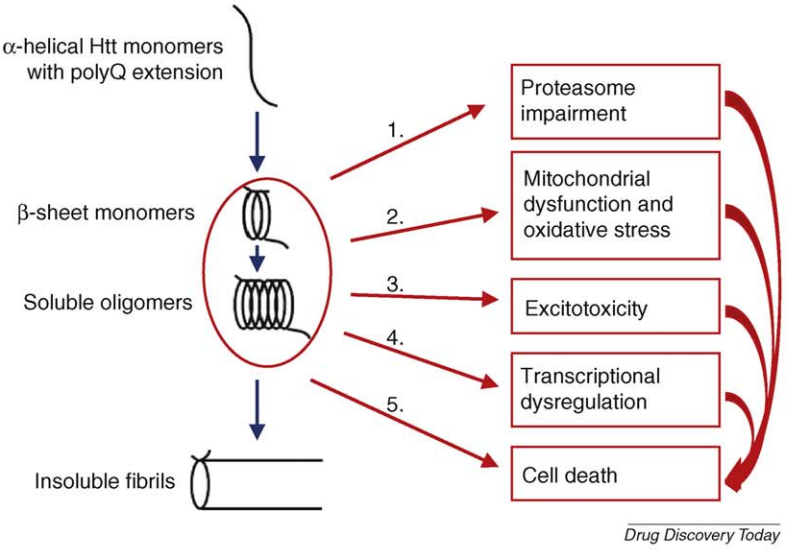

FIGURE 1

Model of pathogenic mechanisms in HD. Huntingtin proteins with >35 glutamine repeats fold into β-sheet structures. This might be facilitated by cleavage of caspases or other proteases. The formation of soluble oligomers and larger insoluble fibrils is aided by transglutaminases that can form intramolecular and intermolecular cross-links. Both monomers and small oligomers are toxic by a variety of mechanisms: 1. The glutamine-containing proteins cannot be cleaved by the proteasome that leads to the accumulation of Htt aggregates. Owing to impaired proteasome function, the level of other misfolded proteins might be enhanced as well. 2. Mutated Htt can interact directly with mitochondria, causing the dysfunction of enzyme complexes of the electron transport chain and decreased calcium buffering. Regulators of important metabolic genes are also repressed; among others are also protective genes against reactive oxygen species. 3. Evidence from animal models and patients supports a role for excessive glutamatergic input in HD pathogenesis but the molecular mechanisms are not completely clear. 4. Beside other proteins, mutated Htt can also bind directly to several transcription factors like p53, Sp1, NF-Y and CBP, sequesters them into aggregates and leads to transcriptional dysfunction and histone hypomethylation. 5. Aggregated Htt can directly initiate pro-apoptotic signaling with activation of caspases and release of Cytochrome *c* by mitochondria, followed by destruction of essential proteins for neuronal survival and ultimately cell death. Either apoptotic or necrotic cell death is a late stage event in the pathology of HD that can also occur as the result of one or more of the events 1–4.

with short N-terminal fragments of Htt showing a much stronger effect than the full-length recombinant protein [8]. The changes in gene expression patterns between these rodent models and human HD post-mortem tissue are comparable [9], underlining the idea that altered transcription is a key mechanism in HD pathogenesis [10]. The polyQ fragments enter the nucleus and mediate transcriptional deregulation by sequestration of transcription factors [11–15] and histone acetyltransferases [16]. Constitutive production of mutated Htt and aggregate formation overcomes the ability of cells to degrade these proteins by the proteasome [17] and autophagy [18] pathways. All these pathogenic mechanisms eventually lead to apoptotic or necrotic cell death [19] (Fig. 1) even if transgenic animal models for HD can show the full symptoms of the disease before any cell death is measurable.

Mutated Htt protein cannot be regarded as a tractable drug target for small molecules itself, mainly owing to a lack of functional activity, unsolved structure, no known and relevant binding sites for small molecules and ubiquitous expression in many cell types. However, alternative approaches using RNAi [20] or intrabodies [21] have shown recent promise in either preventing the production of mutated Htt or the associated toxicity. Companies and academic groups have, therefore, looked for 'downstream' enzymatic targets that might be involved in the pathophysiology of HD. Examples include the use of transglutaminase inhibitors to interfere with aggregation of polyQ fragments [22], the application of creatine [23] and ubiquinone [24] to restore the activity of the mitochondrial electron transport chain, histone deacetyltransferase (HDAC) inhibitors for reversal of transcriptional repression [25–27], and caspase inhibitors to prevent neuronal apoptosis and proteolysis of Htt [28].

In addition, the pathogenic mechanisms themselves (e.g. mutant Htt aggregation and proteolysis, proteasome and autophagy activation, mitochondrial dysfunction and oxidative stress, excitotoxicity, transcriptional deregulation and apoptosis) have been utilized as assay readouts for the identification of novel, potent and HD-specific small molecules for drug discovery. Here we will describe the design and outcome of these phenotypic primary screening assays (Table 1), and also look at some of the innovative technologies and models that are being used to validate hit compounds from these initial screens. Although information on the ultimate fate of the hits is sometimes difficult to obtain, several efficacious compounds in animal models are described and are currently in clinical development [29,30].

In vitro aggregation assays

As aggregation of polyQ fragments is an early consequence of mutated Htt expression, this mechanism has been targeted by many different approaches, often with the aim of identifying inhibitors of the aggregation process. It could be demonstrated that stable amyloid-like aggregates were formed as soon as a glutathione *S*-transferase (GST) tag of an *E. coli* fusion protein, containing 51 glutamines and corresponding to exon 1 of the Htt

TABLE 1

Primary screening campaigns for Huntington's Disease.

Targeted phenotype	PolyQ construct	Assay system	Assay readout	Compounds tested	Hits[a]/hit rate	Secondary assay(s)[b] (hits/tested)	Ref.
Aggregation	HD exon 1 (Q51)-GST	*In vitro*	Filter immunoblot	184,880	~ 300/0.16%	Electron microscopy (2/2), Q51 in 293 Tet-Off cells (2/100)	[34]
Aggregation	HD 1-171 (Q58)-GST	*In vitro*	Filter immunoblot	1040	10/0.96%	Q111 in striatal cells (2/6)	[37]
Aggregation	Ind.HD N-terminal (Q103)-EGFP	Yeast	Growth, EGFP fluorescence	16,000	9/0.06%	Microscopy and PC12-Q103 cells (4/9), COS cells (0/9), bain slices and *Drosophila* (1 analog)	[41]
Aggregation	AR 1-127 (Q65)-CFP/YFP	Transient in HEK cells	CFP/YFP FRET	4000	10/0.25%	HD exon 1 (Q72)-CFP/YFP (10/10), polyQ peptide (2/10), ind. PC12 (Q92) (10/10), *Drosophila* (5/9)	[46]
Aggregation	Q62/Q19-GST	*In vitro*	Phage display	7×20^{10} peptides	350/n.a.	PolyQ turbidity (6/350), CFP/YFP FRET in COS cells (1/1), Cell death (1/1)	[39]
Clearance	–	Yeast	Growth	50,729	12/0.02%	Ind. PC12 (A53T α-synuclein) clearance (4/12), HD exon 1 (Q74)-EGFP (3/4), Autophagy with embryonic fibroblasts, COS and Hela cells (3/3), *Drosophila* (3/3)	[53]
Clearance	Q103-EGFP	Inducible PC12 cells	Protein concentration with EGFP fluorescence	37,200	31/0.08%	α-complementation (1/31), Rescue of Q103-related cell death (1/31)	[55]
Clearance	HD Q72	Neuronal cell line	Protein concentration with TR-FRET	>1 Mill.	~2.000/0.2%	Selectivity/toxicity	(Novartis, unpublished)
Transcript. dysregulation	HD full-length (Q138)	Inducible HEK cells	Luciferase reporter gene	31,000	37/0.12%	Survival of primary striatal neurons transfected with N171-(Q82) (2/2)	(Siena Biotech, unpubl.)
Cell death	AR (Q112)	HEK cells	Caspase-3 activation, fluorescence	1040	15 (no IC_{50}s)/ 1.44%	Rescue of Q112-related cell death by FACS (4/15)	[65]
Cell death	HD exon1 (Q103)-EGFP	PC12 cells	LDH release, absorption	1040	12/1.15%		[66]
Cell death	HD 1-63 (Q148)	Inducible PC12 cells	LDH release, absorption	1040	5/0.48%	Htt aggregation (3/5)	[67]
Cell death	HD 1-548 (Q120)	Temp.-sensitive ST14A striatal cells	Calcein AM cell viability, fluorescence	43,685	29/0.07%	Survival of Q103-related cell death in PC12 (6/29), Survival in yeast (Q72) (0/29), *C. elegans* (2/29), *Drosophila* (2/4), Brain slices (2/4)	[68]

[a] Hits are all compounds with a confirmed concentration response value.
[b] Secondary assays usually also include testing of unspecific compound toxicity.

gene, was removed proteolytically [31]. In this assay the GST tag had the essential role of keeping the recombinant Htt fragments in solution. The aggregating effect was not seen with smaller stretches of only 20 or 30 glutamines, while elongated polyQ sequences of 83 or 122 glutamines in length proved to be too toxic for expression in *E. coli*. On the basis of the behavior of this HDQ51 construct, the authors were able to develop a simple filter retardation assay in which aggregated protein was separated from soluble forms and quantified by dot blot analysis with an Htt-specific antibody. The assay was effective in measuring concentration response values from several, structurally different small molecule aggregation inhibitors, among them Congo red (0.3 μM) and thioflavine S (20 μM) [32]. These *in vitro* data could be confirmed qualitatively both by electron microscopy and in a cellular assay system in which COS cells were first preincubated with the compounds at different concentrations, then transfected with the HDQ51 construct, followed by cell lysis and dot blot quantification of aggregates.

The mode of action of Congo red on aggregation of polyQ fragments was studied in detail *in vitro* and *in vivo* by other groups

[3]. After having established that the compound protected HeLa cells against cell death and caspase activation at 100 μM that had been transfected with a hemagglutinin-Q79 fusion construct, it was also shown that Congo red prevented both Q79-induced depletion of ATP and a general reduction of protein synthesis in this system. The authors went on to develop a cell-free assay with GST-tagged Q19 or Q81 and HeLa cell lysates that also allowed them to rule out any general effect of the compound on chaperone, proteasome and caspase activities. It was then shown in a series of assays that the compound binds directly to aggregated Q79 constructs. In a chemical absorption assay, Congo red showed a significantly higher affinity to GST-Q81 than to GST-Q19 proteins. This was followed by the development of FRET assays both *in vitro* and in transfected HeLa cells using EYFP and ECFP-tagged Q79 constructs that demonstrated that treatment with the compound caused a concentration-dependent inhibition of the FRET ratio between Q79-ECFP and Q79-EYFP, indicating a direct effect on oligomerization, independent of other cellular functions.

The disaccharide trehalose was identified by screening about 200 selected non-toxic compounds in an absorption assay using purified sperm whale myoglobin with 35 glutamine repeats [33]. Although only active at higher concentrations, the activity of the sugar was confirmed in a cellular assay with Neuro2A cells expressing truncated and EGFP-tagged Htt fragments with extended glutamines. Similar to Congo red, trehalose exerts its effect by direct binding to polyQ stretches in Htt. As trehalose does not provoke any toxic side effects, it can be applied *in vivo* at high concentrations. It reduced brain atrophy, improved motor dysfunction and extended the lifespan of R6/2 mice when given orally at 0.2% in drinking water.

High throughput screening for aggregate inhibitors was done when the above-described filter retardation assay was adapted to an automated 384-well format based on a fluorescence ELISA readout [34]. Although consisting of several pipetting, filtering, washing, and incubation steps, including a 16 h incubation at 37 °C to allow for aggregate formation, the throughput of the assay was sufficiently high to screen a library of about 185,000 compounds. Of the 300 hit compounds that inhibited aggregation of the HDQ51 protein in a concentration-dependent manner, a class of 25 benzothiazole derivatives was identified, some of which had previously been shown to be effective in treating other neurodegenerative disorders, such as ALS [35]. Importantly, hit compounds were also tested in a cellular assay and several of the promising compounds had to be discarded owing to unspecific toxicity issues. In this assay, the HDQ51 protein was inducibly expressed in 293/HEK cells under the control of doxycycline. Cells were incubated with compounds under induced conditions for three days, harvested, and aggregates were quantified as before. The compound, PGL135 was shown to be non-toxic and inhibited aggregate formation in cells with a relatively high IC_{50} value of about 40 μM.

In addition to the filter retardation assay, a number of other higher throughput *in vitro* assays were developed for the identification of more potent inhibitors of polyglutamine aggregation. One assay applied chemically synthesized polyQ peptides that were first left to aggregate and then used for coating 96-well ELISA plates [36]. The extension of these aggregates was measured using a TR-FRET readout, through incubation of biotinylated polyQ peptides, followed by Europium-labeled streptavidin. Remarkably, the assay responded similarly with polyQ peptides of both 28 and 40 glutamines. Aggregate binding always followed a two-phase kinetic model with a rapid first phase and a much slower second phase. The assay was found to be suitable for HTS of compound libraries, as it tolerated DMSO up to a concentration of 10% and a pH range between 5.5 and 9, with a sensitivity sufficient for detection of as little as 80 pg synthetic aggregate per well.

A polyQ-GST fusion protein with 58 glutamines was expressed in *E. coli*, purified, and incubated with the NINDS (National Institute of Neurological Disorders and Stroke) compound collection, a small number of FDA-approved and bioactive compounds, at a final concentration of 100 μM [37]. Similar to the gel retardation assay described earlier, the mixtures were filtered 24 h after addition of the protein and quantified by immunoblotting. The 10 best compounds from this initial screen of 1040 compounds had IC_{50} values between 0.7 and 15 μM in this assay. Hits were then validated in a striatal cell model that expresses the full-length wild type or mutant Htt, respectively. These cells are not affected by polyQ-related toxicity. Two compounds were able to reverse the phenotype of the cell line carrying the mutated Htt gene in a concentration-dependent manner, demonstrating that some overlap between results of *in vitro* aggregation assays and perhaps more relevant cellular assays can be expected.

More recently, a high throughput, sensitive polyglutamine aggregation assay was developed that also used an expanded polyQ stretch fused to GST, but in this case aggregate formation was measured fluorescently through the binding of thioflavin-T binding [38]. GST-Q62 and GST-Q19 proteins were expressed in *E. coli*, purified, and the GST tag was removed by proteolytic cleavage with trypsin. Binding of the dye to cleaved Q62 resulted in a fivefold increase in fluorescence emission at 490 nm (excitation at 450 nm) when measured after 24 h. This time period was necessary to ensure that protein aggregation had reached equilibrium. The authors achieved a z' value of 0.56 in a 96-well plate format and demonstrated that IC_{50} values could be detected over three orders of magnitude. Compound autofluorescence was tested by incubating the compounds with the dye in the absence of Q62. The assay was later used for SAR analysis of the peptide inhibitor QBP1 (see below).

While most aggregation assays were aimed at small molecule inhibitors, in one instance phage display was also applied successfully for the identification of a peptide inhibitor [39]. The peptide QBP1 was found by using an 11 amino acid peptide library ligated to the N-terminus of the M13 PIII protein. The library was screened for binding to a polyQ-GST fusion protein with 62 glutamines in the above-mentioned assay. Five out of 350 phage clones were found to bind specifically to expanded polyQ proteins. Peptide binding was then quantified with an *in vitro* turbidity assay using *E. coli*-derived thioredoxin–polyglutamine fusion proteins. In this assay, thioredoxin was chosen to ensure solubility of the polyQ protein and facilitate high expression levels and ease of purification. Again, FRET assays with QBP1-CFP and polyQ-YFP were employed to demonstrate that QBP1 is able to inhibit aggregate formation in COS cells where scrambled control peptides had no effect.

Cellular aggregation assays

Yeast assays

Besides assays that were based on the use of mammalian cells, aggregation of Htt has also been studied in simpler yeast systems. These systems have the advantage of being less vulnerable to polyQ-mediated toxicity, while at the same time being readily amenable to genetic analysis for elucidating the participation of other cellular factors in the aggregation process. In one study for instance, the N-terminal region of Htt with repeats from 25 up to 103 glutamines was fused with GFP and expressed in different yeast mutants, again confirming a strong dependence on the polyQ sequence for intensity and time of Htt aggregation [40]. A yeast strain with increased membrane permeability for compounds was chosen to set up a primary aggregation screen with 16,000 compounds, using a galactose-inducible Htt construct with 103 glutamines tagged to EGFP [41]. Once expressed, this cytotoxic construct caused a reduced yeast growth rate and reduced soluble Htt-103Q expression. Consequently, the assay readout was both an increase in yeast growth by measuring the OD at 600 nm, and an increase in EGFP-tagged protein by measuring fluorescence at 485 nm/520 nm. Nine hits were identified and validated microscopically for an effect on aggregation in yeast cells. The same Htt construct was then applied in PC12 cells to determine the effects of these compounds in a mammalian system; four compounds were found to be non-toxic at 10 μM and inhibited aggregation with IC_{50}s between 2.5 and 10 μM. The same compounds were also effective in a COS-7 cell system with much higher cellular concentrations of an aggregating Htt fragment containing 51 glutamine repeats, although the IC_{50} values were approximately 10-fold higher in this system. Compound structures were chemically modified further until a compound with an IC_{50} of 50 nM was identified (termed $C_{2\text{-}8}$) in the PC12 cell assay. Surprisingly, the activity remained weak in a cell-free filter retardation assay, indicating that the compound did not directly interfere with polyQ aggregation.

Brain slice assays

Attempts were also made to establish assay systems that could very closely mirror the aggregation events seen in mouse models of HD (see below) but would, at the same time, allow a much higher compound throughput [42–44]. Hippocampal slice cultures were prepared from pups of the R6/2 transgenic mouse line and kept in culture for up to three months [45]. Aggregation was quantified with immunohistochemistry using specific anti-Htt antibodies, while cell viability in slices was controlled by staining with propidium iodide. The authors could confirm the anti-aggregating properties of Congo red with an IC_{50} value of 300 nM, a potency similar to the one measured in the *in vitro* filter retardation assay. As the brain slice assay circumvents the hurdle of the blood brain barrier, the predictive value of this assay for the behavior of compounds in animals and patients still needs to be demonstrated. The optimized hit $C_{2\text{-}8}$ from the high throughput yeast screen described above was shown to be highly active in the hippocampal slice culture model after four weeks of incubation at concentrations as low as 10 nM. This compound was also able to delay significantly neurodegeneration in a *Drosophila* model of HD.

Mammalian cell assays

Another cellular FRET assay was developed with an N-terminal fragment of the androgen receptor containing either 25 or 65 glutamine repeats, fused to either CFP or YFP fluorescent proteins [46]. Both non-neural COS-7 and 293/HEK cells and C17-2 neural precursor cells were transfected with these constructs. Photobleaching of the YFP acceptor molecule increased the FRET emission signal of the donor molecule in cells with cytoplasmic or nuclear inclusions, while no FRET was detected in diffusely distributed cells expressing only unexpanded protein. The authors chose 293/HEK cells for development of the HTS assay owing to their high transfection rates and protein expression levels, and their ability to achieve high degrees of nuclear aggregation. Fluorescence was quantified with measurements at three excitation/emission windows for CFP (435 nm/485 nm), YFP (485 nm/530 nm) and FRET (435 nm/530 nm). The assay was then configured for 96-well plate format, which achieved a z' value of 0.67 [46]. To correct unspecific compound effects as a result of decreased protein expression or increased protein turnover, the researchers created a stable 293/HEK cell line expressing a red fluorescent protein. The ROCK kinase inhibitor Y-27632 was active in a small validation screen using this assay, with a specific EC_{50} of 5 μM on polyglutamine aggregation. There are two distinct advantages of this cellular aggregation assay versus any *in vitro* assay; first, a FRET-based assay produces a signal even if aggregates consist of only oligomeric structures that would otherwise escape filter or microscopy-based detection methods. Second, the assay also detects molecules, such as Y-27632, which indirectly target polyglutamine proteins by modulating regulatory pathways on protein misfolding and aggregation [47].

The FRET assay with an androgen-polyQ substrate was then used to screen a total of 4140 compounds [48], including the FDA library of 1040 compounds that were described earlier [37]. Toxic compounds were readily identified during the screen on the basis of loss of absolute CFP and YFP signal. A total of 10 compounds were classified as primary hits owing to their reproducible and concentration-dependent inhibition of the FRET signal, with IC_{50}s between 2 nM and 20 μM. All compounds were also active with Htt-polyQ FRET substrates, but no compound showed more than a 30% reduction of aggregation. All hits were then tested in a secondary aggregation assay with an inducible PC12 cell model expressing the GFP-tagged Htt exon 1 with 92 glutamines. The compounds were incubated for 48 h at a concentration of 10 μM under induced conditions in which intracellular inclusions are readily formed. Quantitative Western blotting with anti-GFP antibodies showed that most hit compounds were effective in reducing the amount of aggregated versus soluble forms of Htt exon 1-GFP in a manner that was comparable to the positive control compound, Y-27632. Five out of nine hits were also shown to be effective in a *Drosophila* model of HD, indicating a high predictive value for this cellular aggregation assay. It is worth pointing out at this point that, if results with different aggregation assays were compared, different hits from the same small compound library of 1040 compounds were identified [37,48]. It is likely that several factors are responsible for this discrepancy. Depending on their physicochemical properties such as aqueous solubility, chemical or metabolic stability, and cell membrane permeability, some compounds will show quite different effects in cell culture

systems, as compared with *in vitro* biochemical assays. Compounds might have interfered non-specifically with the fluorescence signal from the labeled androgen polyQ fragment, acting therefore as 'false positives'. Crucial variances in buffer composition, incubation times, size, or structure of the polyQ fragments in the assays could also have contributed to different results with these compounds. It will be informative to compare the results of these hit compounds again, once they have been tested and further validated in various animal models.

Toxicity of Htt aggregates

An important publication appeared in 2004 questioning the rationale behind the search for molecules that could interfere with inclusion body formation [4]. Striatal neurons were transiently transfected with an expanded exon1 Htt fragment labeled with GFP; inclusion body formation, the presence of diffuse Htt, and cell survival was followed in the same neuron over a period of days using an automated microscopic system. Cells that failed to form inclusion bodies had an increased risk of death, indicating that aggregates are not required for polyQ-induced neuronal death. Although visible aggregates seemed to be protective, rather than pathogenic, the study could not rule out that the major toxic species were, in fact, the early precursors or small soluble oligomers of polyQ proteins. These conclusions were to a great degree confirmed in a recent study with an inducible Q74-EGFP construct in PC12 cells [49]. Here, data on inclusion body formation were collected every 10 min for up to 48 h, allowing deep insights into the underlying dynamic processes. For instance, it was shown that although initial aggregate formation takes place within 1 h, it required an incubation time for up to two days to achieve the full phenotype. The authors also demonstrated that, at least for a certain period, this process was also cytoprotective.

Quantification of Htt aggregates

One area in which rapid advances are being made is the development of automated high throughput image analysis algorithms for quantification of Htt aggregates in cells. Using a well-established stable PC12 cell line with an inducible Htt fragment [41] and an automated fluorescence microscope, conditions for the induction of aggregate formation in these cells were optimized and several analysis methods were developed and compared [50]. Depending on the individual method, automated counting of aggregates took between 0.3 and 10 s per image, including cell death analysis, while manual counting of comparable accuracy required, on average, 56 s per image. A comparably simple, but low throughput, biochemical detection method for Htt aggregates of variable size was based on agarose gel electrophoresis [51]. This method was sufficiently sensitive to detect very small aggregates in a striatal neuronal model even several days before the onset of neurodegeneration.

Despite advances in the design of aggregation assays and recent success stories with respect to compounds whose activities could be confirmed in relevant *in vivo* models of HD, the physicochemical properties of the hit molecules should be assessed carefully before embarking on costly and time-consuming studies. A recent report pointed out that many aggregation inhibitors resembled molecules that were known to form promiscuous chemical aggregates themselves [52]. These, often highly conjugated, hydrophobic and dye-like molecules, form small colloidal particles and sequester other proteins non-specifically. Prion proteins were used as a model aggregation system, which was monitored with the thioflavine-T assay. Among other molecules, the well-known inhibitors of Htt polymerization, Congo red and Direct Yellow 20, were clearly shown to sequester prion proteins by non-specific mechanisms, thereby preventing them from aggregating. Addition of 0.01% Triton X-100, or large amounts of a secondary protein like BSA, inhibited the effects of these compounds, which is a hallmark of chemical aggregate formation. The authors could also demonstrate that the activity of the aggregation inhibitors in cell culture was also based on these non-specific effects, presumably because they remained stable and retained the ability to sequester proteins in a biological environment. Luckily, these non-specific mechanisms of inhibition are easy to detect so that false positive compounds can be distinguished from specific inhibitors early on in the screening cascade.

Htt clearance assays

An alternative approach to the inhibition of Htt aggregation is the search for compounds that are able to increase the cellular degradation of soluble or aggregated forms of the mutated protein. Using a Q74 extended and EGFP-tagged exon 1 fragment of the HD gene in both transiently transfected COS cells, and with a stable inducible, PC12 cell line, researchers found accumulation of the polyQ-containing fragments when cells were treated with different inhibitors of the autophagy–lysosome pathway [18]. Until recently, the immunosuppressant, mTOR kinase inhibitor, rapamycin, was the only compound known to upregulate this important pathway. Owing to its effects on several other growth-relevant pathways, however, this compound is not considered to be suitable for long-term treatment. In an attempt to identify novel autophagy-modulating molecules, a high throughput screen of nearly 51,000 compounds was performed in yeast cells that were also growth-arrested with rapamycin [53]. Four compounds were able to increase the effects of 20 nM rapamycin (with EC_{50}s in the low μM range) and were termed SMERS (small molecule enhancers of rapamycin); however, several other compounds could suppress the cytostatic effects. Three SMERS from the primary screen were then validated in a series of experiments with the EGFP-tagged Q74 exon 1 fragment in COS cells and shown to be independent from the pathway regulated by mTOR. Finally, these compounds were able to reverse the toxic effects of expressed N-terminal polyQ fragments of Htt in a fly model of HD.

An HTS assay for compounds that promote clearance of extended polyQ-containing proteins was also established in mammalian cells. The N-terminal 17 amino acids of the Htt gene were fused to 103 glutamines and EGFP and were placed under inducible control of an ecdysone control element in PC12 cells [54]. Expression of the Q103-EGFP construct in both proliferating and differentiating cells led to visible aggregate formation in up to 80% of the cells without a reduction in cell viability. The assay was validated with the transglutaminase inhibitor cystamine and optimized by varying both the inducer concentration and incubation time [55]. A crucial consideration was the rapid degradation of the Q103-EGFP fusion proteins within 24 h following removal of inducer, which might obscure the effects of compounds on clearance. Therefore, compounds had to be added at the same time as the inducer so that protein synthesis and degradation were at

equilibrium during most of the incubation period. Furthermore, in order to identify weak compounds, the lowest possible concentration of inducer was chosen, resulting in an assay with a z' factor of 0.45. Compounds (37,000) were screened in triplicate at 5 μM on cells treated for 72 h. This screen identified 114 primary active compounds that could reduce EGFP fluorescence by at least 35% that passed a preliminary toxicity screen and that exhibited dose-dependant efficacy in a three-point assay. On the basis of a visual assessment of live cells and more rigid assays for non-specific toxicity, 31 compounds were prioritized, among which were the structurally related compounds A28 and A31. These compounds were then tested in an innovative secondary assay designed to establish selectivity against extended polyQ proteins. Both mutated Q97 and wild type Q23 Htt fragments were fused to the α-subunit of β-galactosidase, and complementation of the enzyme lacking the α-subunit was then measured in PC12 cells expressing these constructs, by means of the catalytic activity of the enzyme. Remarkably, compound A31 only decreased enzymatic activity, hence increased clearance, for the mutated Htt fragment, not for the wild-type form. It was then also shown that, at least at high concentrations, the decrease in mutant Htt caused by compound A31 also increased cell viability.

Assays measuring transcriptional dysregulation

Apart from protein aggregation and impaired clearance mechanisms, one other important factor in the pathology of HD is an overall change in gene transcription. For instance, it could be shown that mutated Htt sequesters the cAMP response element-binding protein (CREB) co-activator, CREB-binding protein (CBP) through direct polyglutamine interactions, which then leads to decreased CREB-mediated transcription [14]. Reporter gene assays in PC12 cells transfected with inducible polyQ exon 1 coupled to cAMP-responsive element (CRE) possessing a luciferase reporter, confirmed that mutated Htt is responsible for changes in the observed CRE expression profiles [15]. The authors of these studies further found that toxicity in their cellular models could, at least partially, be reversed by overexpression of CBP, or by treating cells with either cAMP or forskolin. Similar interactions of Htt with transcription factors were also demonstrated for SP1, p53 ([11–13,56], and, more recently, also for NF-Y [57] or with other proteins known to be involved in the transcriptional machinery, for instance the RNA-binding protein TLS [58]. It is important to note, however, that reduction of DNA binding or cellular amounts of SP1, p53 or CREB in transgenic models of HD was found to be neuroprotective ([56,59–61]. These apparently conflicting results might be explained by a different, as yet unknown mode of action of mutated Htt on transcription. For instance, there is evidence that Htt directly binds to DNA, using transcription factors only as transporters to penetrate the nucleus [62,63].

On the basis of these findings, we developed an HTS assay to identify compounds that could reverse changes in CRE-mediated transcription [29]. We chose to design an assay that recapitulated a more disease-relevant phenotype by utilizing the full-length mutated Htt gene, rather than fast aggregating, but artificial, Htt fragments, in a recombinant 293/T-REx™ cell model. A stable cell line was generated with both a CRE luciferase reporter gene and with the full-length mutant Htt gene under control of an inducible CMV promoter. Addition of doxycycline induces expression of the Htt protein, followed by formation of proteolytic polyQ fragments, sequestration of CBP and concomitant reduction of reporter gene activity (Fig. 2). Cell death occurs after 72–96 h of Htt induction. To our knowledge, this is the only available assay system in which expression of full-length mutant Htt leads to cell death. In any case, this cell system represents a unique tool, as it allows two different assays to be performed in one system: transcriptional dysregulation and cell death. Three different reference compounds known to inhibit aggregation (Y-27632, PGL135 and Congo red, described above) were used to validate the assay. All compounds reversed the mutant Htt-induced decrease of reporter gene activity in a specific manner, that is, these compounds did not have an effect on non-induced cells. Subsequently, the assay was used in an HTS format to screen a diversity library of more than 31,000 small organic molecules that resulted in the identification of several hit series with submicromolar potency. The compounds of one series were selective for the mutated form of Htt and reversed reporter gene activity back to a level of between 40 and 60% of the un-induced state. Some compounds are currently being investigated in a rat primary striatal neuron model [64] in which cells are infected with a lentivirus construct carrying the N-terminal fragment of the Htt gene with either 19 or 82 polyglutamines and cell survival is followed over eight weeks.

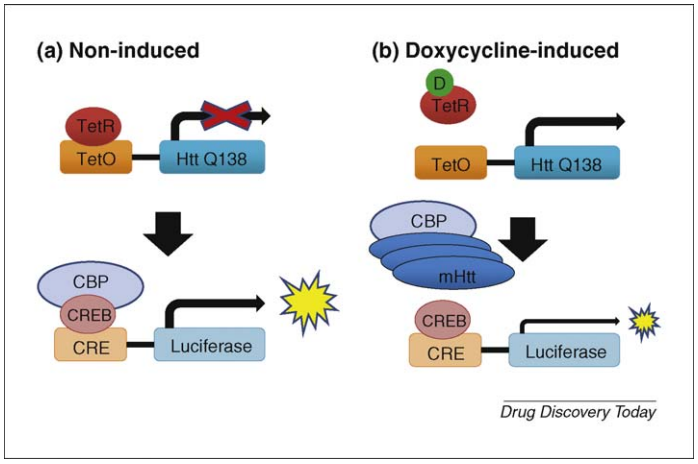

FIGURE 2

Principle of CRE luciferase screening assay. T-Rex 293 cells contain the full-length mutated Htt protein (mHtt) with 138 glutamine repeats under control of the inducible Tet operon and a luciferase reporter gene under control of the cAMP responsive element (CRE) binding site. The CRE signaling pathway can be induced by addition of forskolin that activates the cAMP-generating enzyme adenylyl cyclase (not shown). The transcription factor CRE binding protein (CREB) then binds to the CRE and to the essential co-factor and histone acetyltransferase CREB binding protein (CBP) that leads to transcription of the luciferase reporter gene. **(A)** No mHtt is produced without doxycycline. High amounts of luciferase produce a strong luminescence signal. **(B)** The addition of doxycycline induces expression of mHtt that binds and sequesters CBP. This leads to impairment of CRE-mediated transcription, and only low amounts of luciferase are being produced. Compounds are classified as hits when they interfere with mechanisms at, or upstream of, the interaction between mHtt and CBP, causing again a stronger luminescence signal.

Cell death assays

As polyQ expression ultimately leads to cell death, this downstream consequence is also being used as an assay readout for the

identification of neuroprotective compounds. Caspase-3 activation as a mediator of apoptotic cell death was measured in one screen assessing the effects of a truncated version of the androgen receptor with a 112 glutamine stretch [65], a model system that could be used for other polyQ diseases. The construct was transiently transfected in 293/HEK cells, which resulted in accumulation of aggregates and cell death within three days. Caspase activity was then measured in cell lysates using a fluorogenic substrate. The assay was adapted to a 48-well format to screen (at 10 μM) the NINDS collection of 1040 drugs. The z' factors of such screens ranged between 0.3 and 0.7. Fifteen compounds were identified that consistently inhibited caspase-3 by more than two standard deviations or 71%, compared with the standard deviation obtained with DMSO as the negative control. Secondary cell viability assays revealed that only four compounds were not inherently toxic to cells; three of these were structurally related cardiac glycosides. Interestingly, the four compounds could not directly inhibit caspase-3 but prevented activation of the enzyme upstream in the death pathway. Understanding the mechanism of action of these compounds in HD could reveal new targets for screening.

The same compound library was screened in another cell death assay, this time using the inducible PC12 model described previously, with a construct containing 103 glutamines [54]. Upon induction, these cells underwent rapid cell death as estimated by trypan blue exclusion or ethidium bromide staining, by quantifying lactate dehydrogenase (LDH) release or measuring mitochondrial activity with an MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) assay. Although consistent results were achieved with all the four cell viability assays, the authors chose the LDH release assay for their medium throughput screen [66]. Cell death was measured 48 h after addition of compounds and induction of Htt expression. A throughput of 320 compounds per week was achieved, with a good z' value of 0.51. A total of 12 compounds were identified that protected the cells by more than 75% and were also non-toxic, among them a caspase inhibitor and four cannabinoids. It is noteworthy that none of the hit compounds had any effect on the number, size or location of the intracellular aggregates, again strengthening the hypothesis that soluble intermediates might be responsible for toxicity.

A similar screen was performed with a slightly different inducible Htt construct, this time containing 148 glutamines, but again in PC12 cells and with the same library of 1040 compounds [67]. In this case, cells were also differentiated with NGF during a four-day incubation period with test compounds. LDH release was selected as the readout for cell death, and primary actives were further screened by testing for non-specific cellular toxicity and effects on Htt expression levels. Five hit compounds were identified by this protocol; three of these could suppress Htt aggregation. Remarkably, there was no overlap of hits from all three screens, even though the same library was tested and cell death was measured in all cases. The only exception was the caspase inhibitor, which was detected in all three assays. This may indicate the importance of the selected cellular models and polyQ constructs for the design of the assay.

A higher throughput cell death assay was developed on the basis of immortalized striatal neurons (termed ST14A) expressing the N-terminal 548 amino acid fragment of Htt with 120 glutamine repeats [68]. This cell clone undergoes cell death at a higher rate than the parental ST14A cells when cultivated in serum-deficient medium at 39 °C, caused by degradation of a temperature-sensitive T antigen [69], which permits the identification of compounds that could selectively protect cells against Htt-induced toxicity. Nearly 44,000 compounds were screened (using a 384-well format) and 29 primary hits were identified. The compounds were prioritized by subjecting them to additional filters, such as decreases in Htt protein expression and rescue of cell death, in striatal cell lines expressing different Htt constructs. Importantly, they were also tested in an inducible PC12 model, in yeast, *C. elegans* and *Drosophila* HD models. Four compounds emerged with activities in multiple cell death models and two of these were also active in a brain-slice model of HD. The NINDS collection of 1040 compounds was also tested in this cascade of assays, and some of the hits were recognized as known metabolic inhibitors. Although the exact mode of action of these compounds is not yet clear, it is a good example that a phenotypic screen can also help in clarifying the molecular pathology of a complex neurodegenerative disease.

Even more pathophysiologically relevant cell systems are also in use, utilizing neurons prepared from the striata of rats, a brain region most affected by HD. Electroporation, ballistic [70] or viral infection of such striatal neurons – prepared from rat embryo brains – with polyQ fragments harboring up to 480 amino acids of the mutated Htt, followed by measurement of cell survival, have been described for the identification and characterization of compounds [71]. Owing to the use of primary neurons, however, these assays are difficult to implement for high throughput screening. Nevertheless, primary striatal neurons can be produced efficiently in amounts sufficient for screening several thousand compounds, using imaging cytometers that are adapted to high throughput fluorescence measurements of neuronal survival (R. Pruss, personal communication).

The overall advantage of a cell death assay is that it is independent from any preconceptions related to the molecular mechanisms that cause toxicity in HD. In an ideal scenario, drug screening for HD would be carried out in a model system that correctly represented all essential elements of the disease. In the absence, however, of generally accepted cell or animal models, and in the absence of any drug that works well in the clinical setting, the best strategy seems to be to use several different model systems in parallel, and then focus on those compounds that work well in several assays. The ultimate judgment on the predictive ability of screening assays to identify drugs of therapeutic value will only be possible once drugs that are effective in decreasing the neurodegeneration associated with HD are available.

Non-rodent *in vivo* model systems of HD

Many genetic models of the disease have been generated in model organisms, such as the nematode worm *Caenorhabditis elegans* (*C. elegans*) [72], the fruit fly *Drosophila melanogaster* [73,74] and the zebrafish (*Danio rerio*) [75].

C. elegans is an organism commonly used in developmental biology. When expanded polyQ is expressed in sensory neurons, signs of neuropathology and neuronal dysfunction occur. Among

other effects, time-dependent protein inclusions at random locations in the cytoplasm can be seen in these cells while the worms demonstrate severe deficits in the nose touch response test. Moreover, as in human pathology, the age of onset and severity increase with polyQ length, and the toxicity is accompanied by the appearance of aggregates. Several promising compounds have been tested in this model and were compared with activities in other HD models [76].

Different *Drosophila* models based on ocular expression of polyQ proteins have also been described. The fly eye is made up of hundreds of photoreceptor neurons organized in rhabdomers, which makes it possible to identify any mutation that induces a perturbation of this organization such as is the case when polyQ repeat proteins are expressed. Thus, intact rhabdomer number is a readout often used in this model organism. *Drosophila* models have been used to study HDAC inhibitors as potential drug candidates [25,77] Besides several other compounds (see also in Table 1), the peptide inhibitor QBP1 was tested and found to be active in this model [78].

Zebrafish have been engineered to produce a simple vertebrate model of HD [79]. Injection of plasmids encoding normal and expanded polyQ tracts into single-cell embryos induces clear differences in morphology and survival of embryos. Embryos with 35 or fewer polyQ repeats are normal, whereas 56 and 80 polyQ repeats induce morphological abnormalities, developmental delays and patches of brownish opaque tissue indicative of cell death. This model also displays the formation of mutant Htt inclusions, and it is possible to directly screen compounds in this system. A model with an EGFP-tagged 71Q exon 1 construct was used to test novel inducers of autophagy. Zebrafish treated with these molecules had significantly fewer aggregates and substantially enhanced rhodopsin expression than the untreated fish [80].

Although some of the non-rodent *in vivo* models can potentially be used for direct screening of chemical libraries, owing to cost and throughput limitations they are more typically being used to confirm active molecules that were identified in high throughput screens.

Rodent *in vivo* model systems of HD

Evaluating active compounds in rodent models of the disease is usually the last part of a screening cascade for proof-of-concept studies before testing compounds in clinical trials. As opposed to other neurodegenerative diseases, many animal models exist (both pharmacologically and genetically induced) that reproduce at least some of the characteristics of HD. However, despite the availability of several alternatives, there are as yet no data demonstrating correlations between effects in a particular disease model and efficacy in human HD patients. Therefore, we do not yet know which (if any) of the available models will be predictive of outcomes in the human disease.

Pharmacological models

Pharmacologically induced rodent models represent the first vertebrate models established for the study of HD. These models employ chemicals to induce excitotoxicity mimicking an important mechanism leading to neuronal cell death. The first such model is the quinolinic acid (QA) model [81]. Administration of this endogenous metabolite of tryptophan, an agonist of the *N*-methyl-D-aspartate (NMDA) receptor, mimics some characteristics of HD [81,82] including the induction of selective death of medium spiny striatal neurons and often choreic-like movements also. A second widely used model is the 3NP model in which cell death is induced by chronic administration of 3-nitropropionic acid (3NP), an irreversible inhibitor of the mitochondrial respiratory complex II enzyme succinate dehydrogenase [83,84]. 3NP induces transient hyperactivity, general coordination deficits, bilateral dystonia, gait abnormalities, and perseverative abnormal behavior. Despite an increased popularity of genetic disease models, pharmacological models can still represent a useful tool, in particular to investigate those phenotypic or neuropathological features that are not present in some of the genetic models of the disease such as striatal cell loss. However, it is important to keep in mind that the QA and 3NP pharmacologically induced rodent models have essentially no construct validity and limited face validity for HD. Non-striatal regions such as cerebral cortex for instance are clearly affected in HD, and the QA and 3NP injections into wild-type rodents fail to model this.

Transgenic models with N-terminal Htt fragments

A large panel of transgenic and knock-in mouse models of HD exists [85]. Transgenic mice have been generated by random insertion of the mutant Htt gene, either full-length or part of it, into the mouse genome, which leads to the simultaneous expression of the mutant protein and the endogenous wild-type protein. On the contrary, knock-in models carry the mutation in its appropriate murine genomic context under control of the endogenous Htt promoter and thus represent the most precise genetic model of HD.

The most commonly and extensively used transgenic animal model is represented by R6/2 mice that have been generated by random genome insertion of exon 1 of the mutated human Htt gene harboring a trinucleotide expansion of about 150 CAG repeats (corresponding to about 150Q) [86]. One has to bear in mind, however, that owing to the genetic instability of this fragment, most R6/2 colonies now vary significantly from the original repeat length. These mice show a ubiquitous transgene expression that is associated with a robust and reproducible progressive phenotype. Often the animals begin to show motor impairments already after week 4 while an overt behavioral phenotype with weight loss, ataxia, resting tremors, stereotypic movements, and epileptic seizures is usually observed after week 8. Death typically occurs between 13 and 16 weeks of age. However, for the R6/2 mice, as for all other transgenic or knock-in rodent models, one must keep note of the fact that the onset and progression of the disease depends not only on the genetic background but also on housing conditions, both of which can vary significantly from one laboratory to another. These factors are also important considerations for drug screening in animal models [87–91].

At the neuropathological level, R6/2 mice exhibit brain atrophy and cytoplasmic and nuclear polyQ aggregates. Striatal cell death is only detected during the final stages of disease, which is a disadvantage. Another disadvantage is the very severe phenotype as mice show signs of sickness already at birth. Thus, studies of drug intervention in these animals might not adequately reflect their effects in patients who usually develop the HD phenotype over a period of many years. In addition, important potential

intervention points for drug treatment might be lacking as these mice express the mutated exon 1 rather than the full-length human protein.

Another N-terminal fragment-based model is represented by the N-171-82Q mice [92]. These animals carry a longer N-terminal sequence (exons 1 and 2) with 82 polyQ repeats and the earliest abnormal behavior is observed at 12 weeks of age. Nuclear aggregates are detected at three to four months of age. At the neuropathological level, the observed lesions resemble more closely the human pathology with intranuclear aggregates found more prominently in cortex and hippocampus, rather than in striatum, and energy defects and neuronal loss are more selectively found in striatum and defined hypothalamic areas. These mice, however, exhibit a less well-defined and more variable phenotype, including weight loss, tremors, impaired coordination, bradkinesia, abnormal gait, hind limb clasping, and shorter lifespan. Another potential disadvantage is the fact that expression of the Htt fragment is driven by the mouse prion protein promoter, and not the Htt promoter that is included in most of the other models.

A further interesting rodent model is a transgenic rat model carrying 51CAG repeats and the first 582 amino acids of the human HD gene [93]. These animals show weight loss, reduced lifespan, and declined cognitive and locomotor performances. Although more prominent in projections of basal ganglia, aggregates are widely distributed and striatal atrophy is evident starting from 48 weeks of age. This rat model represents an attractive alternative, as all the major proteolytic cleavage sites of the mutated Htt protein are present in this transgene.

Transgenic full-length Htt models

YAC mice carry a yeast artificial chromosome (YAC) with the entire mutated human huntingtin gene sequence [94]. In the YAC128 mice, nuclear localization of mutant Htt appears earlier coinciding with the onset of behavioral abnormalities, whereas selective neuronal degeneration proceeds in a similar pattern, although less dramatic than human HD. YAC128 mice manifest motor hyperactivity followed by hypoactivity, impaired coordination, intranuclear aggregates, severe corticostriatal synaptic function abnormalities, and enhanced NMDA-sensitivity. These mice also demonstrate cognitive impairment in a T-maze test that appears before onset of motor abnormalities and neuropathology. As in the case of N171-Q82, and opposed to R6/2 mice, the phenotype of the YAC mice is less severe and more variable, rendering the use of larger cohorts of animals necessary in order to achieve statistical significance of measurements. As a model system based on the full-length sequence of the mutated Htt protein, it might, however, be the model-of-choice to confirm and characterize active molecules identified in screening assays that employed the full-length Htt sequence.

BACHD mice represent the most recent rodent model. The mice carry a bacterial artificial chromosome (BAC)-expressing the full-length human mutant huntingtin gene, with 97 glutamine repeats, under the control of the endogenous huntingtin regulatory sequences. The model exhibits progressive motor deficits in the rotarod paradigm starting from two months of age, a late-onset neuropathology, including selective brain atrophy from 12 months of age and the presence of characteristic striatal degenerating neurons without any difference in the number of total striatal neurons. Moreover, unlike other genetic models, BACHD mice do not exhibit early and diffuse nuclear accumulation of mutated protein [95].

Htt knock-in models

Originally, knock-in models failed to reproduce an overt phenotype similar to that of HD patients or transgenic mice although these models exhibit nuclear microaggregates and aggregates. Diverse knock-in models have been engineered that harbor between 94 and 140 CAG repeats [96,97] at the 5′-end of the murine HD gene. These mice show only mild functional and histological changes in neurons, with no overt neuronal cell loss and gliosis, and no lifespan reduction. Another model, harboring 150 glutamines, might be a more promising knock-in model, as it reproduces some of the pathological characteristics of human HD [98]. These mice show a phenotype dependent on mutant allele dosage with homozygote animals displaying an earlier onset of symptoms including moderate weight loss, seizure susceptibility, reduced exploratory activity, clasping, rotarod impairment and abnormal gait. At the neuropathological level, mice exhibit brain atrophy, gliosis and intranuclear aggregates. After about 100 weeks of life, a 40% reduction in striatal volume is observed and 50% of neuronal cell loss.

Primate models

The close physiological, neurological, and genetic similarities between humans and primates would make a monkey model very useful for better understanding of human HD. Recently, the important development of a non-human primate model with rhesus macaques was reported [99]. The model expresses the exon1 of the human Htt gene with 84 glutamine repeats. The severity of observed phenotypes depends on the expression level of mutant protein in the different monkey clones; only one Macaques clone survived for more than one month. The clones showed a variable extent of motor dysfunction, difficulty in movement coordination and swallowing, dystonia and chorea, and behavioral and cognitive impairment. Mutant huntingtin is widely expressed and forms aggregates and inclusions in striatum and cerebral cortex whereas no neuronal degeneration in the striatum is observed.

Conclusions

Since it is too early to define the predictive value of the described phenotypic or mechanism-based assays – measured in terms of identified compounds with real disease-relevance – it is important to characterize active compounds in a broad panel of secondary assays in order to provide further evidence for efficacy before investing in long and expensive *in vivo* studies with vertebrate animal models. In addition, as the molecular targets in such screening assays are not known, target deconvolution activities might be necessary [100]. Thus, the overall screening cascade is significantly more complex than with traditional target-based screening projects. To date, several potential drug targets seem to be involved in HD, but no single target is sufficiently validated yet. Mutant Htt expression is accompanied with dysregulation of diverse cellular mechanisms, which makes drug discovery difficult owing to the uncertainty as to which might be the most important disease mechanism to target. Genome-wide RNAi screens [101] with cellular models of HD are being performed in academia and

industry that will, without doubt, increase even further the number of candidate targets. Only some of them will be linked to HD by means of a direct physical interaction with the Htt protein, and only a fraction of these potential drug targets might be causally involved in the disease. It does not improve matters that mutated Htt binds to some proteins seemingly indiscriminately (as discussed above for the association with transcription factors). Targets acting further 'downstream' from mutated Htt could also have relevance for other neurodegenerative diseases. Some candidates might be only useful as biomarkers while the majority will possibly have no significance at all.

Another issue is the poor predictive ability of one assay over others as each of them mimics only one or just a restricted number of pathological aspects. Therefore, each single lead molecule has to be tested in a battery of secondary assays and preferably in diverse animal models. This necessity can be seen, however, as a unique chance to guide investigators in the first steps in understanding the mode of action of the candidate drug that might lead to the identification of new drug targets. Such targets will initiate new research efforts for novel small molecules for which rational drug design can be employed. Even if there are still many obstacles to overcome, recent examples have shown that creativity in assay design and collaboration for access to highly disease-relevant secondary assays and animal models can identify promising drug leads for this devastating disease.

Disclosure statement

The authors declare no competing financial interests.

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