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High Throughput Screening for Neurodegeneration and Complex Disease Phenotypes

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Abstract

High throughput screening (HTS) for complex diseases is challenging. This stems from the fact that complex phenotypes are difficult to adapt to rapid, high throughput assays. We describe the recent development of high throughput and high-content screens (HCS) for neurodegenerative diseases, with a focus on inherited neurodegenerative disorders, such as Huntington's disease. We describe, among others, HTS assays based on protein aggregation, neuronal death, caspase activation and mutant protein clearance. Furthermore, we describe high-content screens that are being used to prioritize hits identified in such HTS assays. These assays and screening approaches should accelerate drug discovery for neurodegenerative disorders and guide the development of screening approaches for other complex disease phenotypes.

Keywords

High throughput; screening; Huntington's disease; drugs; neurodegeneration; high-content; aggregation; cell death

INTRODUCTION

For decades, drug-discovery involved careful testing of a moderate number of compounds in animals and observing effects on phenotypes. These tests were costly, time-consuming and slow. Advances in synthetic, combinatorial and polymer chemistry now allow for the synthesis of tens of thousands of compounds in a short period of time; advances in liquid handling, automation and molecular and cell biology enable rapid testing in miniaturized assays. Technologies developed in the past several decades have altered the paradigm for drug discovery, by enabling high throughput screening (HTS). HTS allows rapid and parallel testing of thousands of compounds in a short time. This has resulted in the development of systematic serendipity, in which the testing of large enough numbers of compounds results in the fairly reliable discovery of active compounds, the precise nature of which cannot be anticipated ahead of time.

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However, limitations in the assays that are amenable to high throughput screening reduce the generality of this new approach. Relatively simple assays that involve purified proteins and have simple readouts are optimal for high throughput testing. However, in most human diseases, phenotypes are complex and the relevant molecular mechanisms to target for therapy are not apparent. For example, psychiatric and behavioral disorders are beyond the reach of HTS, since they necessitate observing whole organisms for effects of drugs on behavior [1]. However, efforts are underway to overcome these problems, in an attempt to capture even complex phenotypes in HTS and high-content screening (HCS) assays. Here, we discuss some successful attempts, largely focused on neurodegenerative diseases, with a few examples based on other cellular and animal-based screens (Fig. 1).

TYPES OF HTS AND HCS ASSAYS

HTS and HCS assays can be organism-based, but are generally *in vitro* or cell-based. *In vitro* assays test interactions or activity of compounds against a purified protein, RNA or DNA, or a simple mixture of a few molecules. Compounds are added directly to this mix and activity or binding is measured using fluorescence, luminescence or absorbance readouts. These assays have the advantage that any hits identified have a known target. One inherent limitation is that a target must exist before devising such an assay. However, even in cases with a known and validated target, the detailed molecular and biophysical mechanism of any identified compounds needs to be determined and confirmed *in vitro* and in the cellular *milieu*. Furthermore, cellular permeability, toxicity, target selectivity and metabolic inactivation are additional barriers to success with compounds identified *in vitro*.

In contrast, cell-based and animal-based assays can identify efficacious compounds using phenotypic endpoints within the complex environment of cell and tissues. However, these assays suffer from a lack of information about the molecular/protein target of the compound identified and its mechanism of action. Furthermore, phenotypic assays are more costly, are difficult to miniaturize and have lower throughput, largely due to the complexity of such assays.

ASSAY DEVELOPMENT IN NEURODEGENERATIVE DISEASES

Some of the more challenging complex phenotypic assays to develop are in the field of neurodegeneration. Drug discovery for the common neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, is daunting [2,3]. Not only are these diseases age-dependent, with prolonged time courses, their etiology is complex and poorly defined. Even animal models recapitulate only limited aspects of each disease; it is unclear how well they recapitulate the human diseases, which involve complex and poorly defined factors [4,5]. In contrast, Huntington's disease (HD) is one of the most well-defined neurodegenerative diseases with a clearly defined genetic basis.

HD results from a mutation that causes an expanded polyglutamine tract in the N-terminal region of the huntingtin (htt) protein [6]. Polyglutamine expansions above a threshold of 36 glutamines in htt result in a progressive neurodegeneration that is eventually fatal. Importantly from a drug-discovery perspective, the HD phenotype is observed to a certain extent in cell-culture and more so in genetic animal models [7,8]; these models provide the opportunity for the discovery of HD therapeutics [9]. Although HD is less common (~30,000 affected persons in US) compared to other more prevalent neurodegenerative disorders, the therapies for HD may well be applicable and instructive for other neurodegenerative diseases.

AGGREGATION BASED ASSAYS

In Vitro Aggregation Assays

Initial observations of HD patients' brain tissue revealed cellular aggregates [10]. Though the role of aggregates in HD pathology is now debated [11], the correlation of aggregates with HD phenotypes led to the hypothesis that aggregates are toxic, and that blocking aggregation would be therapeutic. For example, purified mutant htt protein (exon 1) forms detergent-insoluble aggregates upon incubation at 37°C for 16 h. This property of mutant htt to aggregate *in vitro* has allowed the development of screens to identify compounds that inhibit the formation of detergent (SDS)-insoluble aggregates [12]. Heiser *et al.* developed a “filter retardation assay” whereby *in vitro* aggregated mutant htt protein was retained upon filtration through a cellulose acetate membrane (0.2 μm pore diameter) and the amount of aggregated protein on the filter was subsequently quantified by western blotting using an htt-specific antibody [12]. Individual compounds were incubated with mutant htt during the incubation period for aggregate formation and known aggregation inhibitors such as Thioflavine S and Congo Red were used as positive controls.

Heiser *et al.* screened 184,880 novel compounds and identified numerous compounds including a number of benzothiazoles that inhibited aggregation. Since riluzole, a closely related benzothiazole, had previously shown therapeutic benefit in amyotrophic lateral sclerosis patients [13], the authors pursued this structural class of molecules for further development. Further testing in a cell culture model of aggregation revealed that all primary hits were toxic to cells. However, two analogs of the benzothiazole compounds were found to be non-toxic and these decreased aggregation in the cell-based aggregation assay. One of these compounds, designated PGL-135, was subsequently tested in an HD mouse model [14], but was found to be metabolically unstable in mice and, not surprisingly, ineffective in this mouse HD model. Though, none of the compounds was active in an animal model, this assay was simple, robust and economical, and identified some hits that were active in secondary cell-based aggregation assays (Table 1). However, this screening effort also underscored the major limitation of *in vitro* assays--the difficulty in translating *in vitro* results to cell-based and *in vivo* models.

A similar *in vitro* aggregation assay was performed by Wang *et al.* who screened a smaller collection of compounds (1,040), but with the advantage that a majority of these compounds were FDA-approved drugs. The rationale for this strategy of using the FDA-approved drugs for screening is that any hits identified might progress to clinical trials more rapidly than new chemical entities [15]. This strategy has already been used in various screening efforts in the last several years [16,17] and already identified compounds that have moved to testing in mouse models of disease. For example, nortriptyline, a drug that is clinically used in the treatment of depression, was identified in an *in vitro* screen as an inhibitor of mitochondrial permeability transition [18], a key step in cell death. Further testing revealed the efficacy of nortriptyline in a mouse model of amyotrophic lateral sclerosis, a neurodegenerative disorder that results from death of motor neurons [19]. Though the molecular target of nortriptyline that is responsible for cell death-rescue remains to be elucidated, the extensive clinical knowledge about its safety and tolerability means that it can now rapidly progress to clinical trials for amyotrophic lateral sclerosis. Similar progress from hit identification to clinical trials can be expected for other FDA approved drugs that are identified in various high throughput assays and thereafter validated in animal models of relevant diseases. A major limitation of this approach is the smaller number of compounds that are FDA approved drugs. This translates to a small number of biological targets that are affected by current drugs; estimates suggest that fewer than 500 of the potentially thousands of pharmacologically tractable cellular targets are affected by current drugs [20].

Using this approach of testing FDA approved drugs and known biologically active compounds, Wang *et al.* identified 19 compounds that inhibited aggregation by more than 50% [15]. Further testing in a cell culture model of HD revealed that two of the active compounds, juglone and celastrol, reversed aberrant nuclear localization of mutant htt protein. This assay revealed an interesting correlation between *in vitro* aggregation inhibition and a cellular phenotype of mutant htt. Further work on the underlying mechanistic basis of this htt localization phenotype may provide insight into the activity of these compounds. Although none of the active compounds identified is currently approved for human use, celastrol and meclocyline were interesting from a therapeutic viewpoint. Celastrol has been considered for treating Alzheimer's disease [21] and meclocyline is a tetracycline antibiotic that is closely related to minocycline, a compound that is efficacious in a mouse HD model and is currently in clinical trials for HD [22]. The therapeutic potential of these compounds needs to be assessed by efficacy testing in other HD models.

Cell-Based Aggregation Assays

While *in vitro* aggregation screens are simple and scalable, they are unable to target other cellular mechanisms that cause and/or modulate aggregation. Zhang *et al.* conducted an aggregation screen in yeast, where inducible expression of mutant htt (Q103) tagged with green fluorescent protein (GFP) resulted in aggregate formation and growth suppression of yeast [23]. In this simple assay, yeast growth acted as a surrogate for aggregation and mutant toxicity and could be simply assayed by measuring absorbance (600 nm), and fluorescent GFP could be used to assay the expression levels and the aggregation state of mutant htt. A library of 16,000 compounds (at 10 μ M) was screened in this assay, and 9 hits were found to enhance both yeast growth and GFP fluorescence by 25%; this latter parameter ensured the exclusion of compounds that suppressed expression of mutant htt. Four of these active hits were subsequently found also to inhibit aggregation in a PC12 neuronal cell-based HD model.

Analogs of these active compounds were synthesized and tested and 3 potent analogs (IC_{50} ~0.1 μ M) were identified that inhibited aggregation in the PC12 cell-based aggregation assay, but, unexpectedly, only one of these analogs, designated C2-8, was able to inhibit aggregation in the *in vitro* aggregation assay. The C2-8 compound inhibited aggregation in a HD mouse brain slice aggregation assay and suppressed neurodegeneration in a *Drosophila* model of HD. This study used a high-throughput aggregation based assay to identify a potent aggregation inhibitor that is effective in *in vivo* HD models. However, the study underscores the unexpected discordances in compound effectiveness *in vitro* compared to those in cell-, tissue-, and organism-based aggregation assays, indicating that htt aggregation in cell context may be significantly more complex than in a simplified *in vitro* environment. This also suggests that a greater understanding of the aggregation process *in vivo* will be critical for addressing more efficiently htt aggregation as a drug target in HD therapy.

FRET-Based Aggregation Assay

A Fluorescence Resonance Energy Transfer (FRET)-based approach can also be used to monitor protein-protein interactions and thus be used to directly assay aggregate formation in cells [24]. FRET is based on the principle of energy transfer between two fluorophores that are in close spatial proximity (<100 Ångstrom). A key requirement for FRET to occur is for one of the fluorophores (donor) to emit light energy that overlaps the absorption spectrum of the second (acceptor) fluorophore [24]. Furthermore, the emission of the acceptor fluorophore should be at longer wavelength than emission of the donor in order to separate the signal of the emission of the acceptor from that of the donor.

Pollit *et al.* optimized a FRET-based cellular aggregation assay for HTS in a model of X-linked spinobulbar muscular atrophy, a polyglutamine disorder caused by poly Q expansion in the

androgen receptor (AR) [25]. Mutant N-terminal fragments of AR protein containing unexpanded (Q25) or expanded (Q65) glutamine repeats were engineered, and fused to either Cyan Fluorescent Protein (CFP; the donor) or Yellow Fluorescent Protein (YFP; the acceptor). A mutant form of the glucocorticoid receptor (Δ GR) was co-transfected with these constructs to enhance aggregation. The soluble AR (Q25) did not cause FRET, in contrast to the expanded poly Q containing AR, for which aggregate formation brought the CFP and YFP fusion proteins in close proximity and caused FRET. Based on a decrease in FRET signal in Q65 transfected cells a robust HTS assay ($Z' > 0.6$) was optimized and 2,800 compounds from an Annotated Compound Library [26], a collection of biologically active molecules, were screened. Though numerous inhibitors of aggregation were identified in the screen, the authors pursued a small-molecule inhibitor of Rho-activated serine/threonine kinase (Y-27632) and found that this compound not only decreased mutant AR aggregation in cells and *in vitro*, but also ameliorated degeneration in a *Drosophila* model of HD. However, none of the compounds identified was approved for clinical use by FDA. Encouraged by the results from this screening effort, the same group subsequently undertook another screening effort and tested 1,340 biologically active compounds with a large proportion of FDA approved drugs in the FRET- based assay. Of these compounds, 10 compounds were found to inhibit aggregation by 20-30% in a reproducible and dose-dependent manner.

These compounds were then tested for their ability to inhibit aggregation in a model of a “pure” polyglutamine peptide aggregation. Surprisingly, only 2 of the 10 compounds inhibited aggregation of this peptide in cells, indicating that flanking protein sequences somehow modulate aggregation and that most compounds target aggregation mechanisms that are modulated by the flanking sequences. These active compounds could be useful tools to understand these mechanisms, although such mechanistic work is non-trivial and would take significant effort. Finally, five of the aggregation inhibitors alleviated neurodegeneration in a *Drosophila* HD model. These results suggest that the cell-based aggregation assay had a reasonably high predictive value for *in vivo* activity. Three of the five compounds that were active in the *Drosophila* model are FDA-approved drugs (nadolol, fosfanol and laevonorfedrin) (Table 1). Though none of these three FDA-approved compounds is appropriate for the required long-term therapy in HD patients, largely due to their toxicity, it raises the possibility that less toxic related analogs could be found that are potential drugs for polyglutamine diseases [27]. Future studies for understanding the mechanisms by which these compounds inhibit aggregate formation could shed light on the cellular mechanisms of this process and guide future drug discovery efforts.

Complex Phenotype Based Aggregation Assay

Aggregation-based assays may also be utilized for therapeutic discovery in diseases with more complex phenotypes. Another novel HTS assay that was based on effects of extracellular protein aggregation on cellular function has been developed for Alzheimer's disease. The etiology of Alzheimer's disease (AD) is complex and not well understood [3]. However, the 1-42 amino acid cleavage product of amyloid precursor protein, termed amyloid β protein ($A\beta$) is associated with neuronal toxicity by forming extracellular aggregates that are linked to Alzheimer's disease [4]. An assay to capture the toxicity of $A\beta$ in cell culture was developed by Blanchard *et al.* who adapted $A\beta$ mediated depolarization of neuronal PC12 cells for HTS [28]. In this assay, the addition of purified aggregated $A\beta$ 1-42 caused PC12 membrane depolarization within 5-15 minutes, and was measured as enhanced fluorescence of a voltage-sensitive fluorescent dye, DiBAC₄. Approximately 1,500 compounds were screened in this assay and 10 compounds that inhibited membrane $A\beta$ 1-42 depolarization were identified. These included a number of tyrosine kinase inhibitors such as DAPH1 and tryphostin AG879. However, further testing of DAPH1, the most potent compound identified in this assay, revealed that it inhibited aggregation of $A\beta$ 1-42 and this likely prevented membrane

depolarization [29]. This assay is an example of an effective strategy that can be used to model complex disease phenotype for HTS.

CELL DEATH-RELATED ASSAYS

Neuronal loss occurs ubiquitously in numerous neurodegenerative diseases including HD and Alzheimer's disease and likely contributes to these diseases [3,6]. Furthermore, caspases that are key players in cell death are thought to play key roles in the toxicity of mutant htt. For example, inhibition of caspases using small molecules has shown benefit in cell culture models of HD [30-32]. Caspases also have a role in generating toxic htt fragments by processing mutant htt [33]. Thus there is a strong rationale for undertaking screens for caspase and cell death inhibitors in neurodegenerative disorders.

Caspase Inhibition-Based Assay

Caspases are a conserved family of aspartyl cysteine proteases that cleave protein substrates in the process of apoptosis [34]. Caspase-3 is a terminal “executioner” caspase that is involved in apoptosis. Cellular caspase activity can be measured based on cleavage of peptide substrates and release of a fluorogenic substrates *in vitro* [35]. Piccioni *et al.* adapted a cell culture model where expression of mutant androgen receptor (AR) with expanded polyglutamine (Q112) induces caspase-3 activation and cell death in human cells [36]. This robust medium throughput screening assay ($Z' > 0.3$) used a caspase-3 fluorogenic assay to measure mutant AR cytotoxicity in cells 72 h after transfection.

The authors screened the 1,040 compounds of the NINDS collection and identified 15 compounds that substantially inhibited ($>70\%$) caspase-3 activity. None of these compounds directly inhibited caspase-3, but acted upstream in the caspase cascade. Interestingly, these compound hits included cardiac glycosides, and further studies showed that these and other cardiac glycosides were also able to inhibit cell death in this assay and a related assay based on a motor-neuron-derived cell line. Surprisingly, the mechanism of caspase-3 inhibition, though not revealed in this study, was distinct from their known cardiotoxic effects caused by blocking Na^+ , K^+ AT-Pase. These compounds are promising drug-leads since they are in widespread clinical use and were active at low doses ($\text{EC}_{50} < 40 \text{ nM}$) that are usually achievable in plasma of patients. These compounds are candidates for testing in mouse HD models. An interesting outcome of this assay was that though the assay tested a downstream point in the apoptotic cascade (caspase-3 inhibition), it was surprising that none of the active compounds directly inhibited caspases. This may be explained by more prominent or more numerous targets upstream of caspase-3 itself, or may indicate that blocking caspase-3 alone cannot prevent cell death cascades. In fact, other studies have suggested that cells are still able to undergo apoptosis, albeit at somewhat reduced rates, when individual components of the apoptotic cascade (e.g. caspase-3) are genetically inhibited. Thus, targeting more upstream events is likely to inhibit cell death. Future studies directed to understand the site of action of the cardiac glycosides could reveal new targets for screening efforts.

ST14A Cell Viability Assay

In another cell viability-based assay, a striatal cell line was engineered to express the N-terminal 548 amino acid of mutant htt, resulting in enhanced susceptibility to serum deprivation induced cell death [37]. Using this cell model, a high throughput (384-well format) cell viability assay, that was based on a fluorescent viability dye (calcein AM), was developed [30]. A total of 43,685 novel compounds from diverse sources were screened and 29 novel compounds that selectively rescued cell death in mutant htt expressing cells but not in parental striatal cells were identified. This selectivity is likely a result of pathways that are specifically altered in mutant htt-expressing cells, and thus present as attractive targets for directed drug development.

Four such selective compounds inhibited caspase-3 activation only in mutant but not parental ST14A cells, which may be a consequence of the known increased caspase-3 processing in N548 mutant cells; there is also increased caspase-3 expression in the R6/2 transgenic mouse model for HD [38]. These results support the notion that aberrant caspase-3 activation by mutant htt is involved in HD. Future studies to determine the mechanism of selective rescue by these compounds would shed light on the connection between mutant htt and caspase activation.

One of the obstacles in prioritizing hits for drug development can be overcome by testing hits in diverse models of a disease. Using the rationale that compounds active in diverse models likely affect a conserved disease mechanism and are therefore suitable for further study, the authors went on to test the 29 hit compounds in 3 additional models of HD, based in PC12 cells, yeast and *C. elegans*, finding 4 of the 29 compounds to be active across multiple models (Fig. 2) [30]. A further class of related compounds was developed (R1 and R2) and were tested and found to be directly neuroprotective in a brain slice-based explant model for HD (see below).

More recently, by testing biologically active compounds in the ST14A model, we identified that metabolic inhibitors such as rotenone, sodium fluoride and oligomycin could prevent cell death in this HD model and in *C. elegans* and *Drosophila* HD models. Furthermore, these inhibitors did not alter cellular ATP levels but activated ERK and AKT survival signaling in the N548 mutant cells and the activation of pro-survival signaling could partially explain the protective effect of the metabolic inhibitors [39]. This work underscores the value of using biologically active small molecules and their use to identify novel and unanticipated mechanisms that could serve as therapeutic targets.

Though we have discussed these two cell viability assays, other cell viability assays have been developed in PC12 cells, where expression of mutant htt leads to cell death [31,32]. Cell death in these assays is readily detected by a simple readout (lactate dehydrogenase release) and these have been optimized for medium throughput. Using these assays a number of suppressors of mutant htt induced cell death have been identified; however none of these is approved for human use. Future studies that shed light on the mechanism of action of these compounds are awaited and may reveal novel targets for HD therapy.

CELLULAR PROTEIN ASSAYS

Protein-Clearance Assays

In cases of dominant genetic diseases caused by the production of a mutant protein, targeted degradation of the mutant protein is a very attractive therapeutic approach. Two recent studies describe assays that can detect enhanced protein clearance of pathogenic proteins. In one study, Coufal *et al.* developed an assay that measures the level of a mutant htt-GFP fusion protein in a HTS mode and tested 16,000 compounds [40]. They identified one compound that selectively enhanced clearance of mutant htt, but without affecting the levels of wild type htt. This assay for selective degradation of mutant htt clearance is notable since decreasing or knocking out the expression of mutant htt has been shown to reverse the effects of HD in a mouse model [41]. In another screen, compounds that enhanced the degradation of mutant synuclein, a protein involved in Parkinson's Disease, were identified based on a yeast screen [42]. Interestingly, these were effective in models of both Parkinson's and Huntington's disease, suggesting the broad applicability of agents that induce toxic protein clearance, as potential therapeutics in neurodegenerative disorders.

Increasing Levels of Protective Proteins

A number of diseases arise from insufficient production of an essential protein. Insufficient levels, as opposed to complete loss, of a protein can occur when only one copy of a gene is deleted or an alternative gene exists which can express the deficient protein, albeit in insufficient quantities. Thus, enhancing the production of the deficient protein from the remaining gene(s) is a therapeutic approach for such disorders. One such disease is spinal muscular atrophy (SMA). This is a fairly frequent genetic disorder (1:40 carrier rate) [43], but due to its recessive nature and rapid lethality, there are few patients with the disease. SMA results from loss of both copies of the normal gene (*smn1*) [44]. However, there exist a number of copies of a related gene (*smn2*) in most individuals. The *smn2* gene transcript is spliced to express a small amount of the normal SMN protein that results in only about 25% of survival of motor neuron (SMN) protein in patients, compared to normal individuals [45]. Such a large decrease of SMN protein causes cell death of motor neurons, resulting in muscular atrophy and ultimately death of individuals; the disease severity is inversely correlated to the amount of SMN protein [45]. In order to enhance SMN protein production from *smn2* gene, Jarecki *et al.* devised a HTS that detects SMN2 promoter activity and screened 550,000 compounds in this assay [46]. The authors identified two drug-like compounds that showed activity in SMA patient fibroblasts by increasing SMN protein levels. Lunn *et al.* have also devised a reporter-based HTS assay to identify compounds that could selectively upregulate expression from the *smn2* gene, but not *smn1* [47]. They screened 47,000 compounds and discovered that indoprofen selectively increased *smn2* reporter levels and SMN protein, in patient fibroblast cells. Though indoprofen is a known non-steroidal anti-inflammatory drug, the effect on SMN protein levels was not related to its anti-inflammatory effects. These screens provide a proof of principle that the levels of specific protective proteins can be increased using small molecules and that this approach can be of therapeutic benefit for other diseases. For example, wild-type huntingtin is reported to have a neuroprotective effect [48]. Thus small molecules that could enhance wild-type huntingtin production may be protective in neurodegenerative disorders.

Microarray and HTS

In certain cases, cellular phenotypes are subtle or not amenable to a HTS readout, especially in the case of complex neurodegenerative disorders. One approach to this problem is to identify molecular markers of a disease state. Microarray technology allows the assessment of a cellular state by measuring the relative abundance of global mRNA species in a cell or tissue [49]. Since complex phenotypes usually involve underlying changes in mRNA expression patterns, these patterns can be used as “signatures” to distinguish between two cellular states. Stegmaier *et al.* applied this approach as a test case to identify compounds that could affect the complex phenotype of differentiation in a cell line derived from leukemic cells [50]. Initially the authors identified surrogate markers by performing microarray analysis on differentiated and undifferentiated white blood cells and identified five signature genes that correlated very strongly with the state of differentiation. They then screened a collection of biologically active compounds (1,739) and identified 8 compounds that reproducibly induced changes in the signature genes to resemble that of neutrophilic differentiation in the leukemic cell line. This study provides a proof-of-principle that complex phenotypes or cellular states can be measured and screened in a HTS. A similar approach could be applied to neurodegenerative disease such as HD, where numerous studies have identified a major role for transcriptional dysregulation [51-53]. Once the “signatures” that accurately predict a neurodegenerative disorder are identified, one could screen for compounds that reverse those signatures from a disease pathology to a healthy state.

HIGH CONTENT SCREENS (HCS)

As could be seen in a number of the example HTS screens described above, the transition from *in vitro* assays hits to efficacious compounds in more complex disease model assays- in cells, in tissues, and most critically in animal models- can be beset with many unexpected, and even seemingly “mysterious” failures. While many such compound dropouts will be due to fundamental chemical issues such as drug availability, metabolism, safety, toxicity, the important challenge in producing a successful drug lead candidate for further preclinical development and, ultimately, clinical trials, is whether efficacy in an *in vitro* assay translates with high predictive value into efficacy in a validated whole-animal disease model.

The *in vitro* to *in vivo* transition can have a very high, and sometimes abrupt, dropout rate. Over the last decade, increased emphasis has been placed on developing “high throughput biology” models in the mid-development process to help bridge the gap between simplified *in vitro* assays and the fully complex biological context and *content* of a whole-animal disease model. Thus, “high-content” screens are designed to provide assays with more predictive value for *in vivo* benefit and clinical potential, but are scalable to evaluate much larger numbers of compounds than *in vivo* animal models. As such, HCS assays are commonly used as secondary screens to help rank order HTS hits for advancement into full preclinical development; they can also be used as primary screens if smaller target compound libraries (typically hundreds to a few thousands) can be identified with particular relevance to the disease state in question.

There are several ways in which the content of drug screening assays can be increased. One obvious, but not always technically easy, approach is to use primary cells instead of cell lines for a given assay. Any cell-based screen arguably has much increased biological content over any *in vitro* assay, but the nature of the cell type in question may have a major impact on its clinical relevance. Indeed, the transformed nature of cell lines makes their relevance for many cell and tissue-types questionable, especially in the nervous system where all neurons in the adult brain are post-mitotic; transformed cell lines are commonly used, of course, for their ease, convenience, and reproducibility. In the context of HD, for example, it would be desirable to use primary striatal neuronal cultures instead of the typical non-neuronal cell line (e.g., HEK or CHO cells) in the design of HCS assays. While for many cell types, especially neurons, the establishment of primary cultures and conditions for their efficient molecular genetic perturbation can be difficult to achieve, the increased biological and clinical relevance of primary cell-based HCS is often considered to be worth the extra effort and expense. The biotechnology company Trophos SA, for example, has developed and implemented a primary striatal neuron HCS assay for HD using image-based endpoints [54].

The use of more phenotypic and functional endpoint measures is the other major way in which the biological content of assays can be significantly increased. For neurodegenerative diseases, for example, this includes the use of image-based assays in which morphological indicators of neuronal “wellness”, such as changes in neurite extension, cell shape, and the translocation/transformation of intracellular disease markers (such as the Htt protein for HD) are used instead of more blunt measures such as overt cell death and clearance. Moreover, these more subtle phenotypic expressions of disease induction are generally thought to reflect earlier stages of cellular pathogenesis, and can potentially reveal drug targets and mechanisms that could be used to intervene at earlier stages in the disease process.

An emerging development in the HCS area takes a further step towards “*in vivo*-ness” by using tissue explants instead of cell lines or primary cell cultures in order to retain, at least partially, the complex milieu of the local extracellular and tissue environment for a given disease-relevant cell type. In the sphere of neurological diseases, much effort has been placed in development of brain slice-based explant cultures for ischemic stroke, given that scores of

neuroprotective clinical candidates developed from *in vitro* and cell-based assays for stroke have, without exception, failed in the clinic thus far (in ~100 clinical trials to date) [55]. Thus, even what in past years had seemed to be a relatively straightforward case of cellular oxygen and glucose deprivation, has turned out to involve a complexity of non-cell autonomous processes that cannot be adequately recapitulated even in primary neuronal cultures.

Thus, a number of organotypic brain slice-based assays have been developed for ischemic stroke with cell or tissue-based endpoint for neuronal cell death such as propidium iodide (PI) staining [56,57]. We have recently described a HCS screen for ischemic stroke based on the creation of a “sentinel” population of pyramidal neurons in cortical brain slice explants *via* biolistic transfection of Green Fluorescent Protein [58]. Subsequent to oxygen-glucose deprivation (OGD), these GFP-expressing sentinel neurons provide a living index of the extent and time course of the ischemic damage produced. The majority of cortical neurons degenerate and die by the 3rd day following OGD (Fig. 3a), thus creating a robust assay for the identification of compounds that can provide neuroprotection to neurons in the context of a living, but degenerating, brain tissue explant (Fig. 3b).

Using this brain slice-based stroke assay, ~5,000 synthetic and natural product compounds, including all FDA-approved drugs, were screened producing ~74 primary and reproducible hits. Intriguingly, one of the strongest hits was the cardiac glycoside neriifolin (see discussion of Piccioni *et al.* above [36]), and subsequent studies showed that other members of the cardiac glycoside family including the FDA-approved drugs digitoxin and digoxin were efficacious as well, albeit with lower potencies. These results suggest that some common pathogenic mechanisms may play broad roles in neuronal degeneration and death in both traumatic and neurodegenerative diseases such as HD.

Analogous tissue explant models for neurodegenerative diseases take advantage of the genetic underpinnings of such diseases, a prime example being that of htt for HD. Studies described above already underscore the complex and context-dependent nature of htt aggregation between *in vitro* and cell-based screens; a further consideration is the potential role that tissue context may play in htt aggregation. Organotypic brain slice explants from the R6/2 transgenic mouse model for HD, for example, can be shown to develop htt aggregates suitable for use in an image-based assay over the course of 3-4 weeks in culture [23].

We have taken an analogous approach to the Wang *et al.* experiments described above to use biolistic transfection to introduce, along with the vital marker Yellow Fluorescent Protein, DNA expression constructs based on mutant htt into medium spiny neurons (MSNs) in cortico-striatal brain slice explants. The sentinel MSNs thus produced are used to track the progressive neurodegeneration produced by htt transfection, thus creating a brain slice-based HCS assay for HD that can be used to screen small molecule compounds as well as DNA-based drug target probes such as siRNAs. This assay was recently employed to aid the development of hits from the ST14A cell viability screen described above [30]. Using similar approaches, Murphy and Messer [59] demonstrated the ability of scFv intrabodies to protect against biolistically transfected htt, and Khoshnan *et al.* showed that perturbation of the NF- κ B pathway can alter the toxicity of mutant htt substantially [60].

Whole-Animal-Based HCS

More biological content still can be achieved by developing HCS assays that are resident within living organisms. To date, those assays with sufficient throughput to be regarded as “screens” (able to evaluate appreciable numbers of compounds per unit time) have been based in invertebrate model organisms, such as nematodes, and in small vertebrates such as zebrafish. In a novel example of a high throughput screen that can measure a very complex phenotype in whole animals, Burns *et al.* developed an assay to detect heart rate in a zebrafish model [61].

They generated a transgenic line that expressed green fluorescent protein (GFP) in the heart muscle of developing early embryos (24 hr) at a time when the zebrafish embryos are still transparent. This allowed visualization of the embryos by automated, microscopic identification of the fluorescent hearts and recording of heart rates. They developed software that extracted raw data of heart rate readings from more than 90% of embryos per plate and automatically converted to a spreadsheet. This assay was validated by testing drugs that are known to affect the human heart rate in dose-response and time-course experiments. This assay is useful for identifying compounds that affect heart rate both as a toxicity test for drug-leads and for HTS screens in cardiac disease models. Similar approaches may be useful for developing whole animal based HTS in zebrafish models of polyglutamine disorders that have been recently developed [62,63].

Thus, cell- and tissue- and even whole-organism based HCS screens, can be used to bridge the long divide between *in vitro* findings and the desired *in vivo* efficacy in disease models critical to the drug development process. Critically, with each assay of increasing biological complexity there is a concomitant increase in clinical relevance as assay context and content approach that of the *in vivo* situation. While throughput generally decreases and expenses rise with HCS assays of increasing complexity, these considerations may be minor compared the potential major upside of much increased likelihood of clinical success of drug candidates developed using such approaches.

HTS Achievements

The successful development of numerous HTS assays for neurodegenerative disorders, including assays for aggregation and cell death, promises an increasing role for the use of complex phenotypes in HTS approaches in this therapeutic area. Such assays are identifying a growing list of active compounds (Table 1), of which several are FDA-approved drugs and could have very short times lines to the clinic [27,36]. Though these small-molecules can reverse phenotypes of several complex neurodegenerative disorders in cell culture and some invertebrate animal models, prioritization and testing in more validated mouse models is a serious challenge. More complex HCS assay and testing in primary cell culture models or invertebrate animal models can provide a reasonable means to prioritize these hits [54,58,64]. However, activity in a small range of secondary assays, though useful for prioritizing such active compounds, is by no means a proof that inactive compounds do not merit further study. More extensive testing in a range of secondary assays, where feasible, would be useful for identifying compounds with therapeutic value. These HTS assays also provide scalable core platforms for larger-scale screening campaigns, both for chemical compounds as well as for genome-wide RNAi-based screens that may provide specific drug targets for selective aspects of neurodegenerative diseases such as aggregation and cell death.

Chemical Tools for Understanding Disease Mechanisms

The compounds discovered in the various assays described in this review may also find use as chemical tools for understanding the mechanisms of neurodegenerative diseases, including those that are insufficiently drug-like in other respects (e.g., stability, blood-brain barrier penetration, etc.) for further development as drug molecules themselves. For example, protein aggregates are common neuropathological components of HD and the other major neurodegenerative disorders including Alzheimer's and Parkinson's disease, but their role in disease pathology has been controversial, with both harmful and protective functions being invoked for aggregates [11]. However, using small molecules that affect aggregates, one can test the various hypotheses and also probe the mechanisms involved in cellular aggregation. In fact, a recent screen using cell culture models of HD and Parkinson's disease found protective effects of enhancing aggregation with a small molecule hit compound [65]. Thus, small molecule hits from phenotypic screens can be useful tools for testing hypotheses, and for

protein target identification. Together, these approaches using small molecule chemical tools can enhance mechanistic understanding of disease pathways and identify targets for disease therapy.

FUTURE DIRECTIONS

Most current assays use relatively simple phenotypes, such as aggregation, cell death and reporter protein level. However, as more insight into mechanisms of disease pathology is obtained, more complex assays that target specific cellular phenotypes need to be developed. The challenge for HTS community will be to create assays that target specific molecular phenotypes of complex disease and thereby develop precise therapies for these disorders. Examples of high throughput screens in *Drosophila* cells [66,67] and in whole organisms such as *C. elegans* [68] and zebrafish are already emerging [61]. These are the beginnings of the quest to conduct HTS for complex phenotypes *in vivo*; screening efforts would finally come full circle.

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ABBREVIATIONS

HTS, High throughput screening
HCS, High-content screening
Htt, Huntingtin
HD, Huntington's disease
AD, Alzheimer's disease
PD, Parkinson's disease
SMA, Spinal muscular atrophy
SMN, Survival of motor neuron
GFP, Green-fluorescent protein
CFP, Cyan-fluorescent protein
FDA, Food and Drug Administration
YFP, Yellow-fluorescent protein
Q, Glutamine
FRET, Fluorescence resonance energy transfer
AR, Androgen receptor
GR, Glucocorticoid receptor
A β , Amyloid β
OGD, Oxygen glucose deprivation

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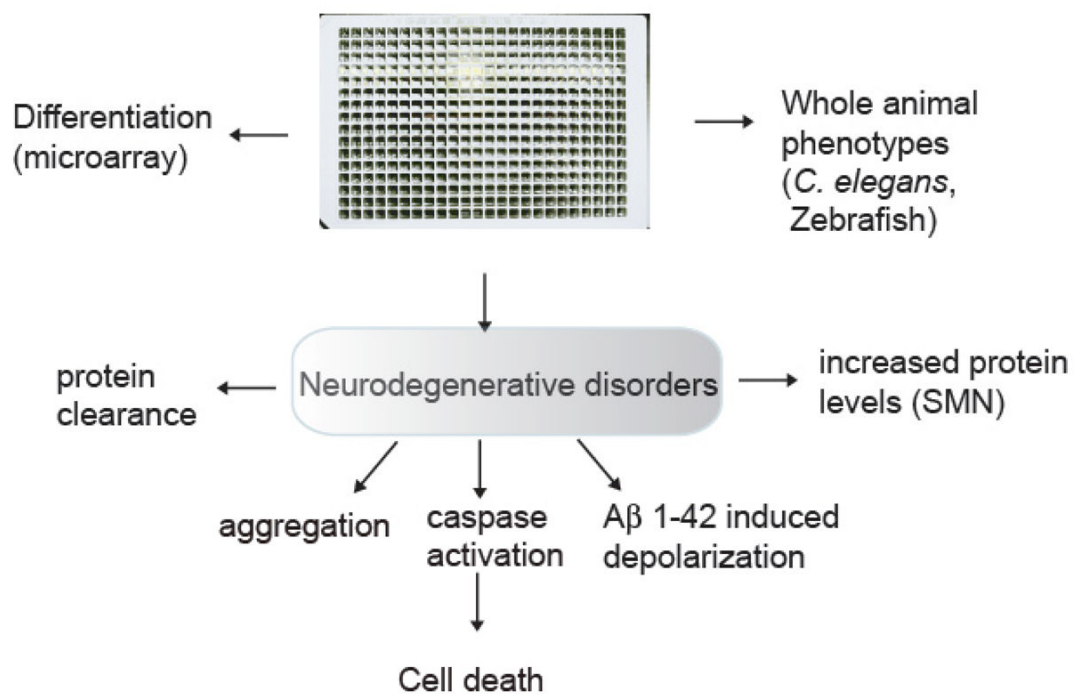


Fig. (1).
Types of complex HTS assays.

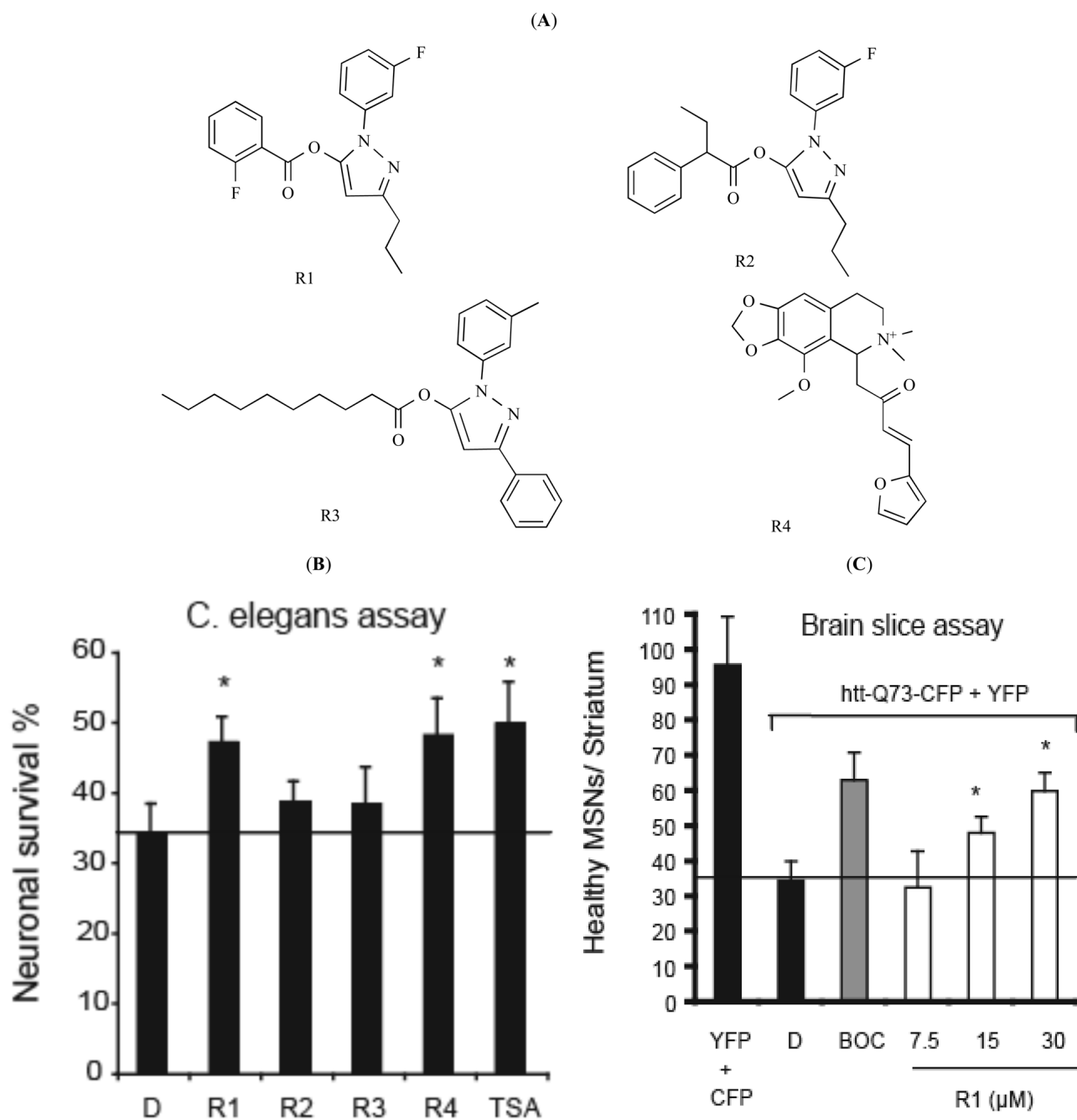


Fig. (2).

Hits identified in a striatal cell-viability assay and their activity in diverse HD models. (A). Structures of four novel hits (R1, R2, R3, R4) that were identified in the ST14A cell culture HD model. (B) Rescue of ASH neuronal death in a *C. elegans* model of HD by R1 (3 mM), R4 (2.2 mM). A histone deacetylase inhibitor, trichostatin A (TSA), was used at a concentration of 1 mM as positive control, and DMSO (D) was the vehicle control. In this model, sensory neurons (ASH) in *C. elegans* that are engineered to express mutant htt (Q150) undergo age-dependent cell death over 2-3 days after hatching. ASH neuronal viability can be monitored in live animals by expression of a Green Fluorescent Protein (GFP) in these neurons. Neuronal death results in a loss of GFP expression. Horizontal line represents the level of death in 3 day-

old DMSO treated animals. *Statistically significant rescue of neuronal death ($p < 0.05$, Student's *t-test*). (C) R1 rescues medium spiny neuronal (MSN) degeneration induced by htt-exon 1-Q73-CFP fusion protein in a rat brain-slice assay. Brain slices were cotransfected with YFP to monitor the morphology of the mutant htt-exon 1 transfected neurons. Loss of typical dendritic morphology was used as an indication of neuronal toxicity induced by mutant htt. Horizontal line indicates the level of degeneration in control DMSO (D) treated cultures. The rescue was significant ($p < 0.05$, ANOVA and Dunnet's posthoc comparison test) for Boc-D-FMK (50 μM) and R1. The results are mean \pm S.E.M. from one representative experiment of at least two independent experiments for each compound (CFP-cyan fluorescent protein, YFP-yellow fluorescent protein. (Reproduced in part from reference [30]).

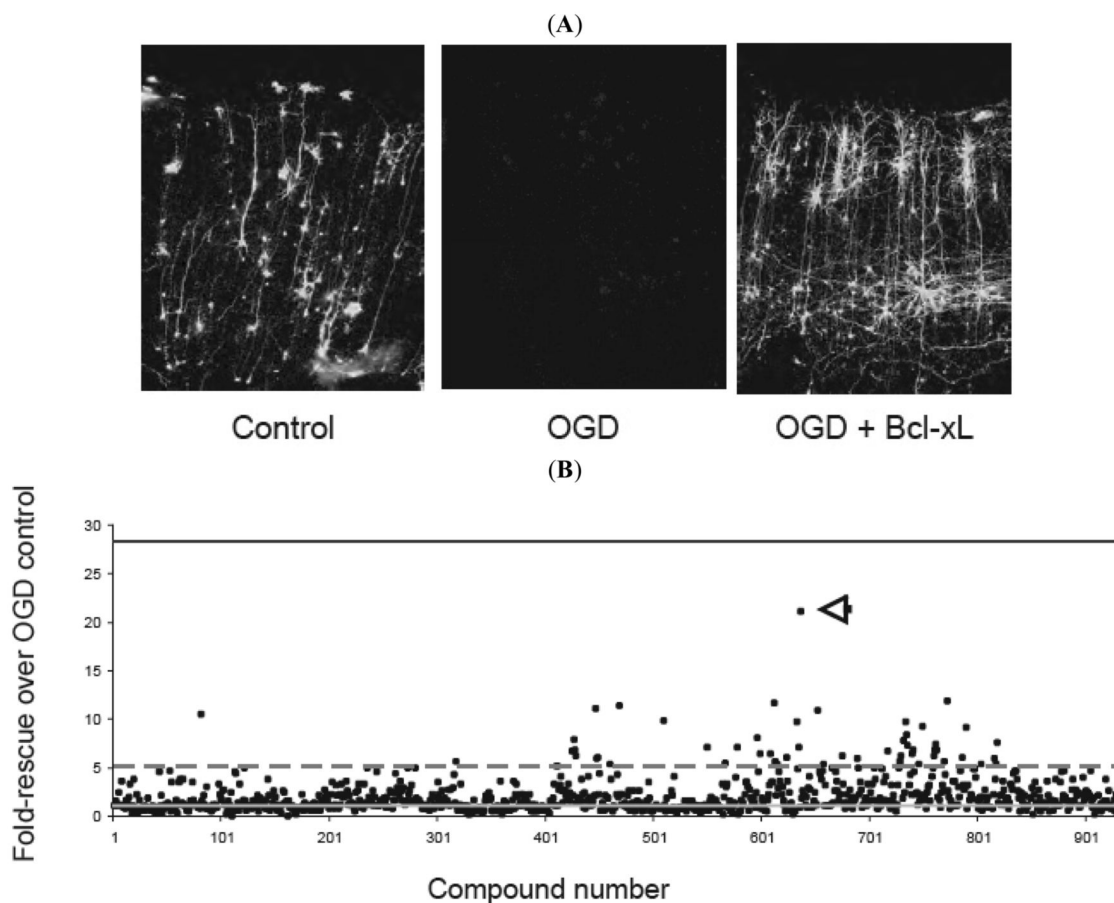


Fig. (3).

High-content screening. Cortical brain slice explant model for ischemic stroke. **(A)** Cortical brain slices biolistically transfected with an expression plasmid for yellow fluorescent protein under normal conditions (*Left*) and 24 h after 7.5 min of oxygen-glucose deprivation (OGD) administered at the time of brain slice preparation and transfection (*Center*). Co-transfection with the anti-apoptotic gene *bcl-xL* rescues against the neuronal cell death induced by OGD (*Right*). Note that neurons in these slices often appear healthier than control neurons in non-OGD slices, presumably because *bcl-xL* also protects against trauma induced in the process of slice preparation. **(B)** Summary of results of a primary screening run of 931 compounds in the brain slice stroke assay. In this summary graph, only the peak value for rescue against OGD is shown for each compound out of the 3-5 concentrations that were tested (3-50 μ M). These values are expressed as a fold-increase or decrease in numbers of healthy pyramidal neurons relative to vehicle-treated OGD brain slices (solid gray bar with a y-axis value of 1.0). Arrow indicates the peak neuroprotection level for neriifolin as discussed in the text. (Reproduced from reference [58]).

Table 1

HTS Assays for Neurodegenerative Diseases

Screen	Protein	Assay	Cpds Tested	Main Hits	2° Screen	Mouse Model Test	Clinical Use/FDA Approval	Ref. ^a
Aggregation <i>in vitro</i>	htt exon-1 Q51	Filter retardation	184,880	Benzothiazole PGL-135	Cell based aggregation	R6/2	No	[12]
Aggregation in yeast	htt exon-1-Q103 EGFP	Yeast growth	16,000	C2-8	PC12, R6/2 brain slice <i>Drosophila</i>	NT	No	[23]
Aggregation in cells	AR N1-127 Q65	FRET	4,000	Y-27632 Fosfosal Nadolol Gefitinib	PC12 <i>Drosophila</i>	NT	Yes	[25,27]
Aggregation	Aβ 1-42 toxicity	membrane potential	~1,500	DAPHI	None	NT	No	[28]
Cell death	AR 1-109 Q112	caspase-3	1,040	Cardiac glycosides	Motor neuron cells	NT	Yes	[36]
Cell Death	htt N63-Q148	PC12 viability	1,040	Cannabinoids	None	NT	Yes	[32]
Cell Death	htt N548 Q120	ST14A viability	43,685	R-1, R-2, R-4	PC12, yeast, <i>C. elegans</i> , brain slice	NT	No	[30]
Protein clearance	Htt-Q103 synuclein	mutant protein clearance	16,000 50,729	A31, A28 SMERs	PC12 <i>Drosophila</i>	NT	No	[40] [42]
SMN	SMN Protein	protein levels	47,000	Indoprofen	Mouse model	Yes	Yes	[47]

NT, not tested. 2° screen, secondary screen.

^a All references in Table 1 have been cited in the main text and the numbers refer to those in the main text.