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Cell death assays for neurodegenerative disease drug discovery

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Abstract

Introduction.—Neurodegenerative diseases affect millions of people worldwide. Neurodegeneration is gradual over time, characterized by neuronal death that causes deterioration of cognitive or motor functions, ultimately leading to the patient's death. Currently, there are no treatments that effectively slow the progression of any neurodegenerative disease, but improved microscopy assays and models for neurodegeneration could lead the way to the discovery of disease modifying therapeutics.

Areas covered.—Herein, the authors describe cell-based assays used to discover drugs with the potential to slow neurodegeneration, and their associated disease models. They focus on microscopy technologies that can be adapted to a high-throughput screening format that both detect cell death and monitor early signs of neurodegeneration and functional changes to identify drugs that the block early stages of neurodegeneration.

Expert opinion: Many different phenotypes have been used in screens for the development of therapeutics towards neurodegenerative disease. The context of each phenotype in relation to neurodegeneration must be established to identify therapeutics likely to successfully target and treat disease. The use of improved models of neurodegeneration, statistical analyses, computational models, and improved markers of neuronal death will help in this pursuit and lead to better screening methods to identify therapeutic compounds against neurodegenerative disease.

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1. INTRODUCTON

Neurodegenerative diseases encompass a wide range of medical disorders with symptoms that range from cognitive deterioration in Alzheimer's Disease (AD) and Frontal-temporal Lobe Dementia (FTLD), to diminished control of motor functions as found in amyotrophic lateral sclerosis (ALS), Huntington's Disease (HD) and Parkinson's Disease (PD)[1]. For most of these diseases, degeneration is restricted to specific neuronal populations. The diseases are progressive and age-dependent, with neuronal loss and the accompanying clinical symptoms occurring gradually, in some cases taking decades to manifest.

To develop therapies to treat neurodegenerative diseases, a key first step is the establishment of reliable assays to monitor neurodegeneration[2]. While the term neurodegeneration can refer to a constellation of phenotypes leading up to and reflecting the loss of neuronal function, the most direct measure of neurodegeneration is neuronal death. In the past, cytoprotective assays using dyes or fluorescent markers have been a mainstay for identifying therapeutics that affect neuronal death[3, 4, 5]. Many of these assays have the advantage of being easily adapted into high throughput screening (HTS) formats and are commonly used by the pharmaceutical industry to test drugs for neurotoxicity as a potential side effect.

Beyond detecting cell death, developing effective therapies requires the ability to detect the events that precede, correlate with and predict neuronal death fate. To this end, assays have been developed that detect biochemical events that lead up to neuronal death such as changes in metabolic activity, mitochondrial function, and DNA fragmentation[5]. Additionally, in live imaging assays, sensitive morphological assays employing robotic HTS fluorescent microscopes have been developed to quantify the early stages of neurodegeneration such as changes in neuronal shape and neurite area[6-89]. Newer imaging technologies have been developed for temporal analysis of neurite area and cell membrane integrity providing assays to monitor neurodegeneration over time[10].

In many neurodegenerative diseases, neurons can become dysfunctional well before they die[11]. Therefore, identifying specific disease-related dysfunction, especially prior to related cell death is an important path forward in generating therapeutics. For example, in the case of AD and ALS, neurons in the most vulnerable cell populations in the nervous system have been found to become hyperactive years before overt clinical symptoms and neuropathology is detected[12-14]. A number of fluorescence cell-based HTS-compatible assays have been developed to measure changes in calcium (Ca^{2+}) transients or electrical activity as a measure of neuronal activity to screen for neuroprotective drugs[15-17], and indeed anti-epileptic drugs, which reduce neuronal hyperactivity, have been shown to slow neurodegeneration in models of AD[18] and ALS[19] and are now being tested in clinical trials[20-23]. While disease-related dysfunctional phenotypes may effectively drive the progression of some neurodegenerative disease, a major challenge remains in identifying specific disease related signatures that are unique and predictive of disease and cell death, to provide a clear screening phenotype. In these cases, cell death also provides a useful marker for establishing the relation of a phenotype to neurodegeneration.

Lastly, progress toward the goal of effective therapeutics requires models that recapitulate disease phenotypes that can be screened for therapeutics. Microscopy provides a common platform for studies of neurodegeneration with single cell resolution that can be used to identify and probe basic cellular mechanisms related to neurodegeneration and can often be scaled up to HTS format to discover drugs to treat neurodegenerative diseases using common models of neurodegeneration. Thus, a great deal of our understanding of the mechanisms of neurodegeneration is based on research using animal models or two-dimensional (2D) cultures of animal cells that are microscopy-amenable. However, in many cases these models imperfectly reflect the human disease[24]. One recent alternative, human induced pluripotent stem cells (iPSCs), can be derived from patients with neurodegenerative diseases, that can be differentiated to generate the specific human neuronal populations (iPSC-derived neurons) most relevant for the disease of interest, and in quantities compatible with HTS. In many cases these human iPSC-derived neurons display spontaneous disease phenotypes, such as increased risk of death, that can be quantified for testing potential neuroprotective drugs. Additionally, three-dimensional models such as organotypic slice culture and 3D brain organoids represent major advances in neurodegenerative disease modeling that are also being adapted to HTS.

Here we will review advances in the development of assays and neurodegenerative disease models that employ advanced microscopy technologies to identify and probe basic mechanisms involved in neurodegeneration that can be used to discover drugs to treat neurodegenerative diseases.

2. MODELS OF NEURODEGENERATION AMENABLE TO LIVE MICROSCOPY

2.1 Rodent models of neurodegenerative disease:

Much of what we know about the mechanisms and etiology of neurodegenerative disease comes from rodent disease models. Genetic mutations have been identified that cause neurodegeneration, and the clinical symptoms of the patients that harbor these mutations can be genetically modeled in rodents[6, 7, 25-27]. Impairments in cognitive, movement, and behavioral outputs from these models can be important in gauging the fidelity of the model to the disease as well as the clinical efficacy of a therapeutic intervention for that disease. However, dynamic analysis of neuronal death in mammalian models of neurodegeneration is rare due to the difficulties of performing microscopy on a live intact mammalian brain. Two-photon microscopy can be used to image the brain in some animal models[28], but live mammals are not practical for drug discovery screens. Instead, primary neurons from genetic mouse models, or wild type primary rodent neurons that have been modified to express disease proteins, are commonly used for studies or screening of compounds in high throughput for neuronal death.

For example, dominant mutations in the gene for transactive response DNA-binding protein (TDP43) expressed in primary rodent neurons induce neurodegeneration and other disease-relevant features of ALS[29]. Similarly, HD and PD can also be modeled by the expression of mutant genes in rodent neurons[30-33]. Using this method, small molecule drugs that increase

autophagy in neurons were able to slow neurodegeneration in primary neuronal models of HD and ALS[31, 34].

Although 2-dimensional neuronal models of neurodegeneration have yielded important insights into disease etiology and mechanisms, the relationship of individual neurons to their surroundings is an important aspect of pathogenesis that is absent from this approach. For example, neuroimmune inflammation, the relationship of non-neuronal cells to neurons during neurodegeneration, hyperexcitible neuronal circuitry, and transmission of neurodegenerative proteins throughout the brain all depend in large part on the 3-dimensional architecture of the brain. As a result, organotypic slice culture has emerged as a complementary approach for modeling neurodegenerative disease and drug screens that preserves much of the structure and connectivity of the brain. HTS assays of compounds that prevent cell death within slice culture have been employed in models of ischemic stroke, and HD[35, 36]. Furthermore, unlike primary cells, this approach offers the unique opportunity to perform screens in mature and aged animal model brain tissue[37, 38].

2.2 Human iPSC derived neurons and organoids:

Presumably because of species-specific differences in neurobiology and physiology, animal models of neurodegenerative diseases have largely failed to accurately predict clinical trial results. The failure rate for neurodegenerative disease is very high, and no disease modifying therapies exist for AD, ALS, FTD, HD or PD that have a major impact on slowing disease progression, despite many failed trials of entities that showed great promise in non-human preclinical models. A major advance that circumvents this limitation is the development of human disease models that employ iPSCs derived from patients, which can provide a relatively unlimited source of neurons for drug screening and discovery.

Human iPSC-derived neuron models have been developed for a number of monogenic inherited neurodegenerative diseases, including AD, ALS, FTD, HD and PD[6, 7, 32, 34, 39, 40]. Patient-derived iPSCs can be directed to differentiate into neuronal subpopulations that are most vulnerable in each disease, for example, motor neurons in in ALS[34], dopamine (DA) neurons in PD[6, 33], and striatal neurons in HD[7, 32]. In addition, iPSCs can be taken from multiple patients with the same disease to study the influence of individual variation, and the effects of specific genetic insults can analyzed before and after correction with gene editing technologies such as CRISPR-Cas9[6]. The use of patient derived iPSCs avoid problems of artefactual overexpression present in transgenic animal models, and the effects of potential neuroprotective agents on iPSC-derived neurons can be correlated with individual donors' clinical histories, allowing for patient stratification during clinical evaluation. In addition, iPSC-derived neurons can be co-cultured with iPSC-derived astrocytes or microglia to increase neuronal maturity and monitor cell non-autonomous phenotypes[41]. Furthermore, monitoring cell death in astroglia generated from patient-derived iPSCs facilitates the analysis of neurodegenerative-disease associated mutations in non-neuronal cell types[41]. Because iPSCs retain the genetic background of the donor, they can also be used to model sporadic cases in which a single genetic cause is not present, which represents the majority of cases in PD[42] as well as most other neurodegenerative diseases. Protocols have now been developed that enable the generation of neuronal populations directly from patient

fibroblasts (i-neurons), rather than passing through a pluripotent stage required to generate iPSCs[43]. These cells maintain more of the patient's epigenetic marks and the age characteristics, thereby aiding in the investigation of sporadic forms of the neurodegenerative diseases which may result in part from the age and epigenetics of a patient. Nevertheless, without a specifically defined genetic insult or other ways to reducing cellular heterogeneity[44], a large number of samples from different patients may be required to overcome variability to define the specific cause of the sporadic disease.

In addition to employing human iPSC-derived neurons in 2D cultures for drug discovery, technologies have also been developed to use these cells to generate 3-dimensional (3D) neuronal organoids and spheroids[45-47]. Culturing cells in 3D may better mimic the microenvironment, cell-cell interactions, and neuronal circuitry found *in vivo* and can result in increased neuronal maturity[48]. Recently human neuronal spheroids modeling AD were adapted into a HTS format for screening assays including neuronal death[49]. iPSC-derived organoids can be engineered to form tissues representing different regions of the brain, such as the cortex[46] for studies on AD, and the midbrain to study nigrostriatal DA neurons[45], the most vulnerable population in PD[50, 51]. Importantly, in addition to expressing more complex neuronal circuity than 2D cultures, organoids have the advantage of longevity, providing an approach to study the gradual neurodegenerative process found in many diseases[52].

Microfluidic and microarray technologies have been developed to employ organoids in HTS formats, using morphological and functional responses of the neurons as phenotypic endpoints for drug screening, including electrical activity and Ca^{2+} transients, neurite outgrowth, and cell viability[47, 53, 54]. Though brain organoids will likely become more commonplace as drug screening platforms as the technology continues to develop, like the approaches described above, there are limitations and drawbacks to using them (Figure 1). These included the lack of uniformity of samples, the relative immaturity of the cells, and the appearance of internal necrotic tissue due to lack of vasculature.

3. CELL DEATH SCREENING ASSAYS

3.1 High throughput cell death screening assays:

Standard cell death assays measure morphological changes of neurons using dyes such as propidium iodide, 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst and calcein acetoxymethylester (calcein-AM)[3, 4]. DAPI and Hoechst are nucleic acid stains, and generally can't enter living cells, but does enter and mark the nuclei of dying cells in which the plasma membrane integrity is compromised. Similarly, SYTOX (ThermoFisher) is impermeant to live cells and will fluorescently label DNA of dead cells[43]. In contrast, Calcein-AM is a cell-permeant probe[35] that easily crosses cell membranes in living cells, where it is cleaved and becomes fluorescent, indicating cell viability. Calcein-AM can be coupled with non-cell permeant red-fluorescent ethidium homodimer-1 that fluoresces when binding DNA to indicate loss of plasma membrane integrity to give a live/dead classification. These assays can be coupled with terminal dUTP nick-end labelling (TUNEL)[4, 55], which monitors the DNA fragmentation that precedes death. TUNEL staining was used as a readout of death in a screen of inhibitors of β-Amyloid induced toxicity in human iPSC-derived neurons[56].

However, the use of these stains to monitor cell death has important limitations. Many dyes, including Hoechst type dyes, have significant non-specific binding that reduces the signal-to-noise ratio. Dyes are generally not well suited for the long-term types of experiments that are often needed in studying neurodegeneration. Fluorescence tends to fade over time because of photobleaching or metabolism by the tissue, requiring periodic additions of fresh dye, which can increase the complexity of the experiment. In addition, nominally cell-impermeant dyes can leak into cells over time, causing false positive signals and potential damage when they intercalate into DNA. Finally, neurodegenerative diseases unfold slowly and neurons may exhibit a continuum of progressive dysfunction before they are considered unequivocally dead and all biological activities cease. Therefore, live/dead cell stains will give signals much later than the point at which the first signs of dysfunction and dysmorphology are evident (Figure 2).

In most drug screens, cell death assays are more frequently used to weed out compounds that produce neurotoxicity rather than to discover drugs that block neurodegeneration[57]. However, cell death assays are almost ubiquitously used in low throughput characterizations of disease models of neurodegeneration. This puzzling discrepancy between the infrequent use of cell death for therapeutic screens and its widespread use as a diagnostic in disease model characterization could be due to a common belief that disease-specific mechanisms well preceding cell death are more viable targets of therapy. It also could reflect the idea that a perturbagen that blocks cell death without correcting any underlying dysfunction may have limited utility. Importantly, neuronal death remains a hallmark of most neurodegenerative diseases, and is often a better correlate of clinical symptoms than other hallmarks of disease such as Lewy bodies in PD[58], or β-Amyloid in AD[59]. Thus, it remains a possibility that better assays to detect neuronal death in a neurodegenerative disease context could lead to the discovery of new therapeutic compounds that reduce cell death by correcting the underlying pathophysiology. More directly, methods for detecting phenotypes that predict cell death are likely to help elucidate which upstream abnormalities and pathways should be focused on to correct the underlying pathology.

3.2 High throughput screening for cell death precedents

3.2.1. Apoptosis.—HTS assays are available that measure activity of caspases that cleave protein substrates involved in apoptosis[2]. The activity can be quantified by immunological methods to detect cleaved activated forms of the caspases, by changes in levels of pro-luminescent or fluorogenic caspase substrates[60], or by Förster resonance energy transfer (FRET) assays that measure changes in proteolysis at caspase cleavage sites between the FRET partners[61]. Annexin V, which binds phosphatidylserine, an extracellular marker of apoptosis, has also been used in a HTS format to measure apoptosis[62]. However, the use of apoptotic readouts can be problematic as cellular events that are commonly associated with apoptosis have also been shown to occur in non-apoptotic cell death and physiological events, and neurodegenerative cell death is not exclusively apoptotic[4, 63, 64].

3.2.2 Metabolic Activity and Mitochondrial Function—Assays that measure diminished metabolic activity of neurons prior to cell death have also been used to monitor neurodegeneration. One measure of metabolic activity is the production of ATP in neurons;

as neurons die, their capacity to generate ATP decreases. The number of viable cells in culture can be determined based on a luciferase reaction to measure the amount of ATP from viable cells. ATP production can also be monitored with a genetically encoded FRET-based ATP sensor composed of the ε-subunit of the bacterial F_OF_1-ATP synthase sandwiched by cyan- and yellow-fluorescent proteins[65]. However, multiple factors can affect ATP production in neurons independent of cell death, which must be taken into consideration when employing this assay to discover drugs to block neurodegeneration[3].

Neuronal survival is highly dependent on mitochondria due to their high energy requirements, as mitochondria produce most of a neuron's ATP[50]. Mitochondria are particularly important for energy-dependent functions in nerve terminals and axons, including maintenance of Ca^{2+} and other ion gradients needed for transmitter release, uptake and storage. Mitochondrial dysfunction plays a key role in ALS, HD, PD and other neurodegenerative diseases[50, 51], and assays to monitor mitochondrial dysfunction could be used to identify drugs that slow early molecular events involved in neurodegeneration.

One measure of mitochondrial function is maintenance of mitochondrial membrane potential (MMP)[66, 67]. Reduced MMP can lead to gradual breakdown of the organelle and reduced ATP production. It can also result in increased production of toxic reactive oxidative species (ROS) and other reactive molecules that are known to cause neurodegeneration and death. Multiple assays are available to monitor MMP and mitochondrial function. The most well-known employs 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[68]. MTT is a colorless salt that is readily taken up by living cells and converted by mitochondrial reductases into colored compounds that can be easily quantified by measuring their absorbance using a spectrophotometer. Reduced MTT readouts are indicative of impaired mitochondrial function, and thus it can be used as a readout of cell viability[69]. MMP can also be measured using the cell permeable dye tetramethylrhodamine ethyl ester (TMRM) which can be adapted to screening assays of neurons[70]. Mitochondrial dysfunction is often paralleled by changes in mitochondrial morphology and fragmentation[71] which can be imaged and assessed by mitochondrial matrix-targeted fluorescent proteins such as Mito-GFP or by transmembrane-insensitive mitochondriophilic dyes, but which is difficult to correlate with neuronal death[72]. Finally, assays are available to monitor increased levels of intracellular ROS as a measure of mitochondrial dysfunction, for example by using the cell-permeant probe dihydroethidium.

3.2.3. Neurite retraction.—Changes in neurite area have also been employed as a morphological readout of early stages of neurodegeneration in imaging assays for neurodegeneration. Neurite outgrowth occurs in healthy neurons during development and is needed for formation of synapses and neuronal circuits, thus neurite retraction can indicate synapse break down and impaired neuronal health. One problem with this hypothesis is that during synapse formation, pruning can result in neurite retraction even though the neuron itself if functional and healthy. Furthermore, neurite area is in constant flux as the activity of the neuron changes, so static assays that only monitor neurite area as a specific time point might miss the dynamic changes in the neurite over time. However, in general neurite retraction has been viewed as a measure of impaired neuronal health and has been suggested as a reliable early marker of neurodegeneration[73, 74].

High content automated imaging technologies have been developed to quantify changes in neurite area or axonal length to discover drugs that can promote neuronal regeneration after injury or block neurodegeneration in disease. Using rodent primary neurons, Al-Ali et al have executed multiple screens of compounds that promote neurite outgrowth[75-78]. In a HT format to screen hundreds of thousands of compounds for the ability to enhance neurite outgrowth from primary neurons, they identified compounds with nanomolar potencies and they were able to use their HTS format to perform chemical deconvolution and structure-function analysis to identify drugs that promote nerve regeneration[78]. Similarly, Claus-Peter's group used an automated imaging technology to screen the 440 compound NIH Clinical Collection on primary rodent cochlear spiral ganglion neurons to identify drugs that facilitate neuronal regeneration after cochlea injury to promote recovery of hearing loss[79]. This study identified a single compound that promoted neurite extension that was mimicked by other structurally similar compounds. Additionally, Joseph Lewcock's group employed an automated neurite retraction assay to screen over 800 compounds for the ability to regulate axon degeneration of rodent primary dorsal root ganglion neurons[80]. A few compounds were identified that increased neurite outgrowth and were also effective *in vivo* in promoting neurite growth after nerve crush injury. With the advent of iPSC technology, HTS of neurite retraction have also been adapted to human neurons. Anne Bang's group[9] employed a HT neurite assay to screen a small molecule library of over 4000 compounds to identify drugs that affect neurite growth of human iPSC-derived neurons. The hit compounds affected neurite area by targeting known pathways involved in neurite extension and retraction. With the ability to generate neurons in quantities compatible with HTS screening, and the relative ease that quantification of neurites provide, HTS screens of changes of neurites in iPSC derived neurons will likely continue to be a popular means of drug discovery.

Neurite assays have also been employed to identify drugs that block neurodegeneration. For example, Gunnar Hargus's group showed that iPSC-derived neurons from patients with FTD exhibited tau pathology and decreased neurite extension as well as impaired mitochondrial function compared to control iPSC-derived neurons [81]. Additionally, Haruhisa Inoue's group generated motor neurons from iPSCs derived from patients with familial ALS harboring TDP43 mutations[82]. The neurons expressed TDP43 aggregates and exhibited neurite retraction compared to controls, and these phenotypes could be reversed by the histone acetyltransferase inhibitor anacardic acid. Taken together, these findings supported the use of the neurite assay to discover drugs that slow neurodegeneration.

3.2.4 Functional assays to monitor neurodegenerative processes.—In addition to monitoring biochemical and morphological changes, automated fluorescent microscopy can be used to detect changes in neuronal prior to their degeneration. A number of studies have suggested that neuronal hyperactivity may play a key role in neurodegeneration in AD, ALS and FTD[13, 14]. Patients with mild cognitive impairment in the early stages of AD display hippocampal hyperactivity and epileptiform activity years before overt cognitive impairment or neuronal loss is detected[14]. Murine models of AD also show epileptic activity prior to overt pathology, and the antiepileptic drug levetiracetam reduces abnormal spike activity, synaptic dysfunction, and deficits in learning and memory[18]. Similarly, altered

activity of motor neurons has been proposed to contribute to neurodegeneration in ALS[12]. Nerve conduction studies evaluating the axonal threshold in ALS patients demonstrate increased axonal membrane excitability, and the degree of hyperexcitability correlates with patient survival[12, 13]. Importantly, human iPSC-derived neurons from patients with ALS and FTD also show hyperactivity that precedes death[19, 40, 83-85], and in the case of ALS motor neurons, treatment with retigabine reduces the hyperactivity and slows neurodegeneration[19]. Thus, screening for compounds that reduce neuronal hyperactivity in cell-based assays could identify compounds that reduce neuronal death and degeneration in multiple neurodegenerative diseases.

Ca^{2+} transients highly correlate with voltage activity in neurons, and dye-based calcium indicators can be used to assay neuronal activity. Fura and Fluo dyes are commonly employed to monitor neuronal activity and have been employed by the pharmaceutical industry to identify neuroactive drugs and test drugs for potential neurotoxicity[86]. The approach is also useful in studying neurodegeneration. Recently, Daniel Ursu's group adapted the use of Fluo4-AM for high throughput screening of neuronal function and synaptic transmission in cultured primary rodent neurons[87]. The application of this screening platform to neurodegenerative disease models could help identify therapeutic compounds by reducing hyperactivity phenotypes.

Genetically encoded calcium indicators (GECIs) provide a targetable, less toxic alternative to monitor calcium transients. Recently developed GCaMP variants are more sensitive at detecting calcium than dyes and can be used for longitudinal Ca^{2+} imaging, whereas dyes need replenishment over time and can lead to toxicity[17]. Because they are genetically encoded, researchers can restrict the expression of GECIs to cell types of interest using cell type-specific genetic enhancers and promoters and can be subcellularly targeted to monitor organelles or microdomains within the neuron such as the cell membrane or mitochondria[88]. Recently, Shi et al. used GCaMP to show that human iPSC derived-motor neurons from ALS patients with C9 mutations have increased Ca^{2+} transients in response to glutamate challenge compared to controls[84]. These authors also found that the enhanced Ca^{2+} response was associated with an increased frequency of action potentials and shorter survival times. Retigabine reduced the hyperactivity of the ALS iPSC derived-motor neurons and increased their survival, supporting the hypothesis that the abnormal activity was related to degeneration of the ALS motor neurons and drugs targeting this hyperactivity may be neuroprotective.

More recently, genetically encoded voltage indicators (GEVI)s have improved in quality and signal allowing more direct imaging of neuronal activity[17]. Furthermore, the addition of an optogenetic protein in combination with a spectrally separated GEVI, a technology called Optopatch[16], provides the ability to both optically stimulate and record from individual neurons, pushing the boundary for imaging technology and phenotypic screening of neurodegenerative disease lines. For example, Adam Cohen's group used Optopatch technology to show that i-motor neurons from ALS patients with the disease-causing superoxide dismutase (SOD1) A4V mutation exhibited higher spontaneous activity and greater firing rate at low stimulation than genetically corrected isogenic lines[85].

While the improvements in sensitivity and signal of GECIs and GEVIs make these sensors amenable to screening assays[89, 90], there remain a number of practical and technical problems with using functional assays as screens for neurodegeneration or cell death. Monitoring neuronal activity with either GECIs or GEVIs requires a rapid frame rate of imaging over time, requiring complex imaging setups that make screening complex. Additionally, although neurons in culture or in 3D organoid models recapitulate some of the hyperexcitibility phenotypes observed in patients and neurodegenerative disease models, it remains unclear whether compounds that affect neuronal activity in culture would have the same effect in the more complex 3D circuitry of the brain. The use of organotypic brain slices could help in this respect, but rapid imaging in three dimensions requires even more sophisticated imaging. Furthermore, the relationship between hyperexcited neuronal activity and cell death remains correlative and complex, and a specific pattern of altered neuronal activity that precedes neurodegeneration or cell death has not been established[91]. General inhibitors of excitotoxicity have not shown much therapeutic potential for the treatment of stroke[92], suggesting gross hyperexcitibility is unlikely to be a therapeutically useful target in a neurodegenerative disease context. A more nuanced understanding of specific signatures of neuronal activity that correlate with neurodegeneration and neuronal death is needed to define disease-relevant activity patterns that could be modulated for therapy.

4. LONGITUDINAL ANALYSIS OF NEURONAL DEATH IN DRUG DISCOVERY

4.1 USE OF ROBOTIC MICROSCOPY FOR LONGITUDINAL SINGLE CELL ANALYSIS

Neurodegeneration is a gradual process, and rates of degeneration may vary depending on cell type (*e.g.,* long projecting motor neuron versus interneurons) and the particular disease in question. Furthermore, even in the same disease and the same neuronal type, neuronal death can be asynchronous. As most methods available to monitor the neurodegenerative processes are static, they only provide snapshot images of neurodegeneration.

To provide a more dynamic snapshot of neurodegeneration, robotic microscopy (RM) was developed to monitor neurodegeneration over time. The RM instrument consists of a custom-built automated platform that collects images from individual neurons[93]. Cells are transfected with fluorescent markers in 96 or 384 well plates and imaged, then automatically returned to a cell incubator. At defined time periods the plate is returned to precisely the same microscope field repetitively, so that fluorescence signals from biosensors introduced into individual cells can be collected at regular intervals in HT and analyzed off-line. Automated analysis programs identify individual cells in each image, assign them a unique identifying number, track them over weeks or months and stitch together the images of each neuron in each well to create a series of montages of the life history of each labeled neuron. Using this technology, small molecule drugs, including autophagy inducers, were shown to slow neurodegeneration of human ALS motor neurons, and human HD striatal iPSC-derived neurons [31, 32, 34]. This supports the utility of the technology for the discovery of neuroprotective drugs.

4.2 KM and CPH analysis to determine risk of death

One of the advantages of RM is the ability deduce the causality of specific cellular mechanisms towards neurodegeneration. With RM, the lifetimes of thousands of individual neurons can be recorded daily, greatly increasing the statistical power, and longitudinal data from each neuron are analyzed by powerful statistics (Kaplan-Meier (KM) for survival and Cox proportional hazards analysis (CPH)). This approach reduces cell-to-cell variability in the data analysis because each cell is its own control, resulting in a 100–1000-fold increased sensitivity to detect the effects of disease causing genes or drug treatments over more conventional approaches[30, 93, 94]. Further, CPH and recently developed Bayesian hierarchical approaches[32, 95, 96] allow for the construction of accurate quantitative dynamic predictive models to identify variables directly from complex datasets rapidly and in parallel, rather than artificially "holding" some variables constant (Figure 3). Because of the statistical power to resolve cause and effect, RM data have called into question the etiology of the progression of neurodegenerative diseases. For example, aggregated forms of mHtt are commonly found as inclusion bodies (IBs) in neurons of HD patients and were thought to be the primary cause of neurodegeneration based on gross correlations between IB formation and neurodegeneration at a population level. RM and longitudinal single cell analysis was used to show that the formation of IBs is a coping mechanism of neurons to slow neurodegeneration rather than a causative factor of HD neurodegeneration[30].

4.3 Protein and mitochondria turnover.

In addition to monitoring cell death and changes in neurite area, RM can be employed to simultaneously monitor practically any other biomarker expressed in a neuron and correlate it with risk of death. This technique has been specifically used to monitor protein and organelle turnover in single neurons using optical pulse labeling (OPL)[32-34]. To monitor protein turnover, the photoswitchable probe Dendra2 is fused to the C-terminal fragment of degron (CL), which targets proteins for proteasomal degradation. To monitor protein turnover, neurons transfected with Dendra2-CL are subjected to brief illumination with short-wavelength visible light to cause an irreversible conformational change ("photoswitch") in green Dendra2, making it emit red fluorescence[32]. After photoswitching, red fluorescence is measured in individual neurons, with loss of red fluorescence used as a measure of degradation of CL over time. Determining the half-life of Dendra2-CL provides an estimation of the proteasome activity in single cells. This method is uniquely suited to RM, because it often requires longitudinally monitoring the same neuron for red Dendra2 over long periods (i.e., a week) which is not easily determined using other optical imaging techniques.

To measure autophagic flux, another photoswitchable probe EOS3.2 is fused to LC3, as the half-life of LC3 is a measure of autophagy. This single-cell OPL assay is sensitive to genetic manipulation and small-molecule regulators of autophagy, and compares well to metabolic pulse-chase methods[32-34]. Thus, RM can monitor the major protein clearance pathways in neurons with single cell resolution. Since risk of death and neurite area can be monitored in the same cell, and RM provides an approach to determine whether there may be links between impaired protein clearance, the buildup of toxic disease-causing proteins and neurodegeneration.

OPL can also be used to monitor the turnover of disease-causing misfolded proteins, such as alpha-synuclein[33], mutant Huntingtin (mHtt)[32] and TDP43[34], in single neurons to study mechanisms by which these proteins induce neurodegeneration. In studies with a mHtt-Dendra2 assay, it was shown that turnover of the HD-causing protein varies with cell type and, using Bayesian regression modeling, it was determined that the mean lifetime of mHtt in neurons was a greater predictor of neurodegeneration than absolute levels of mHtt in a single neuron[32]. This is important because most available technologies can only measure mHtt levels. OPL provides a unique opportunity to determine which cellular actions of disease-causing proteins are most critical in influencing the health of neurons and causing neurodegeneration, and provides an assay format to discover drugs targeting those cellular pathways to slow neurodegeneration.

4.4 Organotypic Slice culture.

RM has mainly been employed to study neurons in 2D cultures. However, recent advances in the technology have provided an approach to study neuronal death of individual neurons in organotypic brain slices[97]. By preserving the 3D structure and connectivity of the tissue, organotypic slice culture enables simultaneous visualization of different cell types, including neurons, neuronal precursors, microglia and astrocytes in a defined circuitry. Moreover, organotypic slice culture can be prepared from primary human tissue and murine models of any genetic background, including models that show reduced postnatal survival. The ability to monitor neuronal morphology and health over 19 days in brain slices provides a unique opportunity to monitor the slow changes in neurons and neuronal circuitry induced by disease-causing proteins. In this study, the 4-Dimensional RM (4D RM) instrument was used to monitor neurodegeneration in mouse hippocampal slices induced by mutant Huntingtin (mHtt), the protein that causes HD. Using time-lapse imaging, the gradual formation of IBs in the neurons expressing mHtt was shown, and over a 14 day period they detected increased risk of death in mHTT expressing neurons, as had previously been shown in 2D culture systems. These findings indicate that with 4D RM, it is possible to track cellular phenotypes in both rodent and human brain slices over protracted time-frames at resolution and scales necessary to deconvolve the sequential events underlying cellular and molecular changes in much the same manner that RM has been used to study neurons in 2D culture, but with the added benefit that cells of interest are maintained within tissue.

With the spatial and temporal resolution of RM, it will be possible to study the synaptogenesis involved in circuit formation *in situ* and investigate how circuitry becomes stabilized via reinforcement. Using the neurite retraction assay of RM, it will also be possible to monitor the breakdown of synaptogenesis in circuitry in diseases such as AD and FTD, and well as the loss of motor unit integrity in ALS. Furthermore, because assays in slice culture can be scaled up for high-throughput drug screens[36] the 4D RM imaging can provide a unique approach to identify drugs that target specific circuitry rather than indiscriminately affect all neurons in the brain.

5. CONCLUSION

Neurodegenerative diseases have a major impact on patients, their families, and society, and with no therapies available to slow the progression of these diseases, new and innovative technologies are necessary to develop effective treatments. The assays that have been developed to discover drugs to treat neurodegenerative diseases fall into two basic categories, those that measure the end stage of neurodegeneration—cell death—and those that monitor earlier stages of degeneration including changes in cell function and morphology. Given the limited regenerative capacity of the nervous system, assays that monitor earlier stages of neurodegeneration might hold greater promise in identifying disease modifying drugs. However, the earlier the stage of neurodegeneration being monitored, the more distant, complex, and less direct the relationship between that phenotype and neurodegeneration. As neuronal death represents a clear disease-relevant end point for neurodegeneration, its relationship to any early stage phenotype should continue be taken into consideration.

The development of new models of neurodegeneration that accurately recapitulate complex neurodegenerative disease-related phenotypes is critical to further our ability to screen for effective therapeutics for these devastating diseases. The establishment of human disease iPSC-derived neurons that show neuronal dysfunction similar to that found in patients has already provided more clinically relevant models systems to discover neuroprotective drugs. Additionally, advances in the 3D culturing techniques and aged organotypic slice culture models could provide needed context for the interpretation of neurodegenerative disease-relevant phenotypes. Finally, identifying cellular pathways linked to neurodegeneration will lead to new molecular targets, and targeting shared mechanisms of degeneration could be effective in treating multiple neurodegenerative diseases.

6. Expert Opinion

While an endless list of neurodegeneration-associated phenotypes can be screened, cell death remains an important translational endpoint for the generation of neurodegenerative disease therapeutics. Even if a successful and potent therapeutic to a neurodegeneration-associated phenotype were to be developed, if it is unable to reduce neuronal death, it would be unlikely to have long-lasting therapeutic potential. Thus, the analysis of the linkage between a neurodegenerative phenotype and its relationship to the risk of death in neurodegenerative disease models is crucially important in assessing the relevance of a phenotypic marker (Table 1).

With the use of advanced statistical models and time lapse microscopy[33, 94, 96, 98], the direct links between neurodegenerative disease phenotypes and cell death can be more clearly established prior to screening. Nevertheless, the current deficiencies of neuronal cell death markers will continue to hamper these efforts. For instance, off-target effects of dyes will continue to confound screens for cell death, and the delay in activation of live-cell neuronal death markers reduces the precision and accuracy of measurements, limiting the ability to statistically link and derive neurodegenerative disease-associated phenotypic markers for screening. A clear need for novel, more accurate and less toxic markers of neuronal death in

live imaging studies is needed to improve these studies. Furthermore, a cell death reporter must be able to multiplex with the phenotypic marker in order to establish the precise relationship between death and that phenotype on a single cell level, which is often limited by the availability of compatible non-overlapping fluorophores in the imaging spectra. Additionally, some common biomarkers such as GECIs will illuminate when a cell is dead, potentially giving a false positive signal and confusing activity with death. The recent application of deep learning artificial intelligence technology to automatically and non-invasively identify dead neurons and phenotypic markers from imaged neurons could provide the missing link in the ability to acutely identify neuronal death in a HTS screening capacity[99]. Furthermore, the application of deep learning analysis to predict future events based on time lapse imaging could provide a more informed link between a neurodegenerative disease phenotype and cell death and improve the ability to screen for therapeutic compounds.

The advent of human patient-derived iPSCs and organoid models of neurodegenerative disease has brought new life to neurodegenerative disease modeling and screening studies. For the first time, neuronal death can be analyzed and HTS screens can be performed on cells with human genetics. In addition, the ability to differentiate iPSCs to cell populations of different human organs and tissues such as liver, cardiac myocytes, kidney and gastrointestinal tract enables researchers to conduct mini-safety trials, preemptively excluding consideration of hit compounds that produce off-target actions[100].

In some cases, disease phenotypes of the human iPSC-derived neurons can only be observed when the cells are stressed, such as when challenged with glutamate[40, 84], which can confound drug screening. Furthermore, as most neurodegenerative diseases are late onset, the ability to mature and age iPSC-derived neurons in culture is a key challenge in modeling. However, the creation of 3D organoids seems to greatly increase the maturity of iPSC-derived neurons, and represents the state of the art of human neurodegenerative disease modeling. Their ability to scale up organoids could provide a new platform for therapeutic drug screening.

While links between neurodegenerative disease associated phenotypes and cell death may be more disease relevant in 3D imaging models such as organotypic slice culture and organoid imaging, they may also be more difficult to define. Imaging in 3D is more difficult in part because higher excitation parameters must be used to image cells within tissue, but these are associated with increased photo-toxicity. Furthermore, light scattering through 3D tissue can create a blur of out of focus and scattered light in imaging that can be difficult to reduce without post-hoc 3D deconvolution. For assaying cell death, cell death reporting dyes have limited permeability into tissue. The adaptation of deep learning analysis to 3D culture will likely play a key role in quantitatively analyzing cell death and the relationship between cell death and its precedents. With these and future technological advances in computer vision, we are optimistic that a more complete picture of the mechanisms underlying neurodegeneration as well as the tools to analyze screens of 3D imaging models will help drive the development of therapeutics for neurodegenerative disease.

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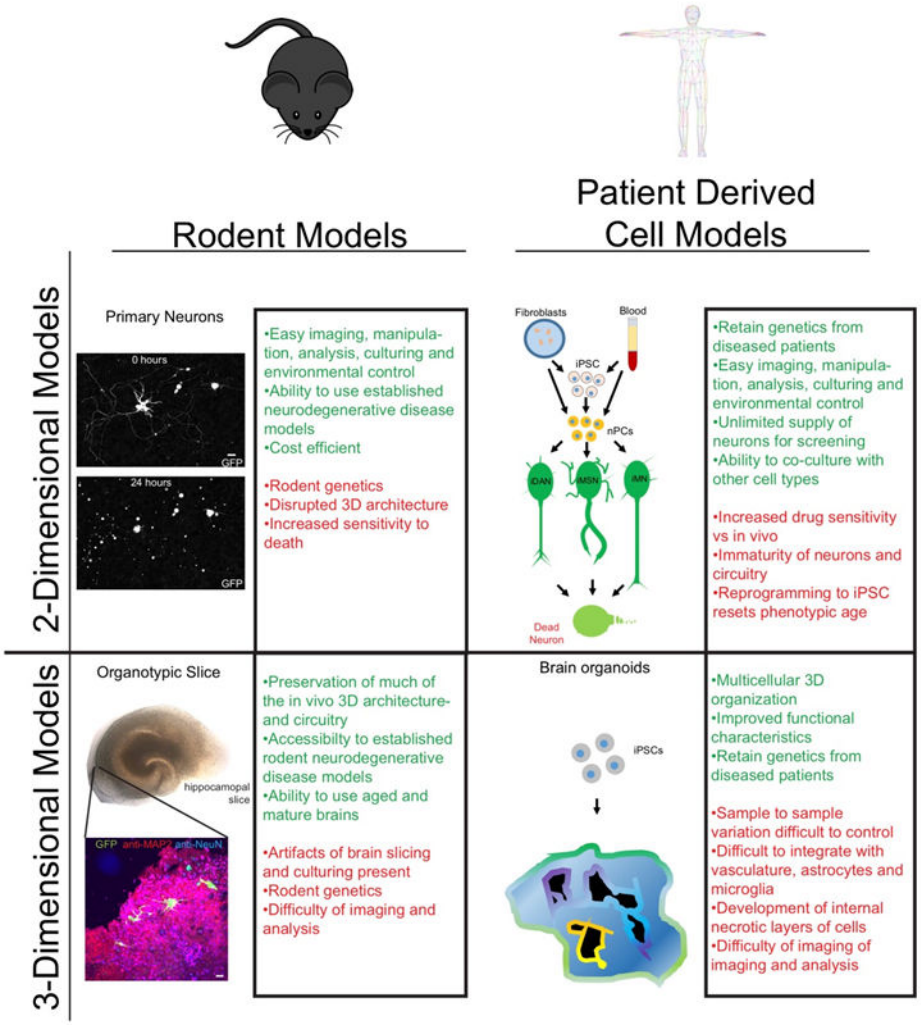

Fig 1.

Advantages and disadvantages of models for cell death assays for neurodegenerative disease drug discovery. Advantages are in green and disadvantages are in red. (Top left) Rodent Primary neuron models of cell death. Image shows mouse primary cortical neurons transfected with GFP and imaged at 0 and 24 hours (Scale= 50μm). (Top right) Schematic of patient derived iPSCs used for cell death assays. (iPSCs= induced pluripotent stem cells, nPCs= neural progenitor cells, iDAN= iPSC derived-dopaminergic neurons, iMSN= iPSC derived-medium spinal neurons, iMN= iPSC derived-motor neurons). (Bottom left) Representative organotypic hippocampal slice (top) and a zoom in of a region transfected with GFP and stained with anti-MAP2 (neurites) and anti-NeuN (neurons) (Scale= 100μm). (Bottom right) schematic of the brain organoids.

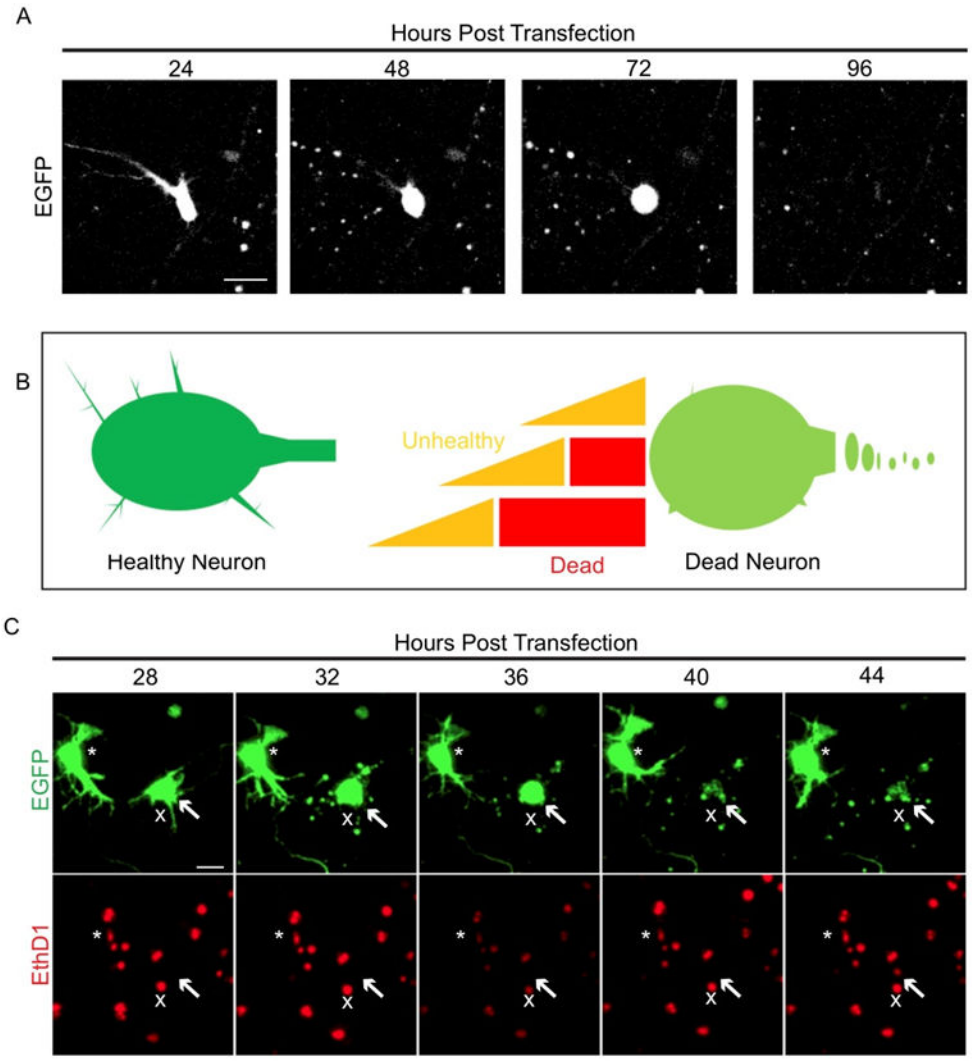

Fig 2.

Difficulty of determining the time of death in cell death assays of neurodegenerative disease. A) A single degenerating neuron transfected with EGFP imaged from 24-96 hours post transfection shows signs of degradation including shortened neurites (asterisk), and rounded soma (x) before disappearing at 96 hours. Scale= 50μm. B) Schematic showing the difficulty in differentiating the unhealthy state versus death in the neuron from (A). There are many different and complex ways a neuron can die, schematized here as “unhealthy”. Yet the physiology of live and dead neurons are intrinsically and fundamentally different, and their conflation while analyzing cellular processes could confound the interpretation of the factors underlying neurodegeneration. C) Two neurons transfected with EGFP and stained with Ethidium Heterodimer D1 (EthD1) imaged every hour from 28-44 hours post transfection. The neuron on right (arrow) shows signs of degeneration beginning at 32 hours post transfection but does not show associated EthD1 signal until 44 hours post transfection. Asterisk marks a second neuron that does not die and shows no EthD1 signal and X marks a untransfected, EthD1+ neuron that is dead before onset of imaging. Scale= 25μm.

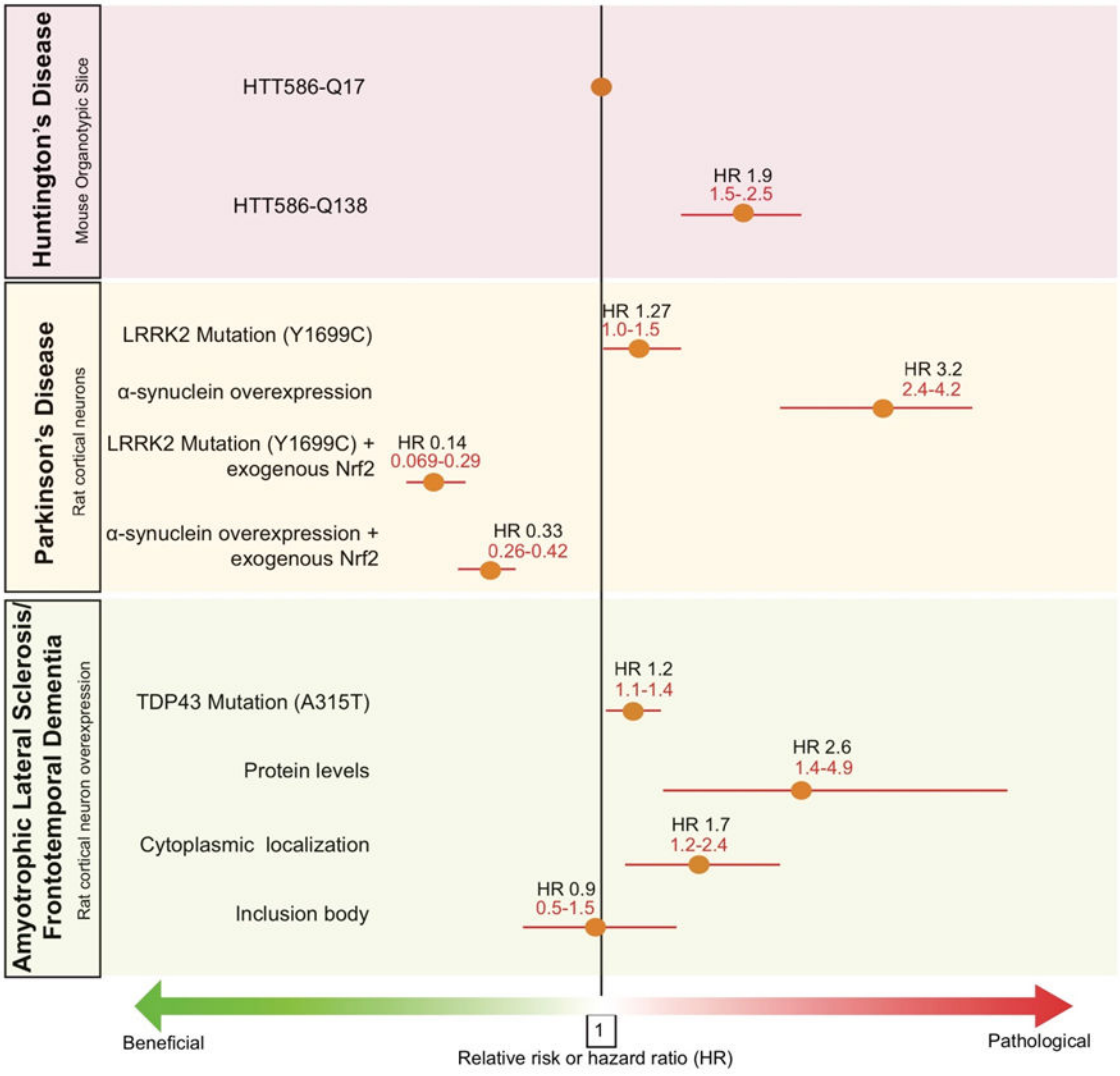

Fig 3.

Quantification of factors and treatments important for neuronal survival in neurodegenerative disease models. Schematic of hazard ratios (HR) of individual factors, red bars and intervals represent 95% confidence intervals. (Top) Quantification of the impact on neuronal survival in organotypic slice culture expressing a disease-associated N-terminal fragment of huntingtin protein containing 138 polyglutamine (polyQ) expansions associated with pathology (HTT586-Q138) compared to a normal 17 polyQ (HTT586-Q17), as control[97]. (Middle) Quantification of the impact on neuronal survival in rat cortical primary neurons in Parkinson's disease models expressing leucine rich kinase-2 (*LRRK2*) (Y1699C) mutation, overexpressing α-synuclein, or overexpressing nuclear factor erythroid 2-related factor (Nrf2) with LRRK2 (Y1699C) mutation or α-synuclein[33]. (Bottom) Factors important for neuronal survival in a model of ALS/FTD. Schematic of hazard ratios (HR) of individual factors relating to survival of a primary rodent model of TDP43 A315T overexpression[34].

Table1.

Referenced studies using cell death and functional assays for neurodegenerative disease drug discovery.

Direct Link to Neurodegeneration (Death)	Use in studies of Neurodegenerative Disease	Screening Assay
Death-DNA accessibility Dyes		
-TUNEL		56
-Hoechst/DAPI/PI/SYTOX/EthD/Calcein	40,43,82	43,47,70
Loss of transfected FP fluorescence/Morphological Features	97,29,30,95,32,33,41	31,34,35,36
Indirect Link to Neurodegeneration	**Use in studies of Neurodegenerative Disease**	**Screening Assay**
Metabolic activity and Mitochondrial Disfunction		
-TMRM	66,67	70
-MTT		69
Neurite Retraction	10	9,76-82
Protein and Mitochondria turnover	32-34	31
Functional Assays		
-Calcium Imaging	84	87
-Voltage Imaging	85	

Due to limitations of space, this table represents references used in the manuscript that are representative of the categories, and is not meant to be comprehensive. TUNEL= terminal dUTP nick-end labelling, PI= propidum iodide, DAPI= 4′,6-diamidino-2-phenylindole, FP= Fluorescent protein, TMRM= tetramethylrhodamine ethyl ester, MMT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.