Title

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Journal

FEBS Letters, 587(8)

ISSN

0014-5793

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Publication Date

2013-04-17

DOI

10.1016/j.febslet.2013.02.043

Peer reviewed

FEBS Letters 587 (2013) 1139–1146

journal homepage: www.FEBSLetters.org

Review

Longitudinal measures of proteostasis in live neurons: Features that determine fate in models of neurodegenerative disease

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ARTICLE INFO

Article history:
Received 6 February 2013
Accepted 21 February 2013
Available online 1 March 2013

Edited by Wilhelm Just

Keywords:
Longitudinal
Survival
Proteostasis
Primary neuron

ABSTRACT

Protein misfolding and proteostasis decline is a common feature of many neurodegenerative diseases. However, modeling the complexity of proteostasis and the global cellular consequences of its disruption is a challenge, particularly in live neurons. Although conventional approaches, based on population measures and single "snapshots", can identify cellular changes during neurodegeneration, they fail to determine if these cellular events drive cell death or act as adaptive responses. Alternatively, a "systems" cell biology approach known as longitudinal survival analysis enables single neurons to be followed over the course of neurodegeneration. By capturing the dynamics of misfolded proteins and the multiple cellular events that occur along the way, the relationship of these events to each other and their importance and role during cell death can be determined. Quantitative models of proteostasis dysfunction may yield unique insight and novel therapeutic strategies for neurodegenerative disease.

1. Introduction

Proteostasis involves a dynamic and highly integrated network of millions of proteins. Multiple cellular processes, intricately integrated, ensure homeostasis [1]. Breakdown of the network leads to cellular dysfunction and cell death [2]. Much effort has focused on determining if disruption of proteostasis is causally linked to neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) [3]. Neurodegenerative diseases normally present late in life with different symptoms, but they all involve deposits of insoluble protein in the brain [4]. At a molecular level, these diseases, also termed proteinopathies, are caused by distinct proteins, but they all undergo protein misfolding, show similarities in the multiple cellular pathways that are disrupted, and eventually lead to neuronal death [5]. Despite this convergence in cellular consequences, strategies to enhance proteostasis have not been translated into therapies.

Recently, considerable interest has been directed towards modeling disease to capture early changes and the temporal and spatial progression of dysfunction and adaptive responses, and ultimately, to relate these events to cell death [6,7]. Models that more faithfully recapitulate the complexity of the disease may improve the success rate of biomedical drugs [8]. Here, we will discuss the properties of proteostasis and neurodegeneration that make them difficult to model and describe a "systems" biology approach to model their complexity.

1.1. Modeling the complexity of proteostasis in single cells

Given the complexity of proteostasis, determining how proteins misfold and why cells fail to handle them is a challenge. The presence of misfolded proteins is probably a consequence of opposing pressures on structural stability and functional flexibility [9]. As the abundance of a protein imposes a stronger evolutionary pressure on its coding sequence than its actual function [10], the cost of protein misfolding to the fate of the cell may be high. In a cell, the accumulation of misfolded protein reflects a decline in the cell's ability to maintain proteome stability. In some models, only a 4 °C increase is enough to destabilize at least 16% of the proteome [11], suggesting that even small perturbations can greatly affect the proteome. Common causes of neurodegenerative disease favor protein misfolding, including mutations in disease-associated proteins and exposure to environmental stimuli, such as oxidative stress [12,13].

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0014-5793
http://dx.doi.org/10.1016/j.febslet.2013.02.043

Most of our knowledge about the folding and unfolding dynamics of proteins is based on in vitro biochemical approaches and in silico studies [14,15]. These methods have provided a wealth of knowledge and shaped a number of concepts surrounding protein folding, including the role for intermediate species [16] and folding energy landscapes [17,18]. These studies often focus on proteins that fold rapidly and rarely misfold and aggregate [16]. However, in neurodegenerative diseases, conformational instability and aggregation prevail [19] and the folding landscapes and intermediate species of disease-related misfolded proteins may require a different understanding. A remarkable number of disease-associated proteins are intrinsically unstructured and exhibit conformational promiscuity. Although this allows for multitasking, mutations that disrupt function may lead to collapse of a network of cellular processes [20].

In vitro biochemical and in silico approaches also lack the cellular milieu that is essential for protein dynamics. Post-translational modifications and intracellular crowding of macromolecules, including chaperones, affect protein interactions [21] that, in turn, influence the folding rates [22], stability, and function of proteins [23–25]. Ideally, physiologically relevant measurements of proteostasis should be carried out in live cells. There is a growing interest in modeling biological systems through a "middle-out" approach, in which the cell is the basic unit of the system and contains spatiotemporal information at multiple levels [26]. Information quantified at each level of the cell can be used to build predictive models that measure the effects of misfolded proteins on the cell. At a genetic level, modifiers can influence the cell's capacity to cope with misfolded proteins. For example, in an ALS model, temperature-sensitive mutations in various unrelated genes enhance misfolding of superoxide dismutase 1 (SOD1) [12]. At a molecular level, misfolded proteins can be measured to determine how processes, such as transcription, translation, folding, trafficking, and degradation affect their dynamics [1]. Conformational sensors can be used to measure rates of misfolding [27] or the effects of protein misfolding on proteome stability [28]. Reporters can also provide readouts for the activation of adaptive strategies, including the heat shock and unfolded protein responses [29], or mechanisms that target misfolded proteins for degradation [30]. These pathways are critical for modulating protein misfolding and toxicity in multiple models of neurodegeneration, such as ALS, PD, and HD [31–34]. Molecular complexes and organelle dynamics can also be measured in a cell, providing insight into the multiple cell processes that coincide with the build-up of misfolded proteins, including mitochondrial dysfunction, aberrant trafficking, synapse dysfunction and altered signaling [35].

Using the cell as the basic unit of the system to measure the impact of protein misfolding requires the simultaneous capture of both the dynamics of the misfolded protein and the stochastic cellular changes that result. In addition to identifying how misfolded proteins cause cell dysfunction, it is also critical to determine which cellular events drive neurodegeneration. Although some cellular events may be harmful, some changes may be incidental or even adaptive responses to more subtle maladaptive changes elsewhere in the cell.

1.2. Separating pathogenic events from adaptive strategies

Neurodegeneration is progressive and may occur along a single pathway or multiple distinct cellular pathways that arise from the same initial insult [36]. In addition, the roles of the cellular events during neurodegeneration may differ. Some may reflect true pathogenic insults, whereas others may be beneficial, adaptive strategies that are up-regulated to cope with the build-up of misfolded proteins [29] (Fig. 1). The extent to which a true coping response is activated is dependent on the pathogenic event that incites it.

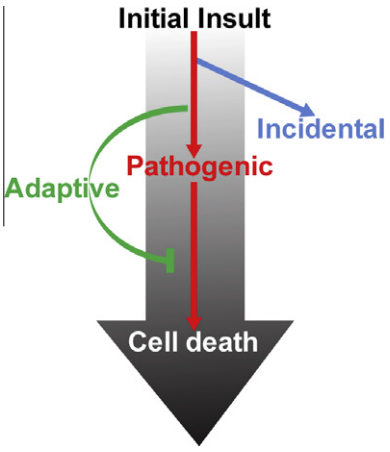

Fig. 1. The different roles of cellular events during neurodegeneration. Schematic shows how, during the course of neurodegeneration, cellular changes may be pathogenic. Alternatively, some changes may be incidental events, while others may be adaptive responses that occur to cope with the pathological events within the cell. As these events may occur in parallel and correlate with cell death, distinguishing their roles can be difficult.

Therefore, pathogenic events and coping responses will occur in parallel, and both will correlate with cell death, making separating their roles during the disease process very difficult.

For example, amyloid-like structures form as a common feature of many neurodegenerative diseases [3]. Early reports implicated amyloid deposits as the toxic species because they were consistently found in the brains of deceased patients. At the time, this seemed to be a reasonable conclusion. The distribution of various pathologies provides temporal resolution of the activities of disease-related proteins within those pathological changes. However, any given sample represents only a single "snapshot" in the life of the protein. In addition, conclusions based on pathological events from postmortem tissue might represent a bias of ascertainment: the tissue comes from patients who have already lost many of the specific neurons affected by the disease. Furthermore, although amyloid deposits were found in many deceased patients, many non-toxic proteins also form amyloid structures [37]. Mounting evidence suggests that amyloid structures sequester toxic misfolded conformers and principally serve as a coping response by the cell [38–40]. This adaptive response is becoming an increasingly common theme in the study of the major neurodegenerative disorders [41].

The unfolded protein response might also be activated in response to rising levels of misfolded protein in the cell [42]. It is mediated, in part, by phosphorylation of the α-subunit of eukaryotic translation initiation factor (elf2α-P), which is found in greater amounts in AD and PD patients than in non-patients and causes the transient shutdown of protein translation, including that of the misfolded protein [43,44]. Protein translation requires a great deal of energy [45], and repressing translation allows reallocation of molecular chaperones to detect and respond to protein misfolding elsewhere in the cell [46] and to promote the selective translation of stress-response genes [47]. However, repressing translation for too long can be detrimental to the cell. Accumulation of the misfolded prion protein is associated with synaptic dysfunction, neurodegeneration and persistent translational repression of global protein synthesis by elf2α-P. Interestingly, irrespective of the presence of the misfolded prion protein, stimulating protein translation preserved synapses and rescued neurodegeneration [48]. It remains unknown if restoring translation prevents neurodegeneration in the long term, even if the initial insult, misfolded prion protein accumulation, is not directly addressed.

To determine whether an event is detrimental, adaptive or simply incidental requires a method that (a) measures levels of misfolded proteins and cellular responses simultaneously in the same cell and (b) links these events to when the cell dies. In addition to determining the role of cellular events, longitudinally tracking cells captures whether an adaptive response is time dependent (e.g., whether the beneficial effect declines or even becomes harmful to the cell when chronically activated).

1.3. Harnessing cell-to-cell variation

The stochastic nature of pathological events often confounds the investigation of proteostasis dysfunction in neurodegeneration. Even within uniform cell populations, stochastic cell-to-cell variation results from intrinsic fluctuations in the levels of gene transcription and translation [49], or extrinsic fluctuations, such as variations in numbers of RNA polymerase or ribosomes [50]. The biological consequences of cellular variation are not clear. Cell-to-cell variation in protein expression is proportional to protein abundance [51] and protein stability [52] and is influenced by transcriptional bursting [53]. Variation may have evolved to promote beneficial diversity [54] and enable the evolution of stable phenotypic switches [55]. The noise in dynamic populations of proteins ultimately leads to the coexistence of different gene-regulatory network states and signaling systems [56,57].

Interestingly, the level of protein variation may be reflected in the function of the protein. For example, levels of non-essential stress-related genes vary more than predicted, potentially reflecting the benefit of variation in this class of genes. In contrast, essential genes involved in protein degradation and synthesis vary less, potentially reflecting their need to be more precise [51]. Alternatively, cell variation may have to be overcome to achieve robust function. Cell variation increases with age and is further exacerbated by oxidative stress [58], two common risk factors for neurodegeneration.

Studying how cells control, tolerate, and harness variability is a challenge. Conventional cell-based approaches often average measurements over populations, whereby cell-to-cell variability is treated as a technical rather than biological variation. As such, proper statistical treatment of variability reduces the sensitivity and power to detect differences in mean responses between populations of cells. Determining the variability between cells with respect to a phenotypic characteristic requires quantitative analysis in a large number of cells over long periods of time [51]. Developing experimental methods that quantify cell-to-cell variation and relate it to biology at a single-cell level would enable the source and dynamics of variation to be harnessed, and would enhance the power and sensitivity to investigate the role of cellular states during neurodegeneration.

1.4. Why is protein misfolding so common in neurons?

Accumulation of misfolded proteins indicates a failure in the network of processes that recognize, re-fold and degrade misfolded proteins. Why neurons are particularly susceptible to this failure is unclear. Although common to neurons, deficiencies in proteostasis are also observed in other cell types leading to diseases, such as type II diabetes, cancer, cystic fibrosis, cardiovascular disease, and multiple myeloma [59], suggesting similarities in the composition of the proteostasis machinery and adaptive responses across different cell types. However, in neurons, components of the proteostasis machinery – including autophagy [60] and the heat shock response [61,62] – are regulated differently. Differences in proteostasis capacity may also extend to different subtypes of neurons. Disease-associated proteins are often expressed in multiple cell types, and often at much higher concentrations than in the specific neuron subtype that is preferentially affected in the disease [63,64]. Also within neurons, changes in synaptic strength are regulated by local control of degradation [65]. Controlling proteostasis at a subcellular level requires directional transport of mRNA and translational machinery to and from the soma to distal dendrites [65], potentially increasing sensitivity to proteostasis dysfunction.

Neurons also influence misfolding by non-cell-autonomous mechanisms. In *Caenorhabditis elegans*, modulating signaling in neurons influences activation of the heat shock response [66], protein aggregation, and toxicity in neighboring somatic cells [67,68]. Neurons are also post-mitotic cells, so misfolded proteins may accumulate due to an age-dependent decline in the proteostasis machinery and quality control mechanisms [59,69]. In transgenic models, accumulation of misfolded proteins is linked to the age of the animal [70], which could explain why most neurodegenerative diseases have a late onset and the aging process itself acts as a major risk factor [71]. Given the unique proteostasis characteristics of neurons, establishing models in primary neurons may be essential to investigate why and how misfolded proteins accumulate during neurodegeneration.

To faithfully study proteostasis in live neurons, we need a longitudinal approach that captures the dynamics of misfolded proteins and their effects on multiple cellular processes. Cell-to-cell variation needs to be harnessed to increase the power and sensitivity in determining which events drive pathogenesis, and which are incidental or adaptive. This will be critical for determining the influence of proteostasis decline during neurodegeneration and for developing therapeutic strategies. A systems cell analysis approach, known as longitudinal survival analysis, may fill this gap.

2. Systems approaches in cell-based models: systems cell biology

Systems biology approaches that combine experimental and mathematical tools are increasingly attractive for capturing the complexity of disease [72,73]. In conventional approaches to study mechanisms of dysfunction, a single cellular feature or biological process is perturbed, while other variables are kept constant, and a phenotypic outcome is observed. There are at least two problems with this approach. Efforts to keep other variables constant often involve highly reductionist systems or constraints that reduce the physiological relevance of the findings. Even then, the relevant biological network is often not fully understood, so critical variables may remain uncontrolled, confounding the interpretation of the results.

Alternatively, systems approaches address biological complexity by gleaning insights from direct measurements and sophisticated mathematical analysis of many components of biological networks, without overly constraining or oversimplifying them experimentally. Modeling cellular systems as a network enables multiple cellular features or transient cell states to be observed simultaneously. Single phenotypic endpoints are replaced by temporal phenotypic profiles [74], enabling genetic or environment perturbations to be reflected as changes in network components, their interactions and overall network structure. Modeling disease as a perturbation of cellular networks is proving to be a successful strategy for dissecting disease mechanisms and developing new therapeutic strategies [75,76]. Unlike conventional single-target strategies, multi-target drug strategies identified by network-based analyses favor therapies that target less essential components of the network, thereby offsetting the potential for side effects [77]. Also, systems approaches might be particularly well-suited for phenotypic screens, which returned to popularity after it was discovered that they are a greater source for FDA-approved first-in-class drugs than more conventional target-focused screens [78].

By using the "middle-out" approach to model biological systems, where the cell is the basic unit of the system [26], information can be collected at multiple scales, including transcription, translation, complexes, organelles and metabolism. In addition, large numbers of individual cells lead to cell–cell and cell–environment interactions that provide information on homeostasis or dysregulation of the cellular community [26]. Unlike traditional "top-down" or "bottom-up" systems biology approaches, "middle-out" approaches overcome cell-to-cell variation, as information is extracted from single cells. A big challenge for systems cellular biology is to develop methods that identify and probe network components and quantify their dynamic interactions after multiple perturbations [6].

3. Longitudinal survival analysis: a systems cell biology analysis approach

Longitudinal survival analysis consists of high-throughput and high-content analysis platforms that enable single cells and their individual characteristics to be longitudinally tracked and linked to when the cell dies [7,79–81]. In parallel, the development of powerful survival statistical tools enables predictive temporal relationships between extracted cellular features and a specified endpoint to be quantified (e.g., determining if a cell with high levels of a misfolded protein is likely to die sooner than a cell with lower levels). Cell death is an obvious endpoint for studying neurodegeneration, but the event could be anything that is observed in a cell, including formation of a protein inclusion, synaptic loss, mitochondrial alterations, changes in neurite structure or activation of a specific pathway. As the cell contains information at multiple levels, once a specified endpoint is chosen, cause-effect relationships across multiple cellular features and the endpoint can be determined simultaneously, enabling the establishment of a cellular systems model. Perturbations of the cellular system by disease-causing mutations or extrinsic factors (including environmental stress or therapeutic agents) are reflected by changes in the importance and roles of the predictive relationships between cellular features and the endpoint. The power, sensitivity, and novelty of longitudinal survival analysis emerges from the integration of physiologically relevant neuronal models of disease, high-throughput imaging and high-content analysis platforms, and powerful statistical survival models.

3.1. Physiologically relevant cell models of disease

A predictive, sensitive, and robust model of neurodegeneration must recapitulate key features of the disease, such as neurite loss, protein aggregation, and neuron death. Establishing models using single cells provides information at multiple scales with significant control and rigor. Although harder and more expensive to handle, the focus has shifted from immortalized cell lines to the use of primary neurons, which are more physiologically relevant [82]. With the discovery of induced pluripotent stem cells (iPSCs) [83,84], multiple subtypes of human primary neurons that display cell-type-specific characteristics can be cultured at will [85–87]. More excitingly, subtypes of neurons can be differentiated from iPSCs derived from patients with neurodegenerative disease. Many studies have already demonstrated that these cells recapitulate features of the disease [88–91]. iPSC technology enables the establishment of human models, even of the sporadic disease. It also allows us to understand the extent to which disruption of proteostasis is a common thread even in cases where the cause of neurodegeneration is not known precisely.

The use of longitudinal survival analysis is not limited to dissociated primary neuron cultures. Organotypic cultures that retain features of multicellular brain tissue, including 3D microculture systems, brain slices or simple organisms, could be used and would provide significant information on non-cell-autonomous mechanisms that may influence proteostasis [67,68]. In vivo imaging by two-photon confocal microscopy has already enabled the longitudinal tracking of cellular events, including aggregation, degradation, and localization of misfolded proteins in models of neurodegeneration [92–94]. However, more elaborate models require more effort to set up and maintain. Furthermore, their throughput is much lower, reducing the sensitivity and predictive power in determining cause-effect relationships.

3.2. Custom-built automated robotic microscope systems

Unlike conventional cellular perturbation studies that extract information at a single point, longitudinal survival analysis tracks individual cells to capture spatial and temporal changes within the cells (Fig. 2). Individual neurons studied using longitudinal survival analysis can be tracked by customized automated robotic microscope systems built in our lab [7,80,81], combined with primary neuron cultures transfected with fluorescent reporters. The low transfection efficiency often associated with primary neurons is optimal for longitudinal survival analysis because it makes it easier to distinguish and therefore automatically identify, track, and characterize each neuron independently. Our microscope platforms and in-house software acquire highly focused epifluorescence images in an automated and high-throughput manner, enabling tens of thousands of cells to be imaged and tracked [81]. One of the unique features of this system is that a fiduciary mark is taken of each plate at the start of the experiment, enabling the plate to be realigned to exactly the same location even after the plate has been removed and then replaced onto the microscope [7]. This is essential if the same field of individual cells is to be followed for days, weeks, or months.

3.3. Defining cellular profiles by fluorescent reporters

As the automated microscope images the same neuron repeatedly during its lifetime, the gradual and stochastic cellular events that occur during proteostasis dysfunction and neurodegeneration are also imaged [7]. Capturing these cellular events in single cells and then recording when or if the cell dies are essential if the role of an event during cell death is to be determined [7,80,95]. As fluorescent proteins (FPs) are expressed diffusely throughout the cell, loss of fluorescence is a read out for changes in morphology, such as neurite loss and cell death, two common features of neurodegeneration (Fig. 2A) [79]. Fluorescence in a cell also indicates the location of a cell relative to others in the field. This could be critical for determining the importance of non-cell-autonomous mechanisms of neurodegeneration, such as whether glia have a protective or destructive role [96,97] or if intercellular transfer of pathogenic proteins is important for disease progression [98]. Loss of synapses is another common feature of neurodegenerative diseases [99]. Numbers of synapses per neuron can be assessed by transfection of fluorescently tagged marker proteins of synapses, such as synapsin-1 or postsynaptic density protein 95 (PSD95). As individual cells are tracked longitudinally, synapse formation, maintenance and loss can be quantified during the cell's life, and the significance of these processes during neurodegeneration can be determined.

Although FPs can be attached to any protein, caution must be exercised to ensure that the FP and its location on the protein does not significantly alter its physiological function, including its subcellular localization and turnover in the cell [100]. However, by attaching a FP to a disease-associated protein, longitudinal survival analysis can be used to capture the dynamics of the protein and determine the role of its transient states, in real time, during neurodegeneration. This approach was used to investigate how

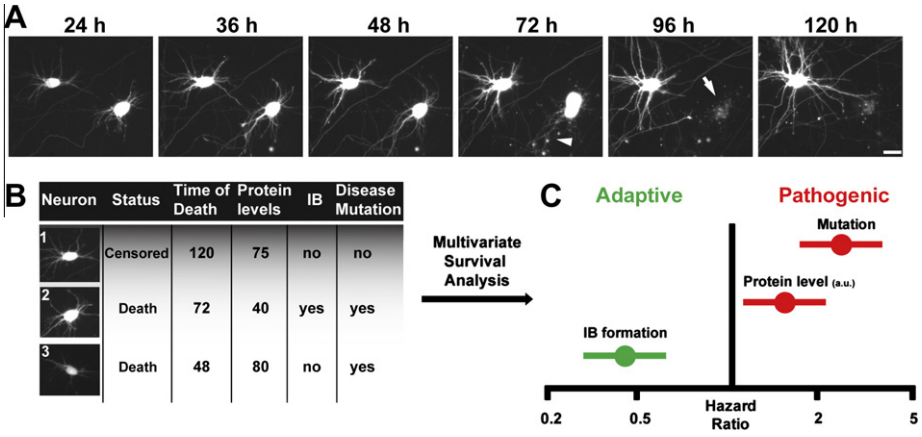

Fig. 2. Longitudinal survival analysis. (A) Representative time-lapse image panel of primary rat neurons transfected 6 DIV with mRFP, which is diffusely expressed throughout the neuron. Images of the same neuron were taken once every 12–24 h. RFP expression indicates neurite retraction (arrow head) and cell death (arrow). Bar is 10 μm. (B) Each individual neuron is tracked and custom-analysis programs extract spatial and temporal fluorescence data for their time of death, along with multiple cellular features. (C) Data from thousands of neurons incorporated into a multivariate regression survival model. For each cellular event, its role (defined in terms of Hazard Ratio (HR) below or above 1) and importance (magnitude of the HR) during neurodegeneration is calculated (IB is inclusion body).

localization, aggregation and dose of disease-associated proteins affect cell survival in models of HD, PD and ALS [32,79,101]. Photoswitchable FPs, such as Dendra2 or Eos2, can also be used to follow a stable intracellular population of proteins, enabling measures of protein turnover within single cells. Analysis of GFP-degradation rates in single cells indicates that they are independent of initial intensity of GFP fluorescence [102]; however, it remains unknown whether this is true for disease-associated misfolded proteins. Furthermore, it is unknown how rates of decay of disease-related proteins predict the fate of the cell.

FPs can also act as sensors to quantify conformational changes and rates of misfolding in proteins. Such sensors, in which protein tetracysteine motifs bind the biarsenical dyes ReAsH and FlAsH with high specificity, have been used to measure conformational changes of mutant Htt in single cells [27]. FPs attached to destabilized proteins that misfold in the presence of disease-associated proteins, such as polyglutamine (polyQ) expanded Htt, or small-molecule inhibitors of proteostasis. Their propensity to misfold is a measure of global consequences to overall proteome stability and can potentially predict widespread cellular dysfunction [28].

FPs can also be used to quantify the size, morphology or function of cellular organelles. For example, longitudinal survival analysis was used to infer the importance of mitochondrial fragmentation during α-synuclein-mediated toxicity [103]. Expression of FPs can be put under the control of promoter regions of specific genes and used to report and quantify the activity of specific pathways. A reporter gene comprising of a gene encoding a FP under the control of the antioxidant response element (ARE) is able to quantify the cell's response to stress. NF-E2-related factor-2 (*Nrf2*) binds to the ARE in multiple genes, activating cytoprotective mechanisms against an array of cellular stresses including misfolded proteins, such as the PD-associated protein α-synuclein [104–106] (Fig. 3).

Finally, photostable and bright fluorophores that span the breadth of the visible and infra-red spectrum [107] enable sufficient spectral separation so that multiple channels can be imaged simultaneously without bleed-through. This allows multiple fluorescent reporters to be co-transfected and followed in each cell. For example, changes in multiple cellular pathways or processes can be captured simultaneously along with changes in localization, aggregation or degradation of the disease-associated protein. Custom analysis programs can then extract spatial and temporal fluorescence data for multiple cellular features, providing each individual neuron with a multiparametric fluorescence profile

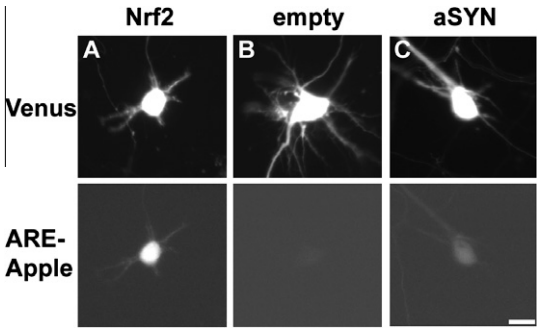

Fig. 3. A fluorescence reporter measures ARE activity in live neurons. Representative images of rat neurons transfected with Venus (to mark neuronal morphology) and ARE-Apple, a reporter of ARE activation. (A) Cells overexpressing Nrf2 have a strong activation of the ARE-Apple reporter compared to cells expressing the control vector (B). (C) Cells transfected with the PD-associated protein α-synuclein show an increase in fluorescence of the reporter, indicating that ARE has been activated by stress caused by misfolded proteins. Bar is 10 μm.

[81]. Most crucially, each individual cell profile is linked to the ultimate fate of the cell, enabling statistical models to determine the roles of changes in cellular processes or dynamics of the disease-associated protein during neurodegeneration.

4. Applying statistical survival tools to single cell analysis

Once individual cells have been longitudinally tracked and imaged, spatial and temporal fluorescence data are extracted from the multiparametric fluorescence profiles of individual neurons (Fig. 2A). Extracted data include information on cellular variables that can be discrete (e.g., the presence or absence of a protein aggregate) or continuous (e.g., the amount of α-synuclein expressed in the cell). Statistical tools can be used to model the relationship between multiple cellular features, and their relationship to a defined endpoint, such as death. A distinguishing feature of survival analysis is that it accounts for individuals that may not reach the defined endpoint (e.g., cells that are still alive at the end of the experiment) by censoring them (Fig. 2B). The Kaplan–Meier method and Cox proportional hazard analysis are commonly used in clinical medicine to investigate the role of cellular variables on survival [108,109]. Cox analysis can be used to quantify the difference in hazard, also known as a hazard ratio, between two or more groups (Fig. 2C). For example, cells expressing a disease-associated

mutation in the ALS protein TDP43 had a significantly greater hazard ratio (HR 1.225) than control neurons [101].

4.1. Determining predictive relationships between cell events and an endpoint

In contrast to linear or logistic regression analysis, survival regression models can be used to predict if a cellular event predicts a specific end point (e.g., does the cellular event increase or decrease the chance of death?), and if it does, what is that magnitude of this effect (e.g., how much does the presence of the cellular event increase the risk of death) [110]. Often cellular features are stochastic events, with the same event occurring in different cells at different times. Irrespective of whether a change is pathogenic or a coping response, it would be expected to appear specifically after disease has begun and would track the disease severity pretty closely. Conventional cell models are often based on population or gross correlations that lack the resolving power to tell the two apart. To separate the roles, you need to identify an event that is unequivocally pathogenic (e.g., cell death) and then measure the role of putative contributing factors by determining the extent to which their appearance accelerates or retards the unequivocal pathogenic event. Those that accelerate are predicted to be pathogenic; those that retard are likely coping responses. Thus, the resolving power depends on the ability to measure the time that elapsed between two key events in the same cell. Although defining predictive relationships is a powerful approach, we are still defining statistical associations and so cannot assume certainty for the role of the cellular events on the endpoint. It still remains possible that the cellular event is inextricably linked but not directly involved in the pathway that accelerates or retards the endpoint.

For example, pathological analyses of HD patients demonstrated that the abnormal desposition of mutant huntingtin (Htt) protein into inclusion bodies (IBs) grossly correlated with disease progression [111]. To determine if these deposits are pathogenic, incidental or represent a coping mechanism to handle the more toxic species of mutant Htt, individual neurons expressing mutant Htt were followed throughout their lives by longitudinal survival analysis. Unlike conventional methods, survival analysis can account for intercellular variability to precisely identify which cells form an IB and when, providing increased sensitivity and power that enables determination of the relationship between IB formation and neurotoxicity. Surprisingly, in neurons expressing mutant Htt, those that formed IBs had a lower risk of death then those without [79].

Up-regulating the degradation of misfolded proteins reduces toxicity in many models of neurodegeneration [32,33]; however, improved cell survival may be at the expense of synaptic function [112]. Mutations in leucine-rich-repeat kinase 2 (LRRK2), a common genetic cause of PD, causes age-dependent biphasic changes in autophagy activity [113]. It remains unclear what role autophagic activity plays during neurodegeneration. However, it is possible that its role may be time dependent, with its relationship to death weakening or strengthening with time. Longitudinal survival analysis follows neurons throughout their life, enabling the presence and magnitude of time-dependent effects to be quantified [114].

4.2. Controlling for confounding and competing cell events

An advantage of longitudinal survival analysis is the ability to control for critical variables that, if unconsidered, can conceal the true effect of another cellular event on death [115]. For example, if we want to determine how levels of Htt affect the risk of death of neurons we need to control for IB formation. Cells with IBs have a lower risk of death; however, increased levels of mutant Htt greatly enhance the chance of IB formation [79], making IBs a confounding effect in determining the relationship between Htt levels and neurotoxicity. Survival analysis can account for confounding effects, either by stratifying by this cellular feature or by including it as an independent variable in the survival model. In a model of HD, when IB formation was stratified, Htt levels were found to have a positive relationship with neuron death that saturated at longer polyQ lengths [116].

Defects in protein degradation pathways are linked to a number of neurodegenerative disorders [117]. Many conventional approaches that calculate degradation rates of misfolded proteins disrupt the cell for biochemical analysis and use results from the whole cell population as a read out [100]. In neurodegeneration, many disease proteins lead to increased rates of death and formation of aggregates (which poorly solubilize); this has an overall competitive effect, confounding population-based measurements of protein degradation rates. Alternatively, fluorescence from photoswitchable FPs attached to misfolded proteins can be analyzed with longitudinal survival analysis to capture the spatial and temporal dynamics of a stable intracellular population of proteins. As cell death and IB formation can be captured simultaneously, these competing effects are included and accounted for in the analysis to determine the degradation rate of the misfolded protein [118,119].

4.3. Constructing complex survival models

Imaging and tracking multiple cellular features simultaneously in a single neuron enables more than one cell feature to be included in the survival model. In this way, the effect of individual cellular features on neurodegeneration can be quantified relative to each other (i.e., can be used to determine which cellular feature plays a more important role during neurodegeneration). For example, in a model of ALS, survival analysis identified mislocalization and overall levels of TDP43 as positive predictors of death during neurodegeneration. However, it was only when both were included in the survival model simultaneously that mislocalization was found to be the more important predictor for toxicity than levels of TDP43 [101].

As the number of cellular events that are being tracked increases, modeling the relationships between cellular events becomes increasingly challenging. The relationship between variables may interact in a complex non-linear manner. Statistical tools, such as penalized splines [120], enable a more flexible relationship to be modeled between the variable and death, with both the linear and non-linear terms in the relationship being quantified. Alternatively, more complex survival models, based on Bayesian statistics [121], can be used to model parameter uncertainty by using prior probability distributions [122]. Such approaches can enhance the predictive power of the relationships between cellular features and neurodegeneration. The development of survival models to incorporate multiple cellular features, and the ability to model their complex relationships with each other and a specified endpoint, such as death, enables the emergence of a system cell biology approach to analyze proteostasis dysfunction during neurodegeneration.

5. Conclusions

Dysfunctions in proteostasis give rise to pathogenic and adaptive responses that occur in parallel and correlate with cellular decline. Features of these cellular responses such as their stochastic nature, sometimes confounding relationship to other responses, time-dependency or subtlety can obscure efforts to determine their true role during neurodegeneration. Conventional models

that rely on population-based or qualitative measures sacrifice resolving power, precision, and possibly physiological relevance for simplicity. Alternatively, a systems cell biology analysis approach, such as longitudinal survival analysis, enables the identification, tracking and quantification of cellular components, including the disease protein itself and changes in cellular processes. Through statistical modeling, the interaction and relationship of these components to each other, and to the fate of the cell, can be determined. As more cellular components are tracked, the model becomes more complex, until a sensitive network of cellular events emerges that can reveal more subtle yet significant predictive events during neurodegeneration. Monitoring network differences in the presence or absence of perturbations may reveal important insight into the underlying mechanisms and the discovery of potential genetic modifiers. A transition towards a systems cell biology approach could also enable the development of combinatorial therapies that target less essential parts of the cellular network to increase efficacy and decrease side effects [6].

Acknowledgments

We thank G. Howard and A.L. Lucido for editorial assistance and K. Nelson for administrative assistance. This work was supported by the NIH – National Institute on Aging grant 2P01 AG022074, Taube-Koret Center for Neurodegenerative Disease Research, the Hellman Family Foundation Alzheimer's Research Program, the ALS association, the NIH – National Institute of Neurological Disorders and Stroke Grants 2R01 NS039074 and 2R01 NS045091, and by the Keck Foundation (to S.F.).

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