PERSPECTIVE

Drosophila neuronal injury model allows for temporal dissection of neurodegenerative events

Neurodegenerative diseases are extremely prevalent in today's society and, according to the World Health Organization, are currently listed as the third leading cause of death, following cancer and heart disease (Gammon, 2014). The seminal characteristic of neuro-muscular degeneration is the complete disruption of the circuit between the brain, peripheral neuron, and muscle, which can be caused by toxic insult, genetic diseases, or trauma. The most well studied type of neurodegeneration is characterized by the catastrophic degeneration of the distal end of a severed axon. This type of degeneration was discovered in 1850 by Augustus Waller and is thus termed Wallerian degeneration (Stoll et al., 2002). On rare occasions, during Wallerian degeneration, the severed neuron will attempt to regenerate to repair the damaged circuit but most often does not succeed and results in degeneration. It is well known that the peripheral nervous system has better intrinsic regenerative capacities than the central nervous system, however the physical regeneration of neurons to their target site is rare in humans. Even after its discovery, 150 years into the future, scientists are still focusing on understanding the molecular mechanisms that drive the neurodegenerative process.

The large majority of neurological disorders are progressive in nature and do not have effective treatments, much less cures. One of the key pieces of information necessary for the development of effective therapies is a more complete understanding of the cellular and molecular mechanisms that cause neuronal degeneration and regeneration. Recently, neurodegenerative research has been surrounded by the extensive study of biomarkers for early detection of neurodegenerative diseases. Several cytoskeletal proteins, such as neurofilaments, tubulin, actin, and tau have known roles in maintaining the stability of axons and synapses and have therefore been investigated as early candidate biomarker proteins when the stability of the neuron has been compromised during neurodegenerative events (Höglund and Salter, 2002). It has been suggested that neurodegeneration may be caused by a mechanism specific to these support proteins. Additionally, synaptic makers, such as α-synuclein and N-acetylaspartic acid, have been used as biomarkers to examine neurodegeneration at the neuromuscular junction. However, only after a detailed description of the cellular events initiating and eventually causing neuronal degeneration and regeneration has been established, can researchers really begin to conduct more focused experiments on individual potential therapeutic target molecules.

Drosophila melanogaster have become one of the preeminent model systems to study neurodegeneration due to their short life cycle, exemplar genetics, vast array of available reagents and most importantly the similarities to the human neurodegenerative process (Fang et al., 2013). *Drosophila* provide researchers with several simple model systems from which to study a variety of neurodegenerative diseases ranging from Alzheimer's disease and Tauopathies to amyotrophic lateral sclerosis and prion diseases. Forward and reverse genetic screens, the use of transgenic animals, and the ability to test candidate therapeutics by pharmacological approaches have allowed researchers to make great strides in determining the molecular signaling involved in the neural injury response (Lu and Vogel, 2009). These simple models are capable of reproducing many complex neurodegenerative phenotypes also observed in humans. The trauma seen

Table 1 Temporal sequence of cellular events leading to neurodegeneration after mechanical injury at the neuromuscular junction (NMJ)

Injury phenotype	Timing of phenotype
Microtubule disruption	Immediately after crush
Neuroglian disruption	Immediately after crush
Mitochondria trafficking defects	6 hours post injury
Buildup of ubiquitinated proteins	12 hours post injury
Neurodegeneration	24 hours post injury

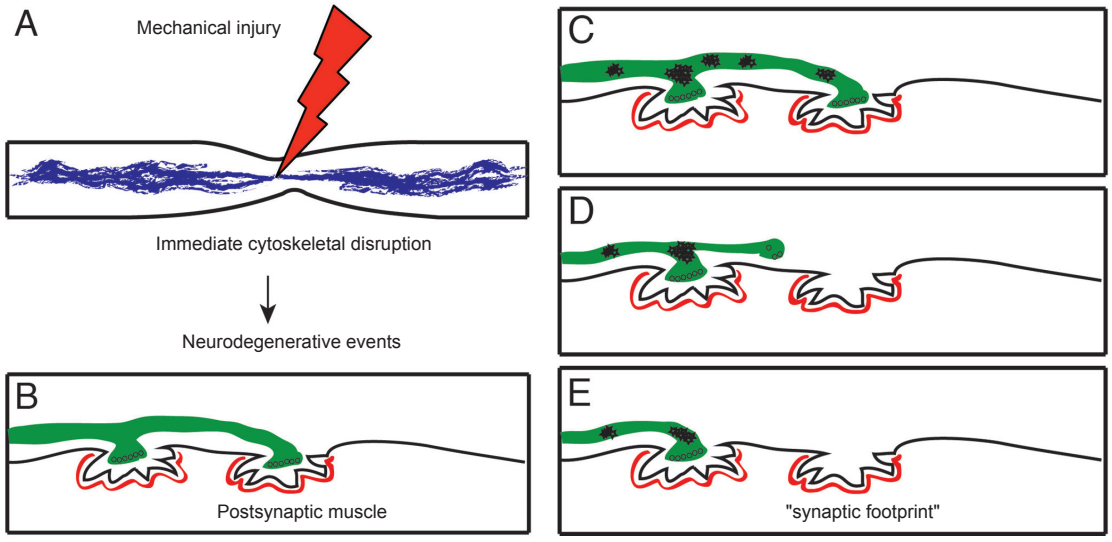

Figure 1 The temporal sequence of cellular events leading to neurodegeneration at the *Drosophila* neuromuscular junction (NMJ).
Our experimental data suggest a temporal sequence of cellular events initiated by mechanical neuronal injury. Mechanical neuronal injury induces an immediate disruption of the axonal cytoskeleton (A) but does not immediately affect the NMJ. The NMJ, composed of the presynaptic neuron (green) and the postsynaptic specialized muscle folds (red), appears normal (B) until 12 hours after injury (C) when ubiquitinated proteins (shown as black puncta) accumulate in the presynaptic compartment. Approximately 24 hours after injury, the NMJ begins to degenerate (D) leaving a "synaptic footprint" (E) which is utilized to quantify the severity of neurodegeneration 24–48 hours post injury.

in spinal cord injuries or traumatic brain injuries can be replicated using both mechanical and genetically induced injuries. The significant progress in neurodegeneration research would not be possible without the technological advances in imaging and staining. The advanced development of fluorescent proteins, live cell imaging technology, and super resolution microscopy have increased the rate of scientific discovery in the fields of neurodegenerative research (Toomre and Bewersdorf, 2010).

We have added to the comprehensive knowledge of how neurodegeneration and regeneration occurs in living animals by focusing our research on determining the spatial and temporal cellular events that occur after neuronal injury. We utilized a modified crush assay originally described by the Collins Lab (Xiong et al., 2010) in conjunction with the GAL4/UAS system in *Drosophila*. Our neuronal crush assay was conducted on second and third instar larvae by rolling them onto their dorsal side and compressing their motor neuron axon bundles through their cuticle using size 3 micro forceps. The larvae were then kept alive for 0, 6, 12, 24, or 48 hours to examine the temporal cytological events of the neurodegenerative process. Larvae were then dissected and stained for a variety of neuromuscular proteins such as the active zone protein Bruchpilot, axonal adhesion and cytoskeletal proteins Neuroglian and Futsch, the postsynaptic protein Discs-Large, and mono- and poly-ubiquitinated proteins. Laser scanning confocal microscopy was then used to characterize neurodegenerative phenotypes at individual axonal nerve bundles along with the synaptic terminals at the neuromuscular junctions (NMJ). Neurodegenerative events were quantified using an established assay, which takes advantage of the fact that presynaptic proteins and membrane disassemble more quickly than their corresponding post synaptic counterparts thereby leaving a "synaptic footprint" (Eaton et al., 2002) (**Figure 1**).

Our experimentation has led to the discovery of a temporal sequence of cellular events leading to neurodegeneration at the *Drosophila* NMJ (Lincoln et al., 2015). We have found that there is an immediate impairment of neuronal membrane at the site of mechanical injury along with a disruption of the microtubule cytoskeleton and a disruption in neuroglian, a conserved member of the L1 family of vertebrate neural cell adhesion molecules (**Figure 1**). After 6 hours post injury, there is an obvious buildup of mitochondria on both sides of the crush site, indicative of disrupted anterograde and retrograde axonal transport. Perhaps the most important discovery was the observation that ubiquitinated proteins accumulate at the NMJ after 12 hours post injury prior to the observation of any neurodegenerative events. The neurodegenerative events characterized at the NMJ did not occur until approximately 24 hours post injury (**Figure 1C–1E**) (Lincoln et al., 2015). The NMJs from uninjured animals show the presynaptic markers in perfect apposition with the postsynaptic markers. However, 24 hours post injury, NMJs exhibited various degrees of missing presynaptic markers at the NMJ ranging from moderate to severe neurodegeneration. Together, these data suggest a spatial and temporal sequence of cellular events originating at the site of axonal injury with immediate cytoskeletal defects inducing axonal transport dysfunction by 6 hours, followed by accumulations of ubiquitinated proteins by 12 hours, and subsequent neurodegeneration at the NMJ by 24 hours (**Table 1**) (Lincoln et al., 2015). The accumulation of ubiquitinated proteins at the NMJ prior to neurodegeneration may provide the scientific community with a novel biochemical biomarker for both diagnostic utility and potential drug development. A careful investigation of the proteasome and whether it is being overloaded by an abnormally large amount of ubiquitinated proteins would be interesting since this is a known stress factor in some forms of neurodegeneration (Lobanova et al., 2013).

The procedures conducted and the findings from our laboratory serve as a model for the detailed observational research necessary to provide the foundation for future studies honing in on the molecular mechanisms involved in initiating and causing neurodegeneration. The complexity and diversity of human neurodegenerative diseases prevent readily available treatments and/or cures however a detailed description of the spatial and temporal cellular mechanisms may lead to unforeseen revolutionary advances in neurodegenerative therapeutics. However, it is worthwhile to mention that these observations may lead to some particularly interesting future experiments. First, it will be very interesting to determine whether the cellular events leading to neurodegeneation observed at the *Drosophila* NMJ are conserved in other neurodegenerative contexts (*i.e.*, during synaptic degeneration within the central nervous system of higher organisms). Second, investigation of stress proteins, cytokines, and activation of the innate immune response after mechanical injury in our system may help address a growing body of evidence suggesting that activation of these proteins can impact neurodegeneration in humans.

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References

Eaton BA, Fetter RD, Davis GW (2002) Dynactin is necessary for synapse stabilization. Neuron 34:729-741.

Fang Y, Soares L, Bonini NM (2013) Design and implementation of in vivo imaging of neural injury responses in the adult Drosophila wing. Nat Protoc 8:810-819.

Gammon K (2014) Neurodegenerative disease: brain windfall. Nature 515:299-300.

Höglund K, Salter H (2013) Molecular biomarkers of neurodegeneration. Expert Rev Mol Diagn 13:845-861.

Lincoln BL 2nd, Alabsi SH, Frendo N, Freund R, Keller LC (2015) Drosophila neuronal injury follows a temporal sequence of cellular events leading to degeneration at the neuromuscular junction. J Exp Neurosci 9:1-9.

Lobanova ES, Finkelstein S, Skiba NP, Arshavsky VY (2013) Proteasome overload is a common stress factor in multiple forms of inherited retinal degeneration. Proc Natl Acad Sci U S A 110:9986-9991.

Lu B, Vogel H (2009) Drosophila models of neurodegenerative diseases. Annu Rev Pathol 4:315-342.

Stoll G, Jander S, Myers RR (2002) Degeneration and regeneration of the peripheral nervous system: from Augustus Waller's observations to neuroinflammation. J Peripher Nerv Syst 7:13-27.

Toomre D, Bewersdorf J (2010) A new wave of cellular imaging. Annu Rev Cell Dev Biol 26:285-314.

Xiong X, Wang X, Ewanek R, Bhat P, Diantonio A, Collins CA (2010) Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. J Cell Biol 191:211-223.