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Quantitative and semi-quantitative measurements of axonal degeneration in tissue and primary neuron cultures

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HIGHLIGHTS

- Quantitative stereological methods are described for measuring axonal degeneration.
- Image analysis methods are described for measuring axonal degeneration.
- Both methods are used in brain tissue sections or primary neuron cultures.

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ABSTRACT

Background: Axon viability is critical for maintaining neural connectivity, which is central to neural functionality. Many neurodegenerative diseases (e.g., Parkinson's disease (PD) and Alzheimer's disease) appear to involve extensive axonal degeneration that often precedes somatic loss in affected neural populations. Axonal degeneration involves a number of intracellular pathways and characteristic changes in axon morphology (i.e., swelling, fragmentation, and loss).
New method: We describe a relatively simple set of methods to quantify the axonal degeneration using the 6-hydroxydopamine neurotoxin model of PD in rats and a colchicine-induced model in primary rat neurons. Specifically, approaches are described that use the spaceballs stereological probe for tissue sections and petrimetrics stereological probe for cultured neurons, and image analysis techniques in both tissue sections and cultured neurons.
Results: These methods provide a mechanism for obtaining quantitative and semi-quantitative data to track the extent of axonal degeneration and may prove useful as outcome measures in studies aimed at preventing or slowing axonal degeneration in disease models.
Comparison with existing methods: Existing methods of quantification of axonal degeneration use densitometry and manual counts of axonal projections, but they do not utilize the random, unbiased systematic sampling approaches that are characteristic of stereological methods. The ImageJ thresholding analyses described here provide a descriptive method for quantifying the state of axonal degeneration.
Conclusions: These methods provide an efficient and effective means to quantify the extent and state of axonal degeneration in animal tissue and cultured neurons and can be used in other models for the same purposes.

1. Introduction

Axonal degeneration is a common feature in several neurodegenerative diseases (e.g., Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis and traumatic brain injury) and an important component of the neural disconnection that occurs in these diseases (Canuet et al., 2015; Luo et al., 2014;

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Shepherd, 2013). For example, the dopaminergic projections from the substantia nigra to the striatum are severely affected early during disease and degeneration of these projections precedes overt loss of cell bodies (Kordower et al., 2013). Similarly, the axons of neurons affected in AD show signs of degeneration early in the disease (Mattson, 2006). There has long been an appreciation that axons are particularly vulnerable to degeneration and that neurons with long projections are often those most affected in neurodegenerative diseases, but the majority of pathological studies focus on the loss of neuronal cell bodies. Axon degeneration appears to precede cell loss in several of these diseases and can proceed in either a retrograde ("dying-back") or anterograde direction from the site of insult or injury (Coleman and Perry, 2002). Interestingly, the molecular events that characterize injury-induced axonal degeneration are distinct from those involved in apoptosis, but the precise mechanisms involved in axonal degeneration in many common neurodegenerative diseases remain unknown (Sagot et al., 1995; Deckwerth and Johnson, 1994).

Axons tend to undergo a relatively consistent pattern of progressive physical changes during the process of degeneration. First, axons become dystrophic, as they swell along their length. Next, segmentation begins, creating thinned segments and larger rounded segments, known as spheroids. Spheroids are readily apparent features of axons that are undergoing degeneration. As degeneration progresses, the axons become physically fragmented, breaking apart at the thinned segments, leaving only the spheroids (Raff et al., 2002). Eventually, the axon debris is degraded beyond detection (Zhou et al., 1998). Other factors independent from the neuron itself can affect the health of the axon. The factors of demyelination associated with traumatic injury and autoimmune disease have significant effects on the health and structure of the axon (Armstrong et al., 2016; Alizadeh et al., 2015). Demyelination of axons can lead to a similar process of fragmentation and axon death. These morphological changes in the axons can be used to study the process and extent of axonal degeneration both in vivo and in vitro.

Despite the rapidly growing interest in studying axonal degeneration, a somewhat limited number of methods exist for quantifying axon degeneration in tissue sections. Axonal loss is often quantified using relatively straightforward image analysis techniques, such as optical density measurements of regional staining intensity for either a phenotype-specific marker or an axonal marker (Knoferle et al., 2010). Unfortunately, this approach does not provide information regarding morphological characteristics of the axons. A recently described method quantifies axons crossing through a single 30 μm thick tissue section using two parallel sampling lines (Cheng et al., 2011). Using this approach, axon degeneration is determined by manually counting all axons that remain intact while crossing both parallel lines. This method provides versatility as a relatively rapid and useful analysis of different axon projections in tissue; however, this method does not utilize random, unbiased, systematic sampling (i.e., stereology). The use of stereology to quantify degenerating axons would provide benefit to the field to standardize the approach to axonal quantification. Stereological quantification is the archetypical method by which degenerated neurons are quantified because of its efficiency in counting an unbiased sample of neurons for an accurate population estimation. The length of axons can be measured in both 3D tissue sections using the spaceballs stereology probe (West, 2013).

A number of methods are used to identify and quantify degenerating axons in cultured neurons. One of the simplest forms of imaging degenerating axons in cultures is to use phase contrast and manually score or count the extent of degenerating and intact axons. Alternatively, neuronal phenotype markers, structural proteins, or axon-specific markers can be useful to identify the process of axon degeneration (Zhai et al., 2003; Park et al., 2010). Use of Campenot chambers or more modern microfluidic device technology facilitates the examination of axons by physically separating somata from axonal projections in primary neuron cultures (Park et al., 2006; Gross et al., 2007). Some of the analysis methods are manual while others have varying levels of automation. For example, the AxonQuant image analysis method is designed for high-throughput analysis of microfluidic devices and uses a custom algorithm to deliver accurate measures of degenerating axons; however, the complicated design of this method makes it less approachable than simpler image analysis methodologies (Li et al., 2014). The length of axons can be measured in 2D monolayer cell cultures using the petrimetrics stereological probe (Howard and Reed, 1998).

Here, our goal was to establish methods for quantifying axonal degeneration using stereological and image analysis-based techniques in both tissue sections and primary neuron cultures. To this end, we chose to use a striatal 6-hydroxydopamine (6-OHDA) rat model and a colchicine-induced model in cultured neurons because each of these models produce clear and robust axonal degeneration (Javoy et al., 1976; Goldschmidt and Steward, 1980). We found that stereological assessment provided a quantitative measure of the overall length of intact and dystrophic/degenerating axons. The image analysis methods provided complementary semi-quantitative data on the extent of axon fragmentation and swelling of the axonal projections present. The methods described here are relatively straightforward and easily implemented to study axonal degeneration in both in vivo and in vitro models.

2. Methods

2.1. Animals

Adult male Sprague Dawley rats (2 month old, 200–250 g, $n = 4$) were used for viral transfection and neurotoxin lesions. Timed pregnant female Sprague-Dawley rats (embryonic day 18, E18) were used to obtain the fetal primary neuron cultures. Animals were obtained from Harlan Laboratories (Indianapolis, IN). The animals were provided rat chow and water ad libitum and housed in a reverse light–dark cycle room (12 h:12 h, Light:Dark). All animal studies were performed in accordance with standard regulations and were approved by the Michigan State University Institutional Animal Care and Use Committee.

2.2. Adeno-associated viral vectors

In an attempt to visualize the axonal projections of neurons in the nigrostriatal pathway independent of phenotype markers, bilateral injections of GFP expressing recombinant adeno-associated viral vector (rAAV-GFP) were made in the substantia nigra (SN). GFP expression was controlled by the hybrid chicken β-actin/cytomegalovirus (CBA/CMV) promoter, which retains high-level of expression during conditions of neuronal injury and stress (Manfredsson et al., 2007; Benskey et al., 2016). The viral genomes were packaged in to AAV2/5 capsids in 293 cells and purified using an iodixanol gradient followed by column chromatography (Zolotukhin et al., 2002). The intracranial injections were performed as described previously (Manfredsson et al., 2007). A volume of 2 μl of AAV-GFP (1×10^{13} viral genomes/ml) was injected in each site (site 1: Anterior/Posterior (AP) −5.3 mm and −6.0 mm, Medial/Lateral (ML) ±2.0, and Dorsal/Ventral (DV) −7.2 mm from dura; site 2: AP −5.3 mm and −6.0 mm, ML ±2.0, and DV −7.2 mm from dura) at a rate of 0.5 μl per minute. Following the injection, the needle was left in place for an additional 5 min in order to improve diffusion and to avoid reflux in the needle tract.