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Quantitative and semi-quantitative measurements of axonal degeneration in tissue and 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.

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Keywords

Axon degeneration; ImageJ thresholding; stereology; Spaceballs; petrimetrics; densitometry; nigrostriatal system; dopamine; substantia nigra

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 [1–3]. 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 [4]. Similarly, the axons of neurons affected in AD show signs of degeneration early in the disease [5]. 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 [6]. 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 [7, 8].

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 [9]. Eventually, the axon debris is degraded beyond detection [10]. 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 [11, 12]. 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 [13]. Unfortunately, this approach does not provide information regarding morphological characteristics of the axons. A recently described method quantifies axons crossing through a single 30µm thick tissue section using two parallel sampling lines [14]. 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 [15].

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 [16, 17]. 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 [18, 19]. 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 [20]. The length of axons can be measured in 2D monolayer cell cultures using the petrimetrics stereological probe [21].

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 [22, 23]. 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, 200–250g, 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 (12h:12h, 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 [24, 25]. The viral genomes were packaged in to AAV2/5 capsids in 293 cells and purified using an iodixanol gradient followed by column chromatography [26]. The intracranial injections were performed as described previously [24]. A volume of 2 μ l 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 μ l per minute. Following the injection, the needle was left in place for an additional 5 minutes in order to improve diffusion and to avoid reflux in the needle tract.

2.3. Striatal 6-OHDA Lesion

The 6-OHDA lesion was made 28 days after rAAV-GFP injection, allowing sufficient time for GFP expression [27]. A striatal lesion was used to cause a dying-back pattern of axonal degeneration that is selective for the dopaminergic axons projecting from the substantia nigra in the midbrain to the striatum [28]. This is a standard model of PD and the time course of striatal denervation and somatic loss in the substantia nigra was well-characterized elsewhere [29]. Briefly, one hemisphere was lesioned via injection of 2 μ l 6-OHDA (Sigma, H116) per site at a concentration of 5 μ g/ μ l (diluted in 0.02% ascorbic acid, 0.9% saline solution) into two sites of the striatum (site 1: AP $+1.6$ mm, ML $+2.4$ mm, DV -4.2 mm; and site 2: AP -0.2 mm, ML $+2.6$ mm, DV -7.0 mm). The contralateral hemisphere was left intact and used as an internal control. Animals were sacrificed 72 hours after 6-OHDA administration, a time at which axonal degeneration is actively occurring in neurons and that precedes significant cell loss. The vast majority of dopaminergic fibers are completely lost in the striatum at approximately two weeks post-lesion in this 6-OHDA paradigm [29].

2.4. Tissue Processing

Animals were transcardially perfused with 200ml of 0.9% saline containing heparin (10,000 U/L), followed by 200ml phosphate buffered 4% paraformaldehyde. The brains were post-fixed in 4% paraformaldehyde for 24 hours. After post-fixation, the brains were equilibrated to 15% sucrose, followed by 30% sucrose. The brains were then hemisected and cut sagittally into 40 μ m thick sections on a freezing, sliding stage microtome. In order to display axons longitudinally, consideration was taken when sectioning the brain by choosing a plane that is approximately parallel to the axonal projections of interest. Sections were stored in cyroprotectant until processed for immunohistochemistry (IHC).

2.5. Primary Neuron Growth and Processing

Primary neurons were generated from dissected E18 rat hippocampi following a similar procedure as described previously [30]. Briefly, the hippocampi of 8–10 pups were dissected and disassociated into a single cell suspension. The neurons were grown in microfluidic

devices (MFDs; n=3 per treatment, Xona, Cat # SND450) to separate the somatodendritic compartment from the axons. MFD culture devices were prepared by rinsing in dH₂O followed by 70% ethanol and 3 additional changes of dH₂O. The MFDs were dried completely. Uncharged microscope slides were incubated in 0.5mg/ml poly-d-lysine overnight at room temperature, rinsed 3 times in dH₂O, and then dried completely. The MFDs were placed carefully on the coated glass slide and kept sterile in large culture petri dishes. Each MFD was plated with 10,000 cells (1,000 cells/ μ l) in the cell body channel. After 15 minutes, the cell chamber was filled with media and the axonal side filled half way to flow media into the microgrooves connecting the cell body and axonal channels. Degeneration was induced by exposing the axonal channel to colchicine (1 μ M) for 2 hours, seven days after plating. Following the colchicine treatment, the cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature, rinsed 3 times in TBS and then processed for immunocytochemistry (ICC) as described below.

2.6. IHC and ICC

A one in six series of 40 μ m sections was processed for IHC as described previously [31]. Sections were stained with a Tyrosine hydroxylase (TH, Millipore Ab152, 1:5,000) antibody for detection of dopaminergic axons in tissue or Green fluorescent protein (GFP, Abcam ab290 1:400,000) to label rAAV transduced neurons/axons. The tissue sections for all animals were processed for TH or GFP IHC simultaneously to maintain consistency between animals. The staining was developed using 3,3'-diaminobenzidine (DAB), sections were mounted on microscope slides and coverslipped with CYTOSEAL 60 (Thermo Scientific, #8310-16).

The cultured neurons were stained using similar methods to those previously described [30, 32]. Briefly, cells were blocked in 5% GS/1% BSA/0.5% Triton-X 100 TBS for 30 minutes, incubated in primary antibody β III-tubulin (Tuj1, 1:10,000, a generous gift from A. Frankfurter [33]) overnight at 4° C. The following day, the cells were rinsed 6 times in TBS, incubated in biotinylated goat anti-mouse antibody (1:500, Vector, BA-9200), rinsed 6 times in TBS, incubated in ABC solutions (made according to the manufacturer's instructions) for 1 hour at room temperature, rinsed 6 times in TBS, developed in DAB (0.5 mg/ml) and then coverslipped.

2.7. Image Analysis

Images were acquired on a Nikon Eclipse 90i microscope at 20x magnification with a Nikon DS-Ri1 color camera. All microscope and camera settings (i.e., light level, exposure, gain, etc.) were identical for all acquired images. Images were analyzed with ImageJ software (v1.45s, <http://rsbweb.nih.gov/ij/>) using the “threshold” function and the same procedures were applied to all images. Images were converted to 8-bit and then axons were identified using threshold algorithms. The Otsu threshold algorithm was applied to identify positively labeled axons [34]. This method determines the threshold setting independently for each image analyzed. The Otsu method is an objectively applied algorithm that weighs the intensity of the background against the positive staining. This results in the identification of a threshold that yields the best separation of each pixel into a binary class. We found that this preset produced the most accurate identification of positively labeled axons in both

tissue sections and neuron cultures. After applying the threshold settings, the “analyze particle” function was used with the pixel size (pixel²) set from 0–∞ and circularity set from 0–1.0 to include all particles. The data output included object count (# of individual objects), total area of detected objects (total pixels²), and average size of objects (pixels/object). In some areas of tissue sections with dense fibers, the individual fibers appear merged after applying the threshold analysis, which must be taken into account when reviewing and interpreting data outputs. Importantly, the threshold settings must be determined empirically for different studies due to the variability in staining intensities, staining patterns, and image acquisition parameters used for various stains and between different research groups.

Tissue sections from control and lesioned hemispheres that contained the nigrostriatal projection system were used to capture the images for each region in each animal (Fig. 1A–C). The control section was matched to a lesioned section in the contralateral hemisphere based on similar medial-lateral coordinates. The nigrostriatal axon projections originate from dopaminergic neurons located in the substantia nigra and they course through the medial forebrain bundle (MFB) as large, relatively unbranched fibers. The fibers then begin to spread in the region of the globus pallidus externa (GPe) before reaching their final destination in the striatum (Str) where they become extensively branched [35]. In the MFB and GPe, 3 sections were used to capture 2 images per section for analysis (total of 6 images/region/animal). The striatum was sampled with 12 images taken in 4 sections (2 images from the most medial and lateral sections and 4 images from two central sections of the striatum). These sampling parameters provided a representative sampling of the extent of degeneration within each region analyzed.

Primary neurons were grown in MFDs to physically separate the somatodendritic and axonal compartments of neurons. This separation facilitates clear examination of axons without the interference of other processes and the somata of neurons. For the MFDs analysis, 3 images were captured within the axonal compartment (one near the top, middle and bottom) for each MFD (Fig. 1D–F).

2.8. Stereology

The stereological method of estimation was used to quantify axonal length. One of two probes were used within this method to count axonal projections depending on the dimensionality of the sample (Supplemental Fig. 1A–F). The spaceballs stereological probe (Supplemental Fig. 1A–D) estimates length in a 3D region while the petrimetrics probe (Supplemental Fig. 1E–F) estimates length in a 2D sample. The spaceballs probe in StereoInvestigator (MBF Bioscience) was used to obtain estimations of total axon length for normal and dystrophic fibers in three distinct regions through the nigrostriatal system axons (i.e., MFB, GPe and Str). The spaceballs probe creates a 3D hemi-sphere embedded within the tissue slice being examined. Each projection that intersects the exterior bounds of the sphere is counted to create an estimate of the total length per region examined. This stereological method was performed using serial sections (1 in 12 series). Sampling grids were chosen for each region to allow for ~200 fiber intersections to be counted for each region in the intact hemisphere. All counting parameters were chosen to yield a Gunderson Coefficient of Error < 0.1 for the control hemisphere. The mean CE for control brains was

0.06 ($\pm$ 0.005 SEM) for Str, 0.1 ($\pm$ 0.008 SEM) for the GPe and 0.08 ($\pm$ 0.003 SEM) for the MFB. A hemisphere probe with a radius of 11 μ m was used to sample sites throughout each region. The mean measured tissue thickness was ~18–20 μ m. A 4x objective was used to outline each region and a 60x oil objective lens (1.35 numerical aperture) was used for all stereological counts.

Dystrophic axons were operationally defined as any TH immunoreactive fiber or fragment that exhibited at least one of three characteristics of degenerating axons, including increased axonal diameter (axonal swelling; Supplemental Fig. 2B, open arrow), fragmentation (clearly beaded or broken fibers; Supplemental Fig. 2B, arrowhead), and/or axonal spheroids (discrete swollen regions that are rounded; Supplemental Fig. 2B, open arrowhead). Axon diameter varies throughout the nigrostriatal system with those in the MFB being the largest, those in the GPe are intermediate, and fibers in the Str are the finest. Measurement of axon diameter provides a relatively easy method for discriminating dystrophic axons from normal axons in many cases (Supplemental Fig. 2E and F). However, individual examination of each fiber for fragmentation or spheroid bodies is necessary for a complete determination of whether a fiber is dystrophic. Axonal swelling, fragmentation, and spheroid formation are the common elements of the axonal degeneration process and using this set of characteristics should be applicable across other studies on axonal degeneration, but parameters such as axon diameter will vary between different axon populations.

The lengths of intact and dystrophic axons of cultured neurons were measured with the petrimetrics stereological probe, a method for quantifying length in 2 dimensional samples (Supplemental Fig. 1E–F). Dystrophic axons were defined using the same paradigm as above, with dystrophic projections being the swollen or fragmented fibers. A sampling grid was drawn in StereoInvestigator (region of 300 μ m $\times$ 1500 μ m) that included all the axons in the axon channel of the MFD. The probe was run using a Merz radius of 19 μ m and a counting frame of 50 $\times$ 50 μ m. A grid size of 2500 μ m² was used because this provided adequate sampling to yield ~200 markers per sample.

2.9. Statistical Analyses

All data were analyzed using Prism software (v6.0). For regional analyses, the intact and lesioned hemispheres were compared using a two-tailed paired t-test for each outcome measure and statistical significance was set at $p < 0.05$. For analyses of treatment and control groups with intact and dystrophic axons, the data were compared using a 2-way ANOVA with significance set at $p < 0.05$. Repeated-measures ANOVA was used for analysis of Spaceball stereological probe in tissue. Post-hoc comparisons were performed using the Holm-Sidak multiple comparison test.

3. Results

3.1. Qualitative observations of axonal degeneration in the 6-OHDA model

The pattern of axonal degeneration followed a dying-back progression along the length of the nigrostriatal pathway. The DA axons originating from the cell bodies in the SNc coalesce in the medial forebrain bundle (MFB) and then course through the globus pallidus externa

(GPe) *en route* to the terminal fields in the striatum (Fig. 1B and C). As a result of the 6-OHDA lesion, the axons of the striatum had the most severe extent of degeneration compared to control (Fig. 2A and D). The total amount of fibers was substantially reduced, with spheroids and mostly fragmented fibers remaining. Few axons remained in the GPe, but those that remained were swollen dystrophic fragments (Fig. 2E). At the greatest distance from the lesion site, the MFB exhibited the least overt axon loss, yet still displayed clear signs of dystrophy and degeneration. When compared to the control MFB (Fig. 2C), much of the axonal processes remained but the processes were very swollen and slightly fragmented (Fig. 2F). Thus, dopaminergic axons exhibit a pattern of retrograde degeneration in the nigrostriatal system following 6-OHDA with the distal portions of the projections in the striatum being most affected, followed by the GPe and the MFB is least affected.

3.2. Stereological estimations of intact and dystrophic axon lengths in tissue sections

Total axon length was estimated using the spaceballs stereological probe that yields unbiased estimates of the entire axon length in the region of interest. Specifically, we estimated the axon length of both intact and dystrophic neurites in the Str (Fig. 2G), GPe (Fig. 2H), and MFB (Fig. 2I). The lesioned Str (Fig. 2D) exhibited significant loss of axons (Axon condition: $F_{1,3} = 124.5$, $p = 0.0015$; Treatment: $F_{1,3} = 9.199$, $p = 0.0562$; Interaction: $F_{1,3} = 21.48$, $p = 0.0189$) when compared to the intact hemisphere (Fig. 2A), with an overall loss of 62% of the total axon length (intact + dystrophic) and 46% of the remaining striatal fibers exhibited a dystrophic morphology. The GPe also exhibited significant loss of axons (Axon condition: $F_{1,3} = 14.26$, $p = 0.0325$; Treatment: $F_{1,3} = 14.20$, $p = 0.0327$; Interaction: $F_{1,3} = 17.11$, $p = 0.0256$) in the lesioned hemisphere (Fig. 2E), with 94% loss of total axons and 98% of the remaining fibers were dystrophic. In the lesioned MFB (Fig. 2F), there was significant loss of axons (Axon condition: $F_{1,3} = 9.332$, $p = 0.0552$; Treatment: $F_{1,3} = 3.436$, $p = 0.1608$; Interaction: $F_{1,3} = 115.0$, $p = 0.0017$), with 14% loss of total axons and 93% of those that remained were dystrophic.

Axon diameter was measured in StereoInvestigator using the quick measure line tool. Fifty axons were measured in each brain region of one animal to establish diameters of intact and dystrophic axons. The dystrophic axons were significantly larger in diameter than the intact axon in all three regions measured. The intact striatal axons were an average of $0.38\mu\text{m}$ (± 0.16 SD) in diameter compared to $0.97\mu\text{m}$ (± 0.28 SD) for the dystrophic axons ($P < 0.0001$; Supplemental Fig. 2E). Intact and dystrophic axon diameters in the GPe were $0.77\mu\text{m}$ (± 0.22 SD) and $2.13\mu\text{m}$ (± 0.56 SD; $P < 0.0001$; Supplemental Fig. 2F), while axons of the MFB were $1.36\mu\text{m}$ (± 0.34 SD) and $3.13\mu\text{m}$ (± 0.67 SD; $P < 0.0001$; Supplemental Fig. 2G), respectively.

3.3. Semi-quantitative image analysis of axonal degeneration in tissue sections

The extent of axon degeneration was semi-quantitatively measured using ImageJ threshold functions. Image analysis produced measures of average object size, number of objects, and total area of objects within the threshold limits. The intact striatum showed robust staining signal because TH+ fibers in the normal striatum are very dense (Fig. 3A). Axonal degeneration in the lesioned striatum led to fragmentation of the projections (Fig. 3D), which was detected as significantly increased object count ($p = 0.0046$; Fig. 3J), reduced

object size ($p = 0.0004$; Fig. 3G), and a decrease in the total area ($p = 0.0056$; Fig. 3M). The intact GPe is comprised of many long thin fibers, sparsely distributed (Fig. 3B). The lesioned GPe was robustly denervated (Fig. 3E) and showed significant reductions in the object size ($p = 0.0282$; Fig. 3H), the number of objects ($p = 0.0046$; Fig. 3K), and total area ($p = 0.0005$; Fig. 3N). The MFB (Fig. 3C) showed a different pattern of changes (Fig. 3F) because of the lack of extensive axon loss, with a significant reduction in object count ($p = 0.0166$; Fig. 3L) and no significant change in object size ($p = 0.3002$; Fig. 3I) or total area ($p = 0.3009$; Fig. 3O).

Image analysis of GFP signal in the nigrostriatal regions produced similar findings (Supplemental Fig. 3). GFP positive fibers in the striatum showed a significant decrease in object size ($p = 0.0201$) and total area ($p = 0.0127$), while exhibiting a significant increase in the object count ($p = 0.0203$; Supplemental Fig. 3E, K, and H). In the rAAV-GFP treated rats, cell bodies can be visualized in the striatum (Supplemental Fig. 3D). Additionally, while significant TH loss occurred in GPe, the GFP positive staining shows no significant degeneration of fibers in the GPe (Supplemental Fig. 3F, I, and L). Importantly, the pattern of GFP fibers in the GPe is not consistent with the normal TH innervation of the GPe (compare Fig. 1B to Supplemental Fig. 3B). Thus, the current approach for rAAV-GFP delivery transduced a large number of non-nigrostriatal cells thereby limiting its usefulness in the current study (see discussion below).

3.4. Stereological estimates of intact and dystrophic axon lengths in primary neuron cultures

Similar to degenerating axons *in vivo*, the degenerating axons in primary hippocampal neurons were dystrophic, fragmented and contained spheroids (Fig. 4). The petrimetrics stereological probe estimates the total length of axons in a 2-dimensional sample, and here we used this method to quantify both intact and dystrophic neurites. Colchicine treatment of the axons led to degenerative phenotypes of almost all neurites (93% of remaining fibers) and overt loss of approximately half of the axons (48% loss; Fig. 4H) when compared to the untreated axons (Axon condition: $F_{1,8} = 56.07$; $p < 0.0001$. Treatment: $F_{1,8} = 4.412$; $p = 0.0689$. Interaction: $F_{1,8} = 8.111$; $p = 0.0215$).

3.5. Semi-quantitative image analysis of axonal degeneration in primary neuron cultures

The image threshold method confirmed that colchicine-induced axonal fragmentation and overt loss of axonal fibers in cultured neurons (Fig. 4C and D). Colchicine treated axons exhibited a significant decrease in total area ($p = 0.0004$; Fig 4E) and a reduction of mean object size ($p = 0.0072$; Fig. 4F). There was not a significant change in the object count ($p = 0.1615$; Fig. 4G) when compared to untreated axons.

4. Discussion

Axonal degeneration is an integral event in the process of neurodegeneration and needs to be studied in the context of neurodegenerative diseases to fully understand disease pathology. A limited number of methods exist to quantify axonal degeneration and none of the previously described methods used stereological methodology. Here, we established a complimentary

set of methods for quantifying axonal degeneration based on a 6-OHDA-induced PD model in rats and a colchicine toxicity model in primary neuron culture. Stereological and image-analysis based techniques were used to evaluate the extent of degeneration in each of these models. The methods described here provide a relatively rapid and straightforward set of approaches that measure the extent of axonal degeneration.

In vivo model systems are useful tools for studying the processes of axonal degeneration because they provide the complex, multicomponent environment that best replicates the human brain. We chose the intrastriatal 6-OHDA rat model because it is well established and produces a clear dying-back pattern of axonal degeneration specifically in the nigrostriatal dopaminergic system [29, 35]. This long axon projection system is particularly advantageous to studying dying-back axonopathy because the insult occurs at the distal end of the axons and subsequent changes along the axon length are easily observed. Another advantageous characteristic is that the dopaminergic axons are easily visualized along their length in sagittal sections.

The spaceballs stereological probe and the petrimetrics stereological probe are robust measures of intact and dystrophic axon length because a systematic, unbiased quantitative approach is utilized. This methodology requires an operational definition of intact and dystrophic axons. In the current experimental paradigms, dystrophic/degenerating axons were defined as fiber that had a larger diameter compared to normal fibers (i.e. swollen), spheroid bodies, and/or fragmentation. The combination of these characteristics provided a clear distinction from intact axons. Other axon pathways may require modifications to some aspects of this operational definition depending on characteristics displayed by the axons when undergoing degeneration. The population of axons in the regions we analyzed had relatively homogeneous diameters (Supplemental Fig. 2E–G); however, an axon population with very diverse diameters normally would make it difficult to use the swollen axons as a reliable metric for degeneration. Using this approach in tissue sections revealed that the dopaminergic projections in regions most proximal to the site of toxin injection (i.e., the striatum and GPe) exhibited the greatest amount of overt axon loss (60%), while the region most distal from the lesion site (i.e., the MFB) showed the least overt loss (14%). However, the vast majority (93%) of remaining fibers in the MFB were dystrophic/degenerating. This amount of degeneration is consistent with a previous study that showed significant loss of striatal TH terminals at 2 weeks post-lesion and remained constant for the following 4 weeks [29]. In cultured neurons, there was a clear loss of axons (51%) and shift to the majority of remaining fibers being dystrophic or fragmented (93%). Thus, this method can provide useful information regarding the extent of axon loss and the condition of axons remaining after an insult.

The threshold analyses provided a set of semi-quantitative measures that were complimentary to the quantitative stereological analyses. Importantly, comparisons between the normal, unlesioned hemisphere and the lesioned hemisphere must be considered when interpreting data from these measures. For example, the striatum normally contains very dense thin fibers that fill the field of view and the threshold method identifies this region as a large continuous mass. After the lesion, the majority of fibers are gone and those remaining are mostly fragmented as indicated by the reduction in total area, increased object number

and reduced object size. In the GPe, the normal fibers are sparser allowing the threshold analysis to better identify individual fibers. After the lesion, there was significant overt loss of fibers as indicated by the reduction in total area and object number, and the remaining fibers are fragmented as indicated by the reduced object size. Like the GPe, the MFB fibers normally are sparse enough to identify individual fibers. After the lesion, there is little overt loss of axons, but almost all of the remaining axons became very swollen (and thus more overlapped when thresholds were applied) causing a reduction in object count, and no change in either total area or object size. Importantly, these analyses provide a semi-quantitative validation of the visually appreciable effects in each nigrostriatal pathway region.

Here, we focused on TH to visualize the nigrostriatal dopaminergic axons. The short post-lesion interval in this study (i.e., 3 days) makes it unlikely that downregulation of the dopaminergic phenotype played a confounding role in our ability to visualize degenerating dopaminergic axons because this requires a genetic response and clearing of the existing TH from the cell and its processes (TH half-life is ~68 hours in neurons [36]). Moreover, the actively degenerating processes clearly contained TH at this early time point. To determine whether exogenous GFP expression could act as a surrogate marker of degenerating fibers that was independent of phenotypic regulation we injected animals with rAAV-GFP prior to the 6-OHDA lesion. In general, similar results were obtained using GFP as with TH as an axonal marker for analytical methods of degeneration, but a significant limitation of the approach used here was the transduction of cells outside of the dopaminergic SN neurons. The AAVs used here have a cytomegalovirus/chicken beta-actin hybrid promoter, which is strong and ubiquitously expresses the transgene in neurons. Unfortunately, GFP positive cell bodies were present in the striatum, likely due to retrograde infection of striatonigral neurons, which interfered with clearly assessing nigrostriatal-derived GFP changes. Finally, the pattern of GFP fibers in the GPe did not accurately resemble the normal GPe TH innervation upon close examination. Thus, we focused this report on the data obtained using TH to identify dopaminergic axons. Future studies could utilize fluorescent protein transgenic animals, lower AAV titers, lower injection volumes and/or AAVs with neuron-specific promoters if the activity of that promoter is not reduced after injury [37].

In vitro neuron models have several distinct advantages over *in vivo* models for studying axonal degeneration. Primary neuron cultures provide a relatively rapid and simple model system where variables are more easily manipulated. This lends itself well to studying molecular mechanisms of degeneration. Additionally, the microfluidic chambers isolate axons from the somata, which increases the ease of studying axonal degeneration-related events and allows the treatment of each cell compartment independently [38]. Importantly, some of the limitations of cell culture include a more limited timeframe than *in vivo* models and a lack of the complex environment of the brain. Thus, cell culture findings should be validated *in vivo* when possible.

A previous study used microfluidic devices to study the degeneration of cortical axons in the presence of amyloid- β . This was accomplished by using ImageJ thresholding of tubulin staining to attain a fragmentation index, which is representative of axonal degeneration [39]. Tubulin staining is commonly employed because tubulin reactivity typically remains in

fragmented axons in culture [16, 40]. In the current study, we chose colchicine as a model axonal degeneration of hippocampal neurons. Colchicine causes microtubule depolymerization, a prominent feature of the axonal degeneration process [41]. Using ImageJ thresholding combined with stereological analysis, we were able to quantify axonal loss by fiber length and the amount of fragmentation. Together, these two methods provide a cohesive set of analyses for axonal degeneration in cultured neurons.

5. Conclusions

The methods described here are complementary and provide a useful set of quantitative and semi-quantitative analyses for examining axonal degeneration by directly evaluating axons in tissue and in culture. The image analysis and stereological measures revealed the extent of total axon loss, fragmentation, and swelling. These methods should be easily applicable in other model systems of axonal degeneration and would likely compliment approaches that already exist. Studying axon degeneration may lead to a more detailed understanding of mechanisms of degeneration when used with therapeutic treatments or alternative neurotoxic insults.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Overview of experimental axon degeneration models *in vivo* and *in vitro*. (A) An illustration of a sagittal section through the nigrostriatal tract including the striatum, globus pallidus externa (GPe), and medial forebrain bundle (MFB). The boxes indicate where representative images were taken for image analysis. (B) The intact dopaminergic (tyrosine hydroxylase positive) axons project through the MFB as relatively unbranched larger caliber axons, then through the region of the GPe where some minor branching occurs and then the fibers reach the final destination in the striatum where the dopamine axons undergo extensive branching as thin processes. (C) The remaining projections of the dopaminergic neurons in the lesioned hemisphere showed loss of fibers in the striatum around the injection sites and into the GPe. The MFB showed swelling of the fibers by a higher intensity of dopamine staining in the region. (D) A schematic of the microfluidic device used to analyze degenerating axons in culture with boxes indicating where images were acquired for ImageJ analysis. Images of control (E) and colchicine treated (F) axons (β III-tubulin positive) that have grown across the microgrooves and fill the axonal compartment. Scale bar = 1000 μ m.

Fig. 2.

Quantitative analysis of axonal degeneration in the nigrostriatal system. The axonal degeneration process was evident in the nigrostriatal axons following striatal 6-OHDA delivery. (A–F) The intact striatum contained very dense, highly branched processes (A), but the lesioned striatum (D) exhibited a robust loss of axons and the remaining axons were fragmented and dystrophic. The fibers of the intact GPe (B) showed distinct fine fibers while the lesioned GPe (E) exhibited swollen and fragmented fibers. The MFB of the lesioned hemisphere (F) showed very swollen axonal projections when compared to the intact MFB (C). Spheroids are indicated with arrows in degenerating hemispheres (D–F). It is noteworthy that the dying-back pattern of degeneration is appreciable when comparing the extent of denervation across the striatum (D), GPe (E) and MFB (F). (G) Stereological quantitation confirmed a significant reduction in intact fibers and increase in degenerating fibers in the lesioned striatum compared to the intact striatum. (H) The lesioned GPe exhibited significant loss of all fibers with few dystrophic fibers present. (I) A large amount of fibers remain in the MFB; however, they were largely dystrophic neurites. $n=4$, * $p<0.05$ vs control ; # $p<0.05$ vs intact control. Scale bar = $100\mu\text{m}$.

Fig. 3. Semi-quantitative image analysis of axonal degeneration in the nigrostriatal dopamine fibers. (A–F) The representative images of the intact striatum (A), lesioned striatum (D), intact GPe (B), lesioned GPe (E), intact MFB (C) and lesioned MFB (F) from Fig. 2 show the pixels within the threshold limits that were used for analysis (highlighted with the red overlay). In the striatum, the object count (J) was significantly increased while the object size (G) and total area (M) were significantly reduced in the lesioned hemisphere compared to the intact hemisphere. In the GPe, object size (H), object count (K), and total area (N) were significantly decreased demonstrating extensive axon loss in the lesioned hemisphere. The

MFB showed no significant change in total area (O) or object size (I), but exhibited a decrease of object count (L). The striatum is a good example of a region densely innervated with fine fibers that has undergone significant degeneration. The GPe is an illustrative example of a less densely innervated region that is in the process of undergoing extensive degeneration, and the MFB is a region that illustrates changes that precede significant overt axonal loss (e.g. swelling). n=4, *p < 0.05

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Fig. 4.

Analysis of axonal degeneration in colchicine treated primary neuron cultures stained for β II-Tubulin. (A–D) Untreated axons (A) are continuous and appear healthy, but following colchicine treatment in the axonal compartment (B) the axons were fragmented and contained several spheroids. The red overlays indicate the pixels within the threshold limits that were used for analysis in the untreated (C) and colchicine treated (D) axons. (E–G) The image analysis measures confirmed overt loss of axons as a significant reduction in total area (E) and axonal fragmentation as a reduction in object size (F) without a significant change in object count (G) in colchicine-treated axons compared to untreated axons. Stereological estimations of total axon length (using the petrimetrics probe) showed that

colchicine treatment resulted in significant loss of intact axons and a concomitant increase in degenerating axons when compared to untreated axons (H). n=3, * p<0.05 vs control ; # p<0.05 vs intact control. Scale bar = 50µm.

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