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Modeling mitochondrial dynamics during in vivo axonal elongation Matthew O'Toole^a, Robert Latham^b, Rehan M. Baqri^c, Kyle E. Miller^{b,*}

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ABSTRACT

Many models of axonal elongation are based on the assumption that the rate of lengthening is driven by the production of cellular materials in the soma. These models make specific predictions about transport and concentration gradients of proteins both over time and along the length of the axon. In vivo, it is well accepted that for a particular neuron the length and rate of growth are controlled by the body size and rate of growth of the animal. In terms of modeling axonal elongation this radically changes the relationships between key variables. It raises fundamental questions. For example, during in vivo lengthening is the production of material constant or does it change over time? What is the density profile of material along the nerve during in vivo elongation? Does density change over time or vary along the nerve? To answer these questions we measured the length, mitochondrial density, and estimated the half-life of mitochondria in the axons of the medial segmental nerves of 1st, 2nd, and 3rd instar Drosophila larvae. The nerves were found to linearly increase in length at an average rate of $9.24 \,\mu m \,h^{-1}$ over the 96 h period of larval life. Further, mitochondrial density increases over this period at an average rate of 4.49×10^{-3} (mitochondria μ m⁻¹) h⁻¹. Mitochondria in the nerves had a half-life of 35.2 h. To account for the distribution of the mitochondria we observe, we derived a mathematical model which suggests that cellular production of mitochondria increases quadratically over time and that a homeostatic mechanism maintains a constant density of mitochondria along the nerve. These data suggest a complex relationship between axonal length and mass production and that the neuron may have an "axonal length sensor."

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1. Introduction

Protein synthesis and axonal transport are vital components in the healthy growth of neurons. Newly synthesized cellular materials are necessary for, among other things, elongation and upkeep of the axonal shaft. For a given cell, protein synthesis in this process could either be constant or variable and either predetermined by a genetic developmental program or regulated through external inputs. How neurons control cellular production during the growth of axons shapes the way one approaches current problems such as nerve repair. Other factors which may play a role in the process are microtubule polymerization rates, tension, and axonal transport. The ability to understand the interactions of these qualities (i.e. to develop accurate models) is crucial in the development of techniques for repairing damaged nerves.

In pre-synaptic neurons, axonal elongation is the result of a complex interplay between force generation at the growth cone that pulls the axon forward (Lamoureux et al., 1989; Miller and

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Sheetz, 2006; O'Toole et al., 2008), pushing forces due to microtubule and actin polymerization and depolymerization (Bradke and Dotti, 1999; Buck and Zheng, 2002; Letourneau et al., 1987), and the effects of cytoskeletal dynamics and motor protein activity along the axonal shaft (Baas and Ahmad, 2001; Myers and Baas, 2007). Because inhibiting either protein synthesis or microtubule polymerization blocks axonal elongation (Bamburg et al., 1986; Eng et al., 1999), while disruption of the contractile actin cytoskeleton can in some cases accelerate axonal elongation (Bradke and Dotti, 1999; Lafont et al., 1993; Letourneau et al., 1987), most models of axonal elongation have focused on the role of protein production by the cell body, the transport of material to the growth cone, and microtubule addition at the growth cone as the key determinates of the rate of axonal elongation. A popular assumption in elongation models is that the flux of new materials into the axon from the cell body is an independent variable that presumably could vary over time, but is not a direct function of axonal length or rate of elongation (Graham et al., 2006; Kiddie et al., 2005; Miller and Samuels, 1997; Van Veen and Van Pelt, 1994). These models assume that the level of production at a given time point is set at some predetermined level and drives axonal lengthening.

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In this study we focus on post-synaptic neurons where axons lengthen based on the growth of the organism as a whole (Bray, 1984; Rossi et al., 2007; Weiss, 1941). Thus, adult neurons have no control over the rate of axonal elongation or the nerve's final length. As neurons constantly generate tension that minimizes axonal length both before and after synapse formation (Van Essen, 1997), it follows that increases in the length of the axon are the result of forces generated by an increase in body size. Consistent with the view that neurons respond to forced lengthening by increasing the production of new material are studies that have shown that axons are able to maintain viability and increase their caliber when artificially stretched to remarkable lengths (Abe et al., 2004; Pfister et al., 2004, 2006). For these reasons it would be useful to have a model where influx is allowed to vary over the course of elongation. Thus in this paper we assign mass production as a variable that is dependent on the length of the nerve and aim to model the manner in which flux of new materials into the axons is modified to accommodate changes in nerve length and density.

While most studies have focused on the production and transport of cytoskeletal material in the axon, we focus on mitochondria for several reasons. The first is that they play a central role in the progression of Parkinson's disease (Mandemakers et al., 2007). Future work on this topic, which our lab is pursuing, requires a rigorous characterization of normal mitochondrial behavior. The second is that the transport of mitochondria seems to be a function of the mitochondrial life cycle in the cell (Fig. 1). Thus further characterization of their transport is of broad interest to the basic cell biology of neurons (Chang and Reynolds, 2006; Frederick and Shaw, 2007; Miller and Sheetz, 2004). Third, the transport and distribution of mitochondria, as compared to microtubules, is far easier to study because it is possible to resolve individual organelles along the nerve. Fourth, as mitochondria are "housekeeping" organelles required for normal cellular function, and are uniformly distributed along the axon (Miller and Sheetz, 2004), they make an excellent proxy for studying the general production, transport, and distribution of material along the axon.

In addition to the production of new material to support structural changes, there is a constant demand for new materials to replace those lost in the normal functioning of the cell. Many



Fig. 1. Life cycle of mitochondria: we hypothesize that new mitochondria are made in the cell body and have a high potential. These mitochondria are transported along the axon and then dock in regions with unmet metabolic demand. Following damage to the mitochondria, they lose potential. This induces signals to undock and return to the cell body, where mitochondrial degradation and repair occurs.

models do not factor in degradation along the length, often because the main focus of those studies is transport (Brown et al., 2005; Smith and Simmons, 2001). For modeling over long time periods on the scale of the half-life of a material, degradation will affect both demand on the soma and the transport (flux) profile, and must be considered. In regard to the degradation of mitochondria, there has been no recent study of mitochondrial half-life, so we provide our own analysis for this parameter of our model. What mitochondrial half-life in the axon reflects has yet to be clearly defined. Based on our prior work, which demonstrated a correlation between mitochondrial potential and the direction of transport in the axon (Miller and Sheetz, 2004), one possibility is that mitochondrial half-life in the axon reflects the rate of mitochondrial degradation. Nonetheless, mitochondria with a high potential also undergo retrograde transport and it is possible that mitochondria are repaired instead of degraded in the cell body. It is likely that this variable will be a complex function of the rate of mitochondrial depolarization, degradation, repair, or some other function that has yet to be discovered. For the purpose of our discussion, the major point is that mitochondria do not stay in the axon indefinitely, but are removed over time at a rate that has a significant impact on mitochondrial distribution.

Direct measurements of axonal materials will be able to confirm/dispute the assumptions and predictions of previous models and aid in the development of a model of our own. In an effort to accomplish this, we have directly measured mitochondria and their movements during the development of the medial nerve in 1st, 2nd, and 3rd instar Drosophila larvae. Specifically, we have tracked increases in both nerve length and mitochondrial density during the 96 h developmental period. Using data from this system and a newly developed mathematical model, we answer the following questions about axonal elongation in Drosophila: (1) Does nerve diameter vary spatially, and does it increase during the lengthening of mature neurons? (2) What is the half-life of a mitochondrion in medial segmental nerves? (3) What profile of protein synthesis is necessary to sustain the observed behavior of these neurons? Our results imply that the neuron responds to lengthening and its absolute length such that uniform density of material is homeostatically maintained by active regulation of the production of cellular materials during maturation.

2. Materials and methods

2.1. Drosophila stocks and culture

Standard cornmeal fly media was used and all stocks maintained at 25 °C. The UAS-mtGFP line was a gift from Dr. William Saxton, University of California, Santa Cruz.

2.2. Image acquisition and analysis of axonal transport

Crawling 1st, 2nd, and 3rd instar *Drosophila* larvae were selected and anaesthetized in halocarbon oil 700 (Sigma) with 10–25% chloroform, titrated to levels just sufficient to inhibit significant muscular contraction. The larvae were then mounted between a slide and coverslip and were imaged for no more than 15 min at \sim 25 °C. With the exception of Figs. 2 and 6, all images were acquired on a swept field confocal microscope with NIS software using a Nikon TE2000-E inverted microscope and a PlanApo 60X oil objective, NA 1.4. The aperture and exposure were set at 70-slit and 100 ms, respectively, and images were captured at 2 s intervals for total time of 7 min for a time-lapse series. NIS files were opened in ImageJ, and frames were aligned using StackReg plugin with rigid body settings. The two medial nerves at



Fig. 2. Mitochondria can be easily and fully visualized along the entire length of motor neurons in an intact *Drosophila* larva: larvae expressing mtGFP driven by the D42 driver in motor neurons were anesthetized using 15% chloroform in halocarbon oil and directly mounted between a slide and coverslip. The volume of the larva was imaged and 3D stacks were z-projected and assembled into a montage to provide a complete overview of mitochondrial distribution in larval motor neurons.

the base of the ventral nerve cord were selected for each analysis. The images were cropped and rotated using TJ Rotate with cubic-B-spline interpolation so that the nerves were always oriented horizontally with the cell body on one side and the synapse on the other. These images were re-sliced and z-projected using the sumslices option to generate kymographs. The kymographs were opened in Adobe Photoshop, image color depth was converted from 16 to 8 bits pixel⁻¹ and color inverted to facilitate better visibility of transport events.

For Figs. 2 and 6 the images were acquired with a spinningdisk confocal fluorescent microscope controlled by MetaMorph software (Universal Imaging) with a $20 \times air$ or $60 \times oil$ objective (Nikon) and a cooled CCD camera (model ER; Hamamatsu). Confocal stacks were acquired, opened in ImageJ (National Institutes of Health), z-projected, and then assembled into a montage in Photoshop. Timelapse series were acquired at 2 s intervals and processed as described above.

2.3. Nerve length and mitochondrial density

The length of each nerve was calculated as the measured distance from the ventral nerve cord to where the nerve bifurcates close to the region of synapse formation (see Fig. 2). To determine the densities of mitochondria along the length of nerves, 3D reconstructions of nerves where mtGFP was expressed were analyzed and mitochondria were counted (Fig. 3). Mitochondria were grouped in 100 μ m bins and the densities were plotted for each instar (Fig. 4). Average mitochondrial density was calculated for each instar (Fig. 5B) to estimate the manner in which density changes during development.

2.4. Measurement of flux and mitochondrial half-life

Multi-kymographs of each trial were generated to analyze retrograde flux of mitochondria in the nerve (Fig. 6). Each row of the multi-kymograph represents one z-slice (height level) of the nerve. Arbitrary lines were traced down the multikymograph and mitochondria moving right to left that crossed these lines were totaled. This total was divided by the number of horizontal rows used to give the number of retrograde moving mitochondria, and this was divided by the length of the observation to give a value for retrograde flux. Anterograde flux of mitochondria was also measured, but the utility of those measurements are beyond the scope of the manuscript and are not reported here.

When measuring half-life we assumed degradation to be proportional to the total amount of mitochondria; $dM_T/dt = \tilde{g} = -M_T/\tau$, where \tilde{g} represents the retrograde flux of mitochondria



retrograde transport docked mitochondria



Fig. 3. Estimation of mitochondrial size using fluorescence levels: to determine the half-life of mitochondria in *Drosophila* larvae, we first estimated the average size of docked and transported mitochondria. Using movies of transport we measured the total intensity of a mitochondrion using an ovular tool and the measure command in ImageJ (background intensities were subtracted from this measurement). Arrows point to the same mitochondria at different times (min:s).

(treated as negative), M_T represents the total amount of mitochondria, and τ is the characteristic time constant. In vivo movies of mitochondrial transport were analyzed to derive values for \tilde{g} and M_T , from which a value of τ and thus half-life were found. As docked mitochondria were noticeably larger than mitochondria undergoing transport, we used total intensity of all mitochondria as our measure of M_T :

- $M_T = (\# \text{ of docked mito.})$
 - \times (avg. intensity of docked mito.)
 - + (# of transported mito.)
 - \times (avg. intensity of transported mito.).

The average intensities of docked and transported mitochondria were found by averaging the intensities of a sample of mitochondria from the images. To eliminate bias, the first 10 mitochondria to the right of the spatial midpoint of the trial were used. In the case of anterograde and retrograde moving mitochondria, if less than 10 were found then all were used. In measuring the intensity, an ovular tool was implemented in ImageJ to surround each mitochondrion. Using the Measure command, the mean intensity of the region was found. A region of equal area, but with no fluorescence, was then measured in a similar way to estimate the average background intensity. Actual intensity of the region by the difference of these two intensities. The flux term *g* was also multiplied by the average intensity of retrograde moving mitochondria:

 $\tilde{g} = -$ (avg. intensity of retrograde mito.) × (retrograde flux of mito.).

The units of M_T are intensity, and the units of \tilde{g} are intensity h⁻¹. In this way we were able to estimate the relative amount of mitochondria moving toward the cell body (i.e. the mitochondria

A 1.2 1 density (mito. / µm) 0.8 0.6 0.4 0.2 0 400 800 1200 0 length (µm) В 1.2 1 density (mito. / µm) 0.8 0.6 0.4 0.2 0 400 0 800 1200 length (µm) С 1.2 1 density (mito. / µm) 0.8 0.6 0.4 0.2 0 400 800 1200 0 length (µm)

Fig. 4. Mitochondrial density profiles for 1st, 2nd, and 3rd instar larvae: density measurements were binned into 100 μ m segments for 1st (A), 2nd (B), and 3rd (C) instar *Drosophila* larvae. The averages for each bin are plotted where the error bars represent the standard deviation. Average density is seen to increase during development, with insignificant variation from bin to bin.

that was degraded). The value of τ was then easily determined and mitochondrial half-life for each trial was calculated as $T_{1/2} = \ln 2 \times \tau$.

3. Results

3.1. Axonal length and mitochondrial density increase with time

Analysis of the medial segmental nerves in 1st, 2nd, and 3rd instar larvae during normal development revealed that axons lengthen in a linear fashion (Fig. 5A). Average values for nerve lengths were $468 \pm 124 \,\mu$ m (mean \pm SD, n = 12) for 1st instar larvae, $631 \pm 262 \,\mu$ m (mean \pm SD, n = 11) for 2nd instar larvae, and $963 \pm 163 \,\mu$ m (mean \pm SD, n = 11) for 3rd instar larvae, and $963 \pm 163 \,\mu$ m (mean \pm SD, n = 11) for 3rd instar larvae. The associated growth constant was calculated to be $\gamma = 9.24 \,\mu$ m h⁻¹. Average mitochondrial densities were 0.34 ± 0.06 mitochondria μ m⁻¹ (mean \pm SD, n = 12) for the 1st instar, 0.37 ± 0.05 mitochondria μ m⁻¹ (mean \pm SD, n = 11) for the 2nd instar, and 0.58 ± 0.08 mitochondria μ m⁻¹ (mean \pm SD, n = 11) for the 3rd instar. The constant of density increase α was hence found to be 4.49×10^{-3} mitochondria μ m⁻¹ h⁻¹ (Fig. 5B). We found the distribution of mitochondria in each instar was not significantly different from uniform (Fig. 4).

3.2. Mitochondrial half-life in the nerve

The half-life of mitochondria in 3rd instar larvae nerves was estimated using measurements of total mitochondrial intensity and retrograde flux, the method which is explained in Materials and methods. Using measurements from six different nerves we found the average half-life to be $T_{1/2} = 35.2 \pm 17.6$ h (mean \pm SD, n = 7). This value was derived from a measured time constant $\bar{\tau} = 50.8 \pm 25.4$ h.

3.3. Derivation of model

We use the results obtained by imaging to derive a model for the system which predicts the rate of protein synthesis required to satisfy the needs of an elongating axon. When studying the change in the amount of a substance in a bounded region *V*, a basic conservation law must be satisfied:

$$\frac{\mathrm{d}}{\mathrm{d}t} \int_{V} P(x,t) \,\mathrm{d}V = \int_{V} g(x,t) \,\mathrm{d}V - \int_{\partial V} \mathbf{J} \cdot \mathbf{dS}. \tag{1}$$

This equation states that the rate of change of total substance in a region V is equal to the difference of the local synthesis of the substance and the rate of flux of materials out of the region, where g gives the local production/degradation of the substance and **J** is the flux of materials (∂V is the boundary of the region V). With the use of the divergence theorem, the relationship can be rewritten as

$$\int_{V} \left[\frac{\partial P}{\partial t} - g(x, t) + \nabla \cdot \mathbf{J} \right] dV = \mathbf{0}.$$
 (2)

Since the relationship holds on an arbitrary bounded region V, we may simply write

$\partial P/\partial t = g - \nabla \cdot \mathbf{J}.$

We consider the two factors, g and J that contribute to changes in the mitochondrial density P. Local synthesis of mitochondria, with regard to the assembly of nuclear encoded proteins, is assumed to be negligible. The function g then solely describes the removal of healthy mitochondria from the axon. Whether this varies over time is unknown and will be important to investigate more thoroughly in the future, but for this study we assume that mitochondria are cleared at a constant uniform rate, and thus the rate of mitochondrial loss is proportional to the concentration. Define a population of mitochondria to have half-life $T_{1/2}$. Then the characteristic time constant τ is given



Flux of mitochondria due to active transport and low velocity transport (LVT) (Miller and Sheetz, 2006) will also cause changes in the concentration along the length of the axon, but diffusion is omitted since mitochondria are docked tightly to microtubules (Hollenbeck, 1996), actin filaments (Chada and Hollenbeck, 2004), and neurofilaments (Wagner et al., 2003). Therefore, the term **J** strictly describes the combination of active transport and LVT. Now if we assume that mitochondrial concentration is radially uniform we may write the equation in one dimension:

$$\frac{\partial P}{\partial t} = -\left[\frac{P}{\tau} + \frac{\partial J}{\partial x}\right].$$
(4)

We make two assumptions for our model which are based on our experimental observations. The first observation is that nerves tend to lengthen at near constant rates (Fig. 5A), though this rate may vary given the placement of the neuron. The length of the neuron may then be written as a linear equation in t, $L(t) = L_0 + \gamma t$, where γ is the elongation rate. The second observation is that mitochondrial density appears to scale with time since innervations (Fig. 5B). There was an observed trend between nerve length and mitochondrial density (Fig. 5C), but this relationship does not hold when considering neurons with differing mature lengths (e.g. different segmental nerves). Analysis also revealed that the distribution of mitochondria is effectively uniform $(\partial P/\partial x = 0)$. We can thus write the mitochondrial density in terms of t by $P = P_0 + \alpha t$.

Using this last equation we can substitute $\partial P/\partial t = \alpha$ into our main PDE. Then we may solve Eq. (4) for $\partial J/\partial x$ as

$$\frac{\partial J}{\partial x} = -\left[\frac{P_0 + \alpha t}{\tau} + \alpha\right].$$
(5)

Since the majority of protein synthesis of nuclear encoded proteins is believed to occur in the cell body (Campenot and Eng, 2000), in order to maintain a uniformly increasing mitochondrial density, new protein required to support this growth must flow into the axon at x = 0 There are three changes in the concentration along the length of the axon that will create a demand for mitochondria. Those needs are (A) replacement of mitochondria that are cleared from the axon, (B) new mitochondria required due to lengthening of the axon, and (C) new mitochondria required to cause an increase in mitochondrial density. Thus new material must enter the axon at x = 0 to satisfy these three mitochondrial needs. The latter two changes correspond to changes in cell volume while the former deals with depletion of axonal mitochondrial. The flux requirement, respectively, for each condition is (A) the rate of concentration decrease times length: $P(t)L(t)/\tau = (P_0 + \alpha t)(L_0 + \gamma t)/\tau$, (B) the rate of lengthening times the mitochondrial density: $\gamma P(t) = \gamma (P_0 + \alpha t)$, and (C) the rate of increase in density times length: $\alpha L(t) = \alpha (L_0 + \gamma t)$.

Fig. 5. Nerve length and mitochondrial density increase over time: both nerve length and mitochondrial density are observed to increase as Drosophila larvae mature. Arrowheads in A, B denote the approximate time, in development, of synapse formation when the mode of growth switches from neurite growth to towed axonal growth. (A) The average lengths of neurons of each instar are plotted at the approximate midpoint time of each instar. Average lengths were found to be $468 \pm 124 \,\mu\text{m}$ (average \pm SD, n = 12) for the 1st instar, $631 \pm 262 \,\mu\text{m}$ (average \pm SD, n = 11) for the 2nd instar, and $963 \pm 163 \,\mu\text{m}$ (average \pm SD, n = 11) for the 3rd instar. The growth rate constant γ is estimated to be 9.24 μ m h⁻¹. (B) Mitochondrial density data is similarly plotted and the average values were found to be $0.34\pm0.06\,mitochondria\,\mu m^{-1}$ (average \pm SD, n = 12) for the 1st instar, 0.37 ± 0.05 mitochondria μ m⁻¹ (average \pm SD, n = 11) for the 2nd instar, and 0.58 ± 0.08 mitochondria μ m⁻¹ (average \pm SD, n = 11) for the 3rd instar. The constant of density increase α is estimated to be 4.49×10^{-3} mitochondria μ m⁻¹ h^{-1} . (C) Mitochondrial density plotted versus nerve length (n = 34). A linear trend is clear, though whether these two factors change independently with time is not clear.





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Fig. 6. Robust fast mitochondrial transport in fly neurons: mtGFP was expressed in motor neurons and a single optical plane was captured once every 2 s using a spinning disk confocal. (A) A wide view where the neuronal cell bodies in the ventral nerve cord and the two A8 segmental nerve branches are shown. (B) Examples of the fast transport of individual mitochondria are shown in frames of the movie. (C) The multi-kymograph with hand drawn traces over the positions of fast transported mitochondria to illustrate the high level of detail that can be resolved. Retrograde flux was used to quantify degradation while anterograde transported mitochondria represent a combination of slow transport and translation of the nerve due to elongation. Each of the six panels spans a 200 s interval. (D) Summations of the traces of anterograde and retrograde multi-kymographs, excluding slow mitochondrial movements.

Thus we choose our boundary condition to be

$$J(0,t) = \frac{(P_0 + \alpha t)(L_0 + \gamma t)}{\tau} + \gamma (P_0 + \alpha t) + \alpha (L_0 + \gamma t).$$
(6)

3.4. Flux profile

Solving Eq. (5) with the boundary condition (Eq. (6)) gives an equation for the flux profile of mitochondria along the length of the axon:

$$J(x,t) = -\left[\frac{P_0 + \alpha t}{\tau} + \alpha\right] x + \frac{(P_0 + \alpha t)(L_0 + \gamma t)}{\tau} + \gamma(P_0 + \alpha t) + \alpha(L_0 + \gamma t).$$
(7)

The same flux profile can be derived using the less direct boundary condition where only the flux at the growth cone is



Fig. 7. Flux predictions of the model: (A) Flux of mitochondria is linearly decreasing with distance from the cell body. Shown are the flux profiles at times t = 0, 24, 48, 72, 96, and 120 h. As the nerve elongates and increases its caliber, both production and flux along the length of the axons increase. $L_0 = 237 \,\mu\text{m}$, $P_0 = 0.229$ mitochondria μm^{-1} , $\alpha = \bar{\alpha}$, $\gamma = \bar{\gamma}$, $\tau = \bar{\tau}$. (B) The flux of new mitochondria into the axon is plotted as a function of time. Production must increase quadratically in order to support the observed increases in length and density. Values used are the same as in (A) except for τ which is varied. Increasing τ diminishes the quadratic effect on the increase in production.

considered:

$$J(L(t), t) = \gamma(P_0 + \alpha t).$$
(8)

Eq. (7) reveals two things; the flux of materials along the axon decreases linearly (Fig. 7A), and the rate of influx of materials must increase in a quadratic fashion over time in order to sustain constant increases in axonal length and mitochondrial density (Fig. 7B). The flux equation can be written as a quadratic in terms of t as

$$J(x,t) = At^{2} + B(x)t + C(x),$$
(9)

where $A = \alpha \gamma / \tau$ and this value, using our estimates, is equal to 8.17×10^{-4} mitochondria h^{-3} .

The model can be applied to axons not only when it is changing in both length and diameter, but also when only one of the two is changing or when neither is changing. Our analyses of length and density in *Drosophila* show that both increase over the three instars. However, as towed growth continues through maturation the diameters of these nerves may remain constant. During this phase of growth the parameter α will be zero, reducing the flux equation to being linear in *t*. Similarly, short neurons will reach their mature length quickly and continue to increase their diameter, also giving a linear influx profile (in *t*). When the axon has reached its mature length and diameter, then influx must be constant to keep the axon supplied with functioning mitochondria. Between synapse formation and the end of the neuron's life the axon will be in one of these states.

4. Discussion

In this study we explore several aspects of axonal elongation in *Drosophila* and derive a mathematical model which predicts the rate of mitochondrial addition to the axon. Length and diameter are shown to increase in a linear fashion in medial segmental *Drosophila* neurons, and a value for mitochondrial half-life is derived. A basis for our model is the mitochondrial life cycle hypothesis (Fig. 1). The model incorporates mitochondrial clearance from the axon and active transport, but excludes diffusion since mitochondria are either attached to microtubule motors or stably docked to the cytoskeleton (Chada and Hollenbeck, 2004; Hollenbeck, 1996; Wagner et al., 2003). Flux of mitochondria decreases linearly with distance from the cell body. Based on the constant rate of lengthening and diameter increase, and on mitochondrial degradation, influx of new mitochondria to the axon was found to necessarily increase in a quadratic fashion.

Analysis of mitochondria in 1st, 2nd, and 3rd instar larvae revealed no significant concentration gradient along the nerve (Fig. 4). This suggests the neuron has an efficient mechanism for organization of mitochondria. Kymographs have revealed that mitochondria are able to dock in regions of low density (Miller and Sheetz, 2004), and docked mitochondria have been observed to undergo short bouts of transport and then re-dock; a sort of mitochondrial redistribution (Brown, 2003; Chada and Hollenbeck, 2004). The ability of mitochondria to reorganize is crucial in maintaining a uniform density given the factors that lead to gradients in distribution. Namely, axons are viscoelastic structures which can be deformed either by growth cone induced stretching (Lamoureux et al., 1989) or towed growth of pre-synaptic (Dennerll et al., 1989) or mature neurons (Abe et al., 2004; Pfister et al., 2004), and mitochondria eventually become depolarized and are transported back to the cell body (Miller and Sheetz, 2004).

The analysis did reveal, however, that mitochondrial density increases throughout larval development (Fig. 5B). When coupled with the lengthening of the nerve, the demand for mitochondria increases in a quadratic fashion (Eq. (9)). Using our estimates for α , γ , and τ , and extrapolated values for the initial length and density of the nerve (Fig. 5A and B), we found that the quadratic coefficient has a value of 8.17×10^{-4} mitochondria h^{-3} while the linear coefficient is 0.146 mitochondria h^{-2} when x = 0. The quadratic effect at the cell body is most noticeable when the flux profile is viewed over the course of days (Fig. 7A). Should either rate of increase (α or γ) go to zero, the flux would be increasing in a purely linear fashion. At steady-state length and density ($L = L_{\infty}$, $P = P_{\infty}$) the flux profile is constant over time. It is also possible that τ increases during development. This would lower the demand for new mitochondria and thus should be investigated.

While analysis of mitochondrial density revealed a uniform distribution along the length of the nerve (Fig. 4), the model suggests that the profile of moving mitochondria is linearly decreasing along the length (Eq. (7)). In order for this to be the case, the profile of docked mitochondria must necessarily be linearly increasing along the length. Since healthy mitochondria, either motile or stationary, are capable of producing ATP, this is consistent with a uniform demand for energy along the length of the axonal shaft (excluding the region directly proximal to the synapse, where mitochondrial concentration has been observed to be higher) (Chada and Hollenbeck, 2003). Mitochondria in the cell body may also contribute ATP to the most proximal region of the axon, which would further reduce the need for docking there. However, if the overall ratio of docked mitochondria to transported mitochondria is high, then a gradient in docked mitochondria would not be observed.

There has been clear evidence that cell bodies are able to respond to changes in length by increasing cellular production. In one such case Pfister et al. showed that extreme towing of mature neurons resulted in longer healthy neurons of increased caliber (Pfister et al., 2004, 2006). Here the change in length was mechanically driven and the cellular density increased as a result. If towing occurred too rapidly, however, the axons ruptured. Hence the cell body's ability to adapt to rapid changes in length is limited. The data in those studies give insight as to how quickly axons may be towed, but to our knowledge no theoretical analysis of the problem yet exists. For the purposes of neuronal repair it would be necessary to understand how an axon is viscoelastically deformed by axial tension (i.e. the increase in protein demand in response to towing) (O'Toole et al., 2008) and also to know the production limitations of the cell body.

Our model is broad in the sense that it studies the behavior of the mitochondrial population as a whole. The main idea is that the cell is working to achieve an optimal density which is uniform along the length but increases with time (Figs. 4 and 5B). Tension on the cell from various sources can lead to local differences in density. How a uniform density is re-established is not specifically addressed. One possible explanation is that new mitochondria travel the entire distance from the cell body to sites of need. Another possibility is that, as new mitochondria are being produced, docked mitochondria are redistributed through fast axonal transport (Brown, 2000). In terms of maintaining uniformity, redistribution through fast axonal transport would be able to achieve this in less time.

An interesting point is that the longer segmental nerves were not observed to be thinner than the shorter ones. This suggests that the level of mitochondrial biogenesis/protein synthesis in the neurons differs, and that two neurons of the same age are able to regulate protein synthesis based on their lengths. If protein synthesis were independent of nerve length, then one would expect shorter nerves to have larger caliber. Little is known about whether neurons can sense how long they are, but this piece of evidence makes a strong case for some sort of signaling pathway for length recognition. While the length of the nerve is determined by the size of the animal, the caliber is controlled by the production of material, making this a vital regulatory process. Another example of selective diameter regulation is seen in the variance of cat retinal axons. Here, a correlation was found between the eccentricity of intraretinal X-cells and their axonal diameters, and also between soma size and axonal diameter (Stanford, 1987b). The entire system, which has the difficult task of processing visual cues, was found to be highly complex, with intraretinal and extraretinal axon sizes varying to produce uniform mean total conduction times (Stanford, 1987a). The regulation of action potentials is known to be dependent on axonal and dendritic diameters (Hodgkin and Huxley, 1952; Rall, 1959), thus this may be the motivation for a carefully managed axonal diameter. Given these examples we propose that the neuron is able to detect its own length and mitochondrial density and, in turn, modulate production and reorganize cellular materials to achieve and maintain a preferred caliber.

Current therapies for treating nerve damage typically involve the application of drugs or growth factors, the splicing of severed nerve endings together such that the distal degenerating nerve provides a track for the regeneration of axons coming from the proximal stump, and/or the insertion of conduits to provide tracks (Pfister et al., 2007). Our model raises questions pertinent to the treatment of peripheral nerve injury. It suggests that the production of materials is a response to increased axonal length. Traditionally, reconnection of served nerves is performed without tension at the site of repair. However, studies in primates have shown that direct repair under modest tension yields better results relative to a tension-free repair, but this remains controversial (Rowshan et al., 2004). Could force application at the stump of a severed nerve, that causes axonal lengthening, be used in combination with current therapies to facilitate regeneration?

Developing rational treatments for chronic neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, is hindered by the weak understanding of the chain of events linking the primary insult and the ultimate manifestation of the disease. A deeper knowledge of the mitochondrial life cycle (Fig. 1) will provide a foundation for the study of these maladies, not unlike the way Harvey's discovery of blood circulation (Zareba, 2007) laid the groundwork for the treatment of heart disease. The predictions made by our model add to the overall understanding of mitochondrial dynamics, and further studies of mitochondria in the axon will be essential to the goal of curing neurodegenerative diseases.

While lengthening in post-synaptic neurons is governed by the rate of bodily growth, it is yet unclear how tension changes during this process. On the surface, stretching of the axons would seemingly increase tension. However, the addition of new material to the axons will counteract the stress that is caused by stretching. Further, it is unclear whether mass addition occurs in preparation for or in response to lengthening-induced tension. How these two processes interact, which we cannot elucidate from this study, is highly significant in understanding mitochondrial biogenesis and transport and demands further examination.

4.1. Model comparisons

While production of cellular materials is shown to necessarily increase with time, the profile of flux at any fixed time is decreasing with distance from the cell body (Fig. 7). The result is in qualitative agreement with studies that observed a decline in transported mitochondria in growing axons (Miller and Samuels, 1997; Morris and Hollenbeck, 1993). This flux gradient is present in models of transport which consider protein degradation (McLean and Graham, 2004) and is absent in models which do

not (Friedman and Craciun, 2005). Smith and Simmons reported a steady flux in their unidirectional transport model and a linearly declining flux in their bidirectional transport model (Smith and Simmons, 2001). Our flux results are in agreement with their bidirectional model when a maximal efficiency of cargo loading onto microtubules is assumed. The declining profile can be explained in that any new mitochondrion, regardless of destination, must be transported through the most proximal region of the axon but fewer and fewer are transported to increasingly distal regions. Mitochondria are believed to dock in regions where ATP levels are low (Hollenbeck, 1996; Miller and Sheetz, 2004), which means that transported materials can leave the transported phase at any point along the axon. This differs from other models, where all transported materials must be delivered to the growth cone (a one sink model versus a multi-sink model). Hence different models should be used for transport of different intracellular cargos (e.g. mitochondria versus synaptic vesicles).

A significant difference between our model and previous models (McLean and Graham, 2004; Miller and Samuels, 1997; Van Veen and Van Pelt, 1994) is that we consider axonal length to be independent and treat protein synthesis as a dependent variable. At the foundation of this choice is the debate over what drives axonal elongation. While some have presented arguments for production-driven elongation, it may be the other way around where production responds to changes in axonal length. We have concentrated here on mature neurons, yet the idea applies to presynaptic axons as well. Tension has been shown to cause lengthening in both types of neuron, and in those cases the cell bodies have responded by increasing production to restore the axon or nerve to a viable diameter (Abe et al., 2004; Dennerll et al., 1989; Lamoureux et al., 1989; Pfister et al., 2004). For these reasons we hold changes in length as independent and study how production must change to sustain the axon and avoid rupture. Put another way, production does not increase to cause changes in length, but to support them. It is likely that there is an upper bound on material production by the cell body, but such a bound is yet unknown.

Redistribution of cellular materials likely plays a large role in maintaining the observed uniform cellular density. Brown et al. derived a stochastic model to address stop-and-go transport of neurofilaments (Brown et al., 2005). In this study, transition probabilities of neurofilaments changing between the various states of transport (paused or moving at various velocities) were experimentally determined. To maintain a uniform concentration of cellular materials, stop-and-go transport would need to preferentially occur in regions with a local concentration gradient. A possible way to apply this stochastic model to one that favors a uniform mitochondrial distribution would be to use transition probabilities which are functions of $\partial P/\partial x$.

5. Conclusion

In this paper we have developed a model for elongation that suggests that the flux of mitochondria in the axon decreases linearly along the length of the axon and increases quadratically with time. The model is based on the observations that axonal length and mitochondrial density tend to increase at a linear rate during development. Using an analysis of retrograde flux of mitochondria we derived an updated estimate for mitochondrial half-life in *Drosophila*. Our model suggests that mitochondrial production is modulated by the cell body based on axonal length, axonal diameter, and mitochondrial half-life. If neurons are able to detect their lengths, as we suggest, then uncovering the means by which they do this opens many exciting avenues for future work. To test these predictions we plan to measure anterograde and retrograde mitochondrial flux during each of the three instars of development. In this process we may also assess whether mitochondrial half-life is constant through development or whether it varies, and also test the expected linear decrease in flux along the length of the nerve.

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