A Motor-Driven Mechanism for Cell-Length Sensing

Ida Rishal,1,5 Naaman Kam,1,5 Rotem Ben-Tov Perry,1 Vera Shinder,2 Elizabeth M.C. Fisher,3 Giampietro Schiavo,4 and Mike Fainzilber1,*

1Department of Biological Chemistry
2Department of Chemical Research Support
Weizmann Institute of Science, 76100 Rehovot, Israel
3Department of Neurodegenerative Disease and MRC Centre for Neuromuscular Diseases, Institute of Neurology, University College London, London WC1N 3BG, UK
4Molecular Neuropathobiology Laboratory, Cancer Research UK London Research Institute, Lincoln’s Inn Fields Laboratories, London WC2A 3PX, UK
5These authors contributed equally to this work
*Correspondence: mike.fainzilber@weizmann.ac.il
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SUMMARY

Size homeostasis is fundamental in cell biology, but it is not clear how large cells such as neurons can assess their own size or length. We examined a role for molecular motors in intracellular length sensing. Computational simulations suggest that spatial information can be encoded by the frequency of an oscillating retrograde signal arising from a composite negative feedback loop between bidirectional motor-dependent signals. The model predicts that decreasing either or both anterograde or retrograde signals should increase cell length, and this prediction was confirmed upon application of siRNAs for specific kinesin and/or dynein heavy chains in adult sensory neurons. Heterozygous dynein heavy chain 1 mutant sensory neurons also exhibited increased lengths both in vitro and during embryonic development. Moreover, similar length increases were observed in mouse embryonic fibroblasts upon partial downregulation of dynein heavy chain 1. Thus, molecular motors critically influence cell-length sensing and growth control.

INTRODUCTION

Size homeostasis is one of the most fundamental aspects of biology, with distinct size limitations for individual cell types (Hall et al., 2004). Neurons exhibit the most marked size differences of any class of cells, having process lengths ranging from a few microns in central interneurons up to a meter in human peripheral neurons. Early work suggested that the intrinsic growth rates of embryonic sensory neurons in vivo differ according to the distances they must cross in order to reach their specific targets (Davies, 1994) and that neurons modulate protein biosynthesis and degradation rates to maintain size homeostasis (Franklin and Johnson, 1998). Individual axonal growth rates can vary considerably at different stages of elongating growth in the embryo (Rossi et al., 2007), and interstitial growth rates of axons that have connected to their targets can be remarkably enhanced by stretch growth along with the organism (Smith, 2009). How then can a neuron coordinate between the output of transcriptional and metabolic programs controlled by the nucleus and differential growth and maintenance needs of different lengths of axons?

A fundamental prerequisite for intracellular coordination on axonal scales should be an intrinsic capacity to monitor process lengths. Indeed, intrinsic length sensors in neurons are supported by observations on axonal initiation in hippocampal neurons (Goslin and Banker, 1989), sprouting capacity in motor neurons (Pestronk and Drachman, 1988), mitochondrial content of Drosophila medial neurons (O’Toole et al., 2008), dendritic arbor length in cortical neurons (Samsonovich and Ascoli, 2006), myosin effects on soma and minor process size (Kollins et al., 2009; van Diepen et al., 2009), and distance sensing of axotomy sites in a wide variety of neurons (Kam et al., 2009). Theoretical analyses also support the existence of intrinsic constraints on maximal lengths of neuronal arbors (Brown et al., 2008). Thus, both modeling and experimental approaches support the existence of intrinsic length sensors in neurons, but the underlying mechanisms are largely unknown.

Recent studies have proposed length-sensing mechanisms based on diffusion gradients in cell division control in fission yeast (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009) and in polarity determination in hippocampal neurons (Toriyama et al., 2010). Although such diffusion-based mechanisms may allow length sensing over restricted distances, the range limits of intracellular gradients fall far short of those needed for process lengths of more than 100 μm (Stelling and Kholodenko, 2009). Intrinsic “molecular rulers” based on microtubule dynamics or length-dependent modulators have also been described (Picone et al., 2010; Varga et al., 2009), but given the plasticity and lengths involved, a simple microtubule “ruler” is not a likely length-sensing mechanism for large cells. Active transport on molecular motors allows rapid translocation of signals between neurite tip and cell body (Ibáñez, 2007). We
Figure 1. Motor-Based Models for Cell-Length Sensing

(A) A gradient-based model wherein length-encoding signals are actively transported by dynein from axon tip to cell body, with a constant rate of signal loss en route. At an early time point (T1), axons are short and signal levels at the cell body are high. At later time points (T2 and T3) the accumulating signal loss along longer tracts will reduce signal levels in the cell body.

(B) Retrograde signal levels at the cell body during axon elongation from simulations of the gradient model at high (blue), medium (red), and low (green) dynein levels, respectively. Reduced dynein levels result in shorter axon lengths (e.g., using threshold indicated by horizontal line in main graph; inset).

(C) A bidirectional mechanism wherein anterograde signals are transported by a kinesin from cell body to axon tip, where they activate dynein-dependent retrograde signaling to the cell body, which then represses the anterograde signal via negative feedback.
therefore examined the possibility that motor-based signaling might enable distance sensing between cell center and axon endings on a continuous basis.

**RESULTS**

**A Motor-Dependent Frequency-Encoded Length-Sensing Mechanism**

Models were constructed using a previously described approach (Kam et al., 2009), representing motor-transported signals (MTS) as moving units in a computer simulation. Each such MTS moves in simulation space according to a velocity assigned to it on the basis of known velocity distributions for molecular motors (Figure S1). We first attempted to model a gradient generating mechanism that would be similar in principle to the diffusion gradient mechanism in yeast, by simulating active transport of length-encoding signals by the dynein motor from axon tip to cell body, with a constant rate of signal loss en route. In this scenario, signal levels at the cell body remain high as long as axons are relatively short. Upon sufficient axonal extension, the accumulating loss along longer tracts will reduce signal levels in the cell body (Figure 1A). Simulations of this model predict that reducing levels of the dynein motor—hence, reducing amounts of transported signal—should result in shorter axon lengths (Figure 1B). One limitation of this gradient-generating model is that it does not consider the source of the axonal tip signal; therefore, we examined a second family of models wherein a cell body signal is anterogradely transported by kinesin motors to the neurite end, where it activates dynein-mediated retrograde transport of another cargo (or a modified version of itself) to the cell center. Different versions of this latter model were tested by simulation, including single and dual positive feedback loops (Figure S1), and a composite negative feedback configuration wherein the retrograde signal represses the original anterograde entity, thus periodically resetting the system (Figure 1C and Movie S1). The latter configuration generates an oscillating retrograde signal, with frequencies that decrease as a function of increasing cell length (Figures 1D and S1). It is noteworthy that this frequency-encoded model showed much greater robustness in repetitive runs of the simulations than the gradient model or previous quantity-dependent models for sensing injury distance (Kam et al., 2009). It is interesting that frequency-encoded signals are utilized for distance determination in radar technology, where they are commonly termed “chirp” signals (Bloch, 1973). Reducing levels of either anterograde or retrograde motors (thereby reducing signal levels in each phase of the mechanism) causes a slowing in frequency reduction with growth time in the system (Figure 1E). If elongation rates are correlated with retrograde signal frequency, this leads to the counterintuitive prediction that an increase in axon length should occur in both cases (Figure 1F). Thus, cell lengthening or shortening upon partial knockdown of a candidate motor provides a way to discriminate the alternative models shown in Figure 1.

**A Targeted siRNA Screen Reveals Motors Affecting Sensory Neuron Length**

Adult sensory neurons from the dorsal root ganglia (DRG) extend axonal-type processes in culture with a unidirectional microtubule cytoskeleton (Zheng et al., 2001). All anterograde transport in these processes is kinesin-based, while retrograde transport is dynein-based, thus providing an appropriate system for testing of the models outlined earlier. We used siRNA transfection of yellow fluorescent protein (YFP) transgenic sensory neurons followed by automated live cell fluorescence microscopy to assess the effects of partial knockdown of 36 microtubule motor heavy chains. These experiments revealed clear axon length increases upon transfection with siRNA against five kinesin heavy chains, including the three Kif5 isoforms, and dynein heavy chain 1 (Dync1h1), without significant effects on soma size (Figures 2A and 2B). These effects are in accordance with the predictions of the frequency-dependent model. Strikingly, combined downregulation of Kif5B and Dync1h1 caused a greater length increase than with each siRNA alone (Figures 2C and 2D), as predicted by the frequency-dependent model in the case of partial downregulation of both motors. Validation of the siRNA effects for Kif5B and for dynein confirmed reductions of 40%–60% in levels of the targeted proteins in neuronal cultures exhibiting the observed length increases (Figures 2E–2H). Since dynein is a potential cargo of Kif5 complexes, we quantified the degree of reduction in both Kif5B and Dync1h1 after treatment with siRNAs against Kif5B, siRNA treatment leading to 40% reduction in Kif5B levels had no effect on Dync1h1 expression (Figures 2I and 2J). Thus, the increase in axon length observed upon Kif5B reduction is not due to an indirect effect on dynein levels.

The length increase observed upon reduction of Dync1h1 is striking since this ATP-binding subunit is indispensable for all dynein-based axonal transport processes. It is important to note that microtubule network and growth cone morphology were normal in neurons treated with Dync1h1-siRNA using this protocol (Figure S2). Greater depletion of Dync1h1 by longer siRNA treatment was previously shown to disrupt microtubules, cause aberrant growth cone morphology, and reduce neurite outgrowth (Ahmad et al., 2006). Partial depletion of the motor as achieved here does not cause these drastic effects, thus enabling detection of the length increase phenotype.

**Increased Process Lengths in Sensory Neurons from a Dynein Mutant Mouse**

To verify the siRNA results by an independent approach, we then carried out experiments on sensory neuron cultures from the Loa mouse, which harbors an F508Y mutation in Dync1h1 that is lethal in homozygotes but tolerated in the heterozygous background over normal lifespan (Hafezparast et al., 2003). Adult Loa heterozygote sensory neurons reveal a reduction in Dync1h1 levels specifically in axons, while overall cell body levels are not affected (Figures 3A and 3B). Transport velocities for both
Figure 2. Partial Downregulation of Certain Microtubule Motor Heavy Chains Increases Neurite Length

(A and B) siRNA screen for 34 kinesin and two dynein heavy chains (n > 100). Positive hits were validated in at least three independent experiments (n > 500), showing that partial downregulation of KIF5A, KIF5B, KIF5C, KIF1B, KIF23, or DYNC1H1 increases process length up to 50%. *p < 0.05, **p < 0.01, and ***p < 0.001 (Student’s t test and one-way ANOVA).

(C) Fluorescence images of cultured DRG neurons from adult Thy1-YFP mice treated with the indicated siRNAs. Neurons were replated 1 day after siRNA transfection, and images were acquired 48 hr after replating. Scale bar, 200 μm.

(D) Combined downregulation of both KIF5B and DYNC1H1 causes a greater increase in axon length than observed upon downregulation of each motor separately (n = 80; p < 0.01, ***p < 0.001, Student’s t test and one-way ANOVA).

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retrograde and anterograde transport do not differ significantly between wild-type and Loa heterozygous neurons (Figure S3); thus, for our purposes, sensory neurons from the Loa heterozygote mouse provide a model with specific dynein reduction in the axon that does not significantly impact housekeeping transport requirements. Again, the frequency-dependent model prediction for this case is that cultured mutant neurons should extend longer neurites than the wild-type. Indeed, neurite lengths in cultured adult sensory neurons from heterozygous Loa mice are significantly longer than those observed from wild-type littermates (Figures 3C and 3D). Moreover, there is a greater length increase in Loa heterozygous neurons than in wild-type neurons upon treatment with Kif5B siRNA (Figures 3E and 3F). This finding corroborates the result obtained with dual siRNAs treatment (Figures 2C and 2D), and is in complete accordance with the frequency-dependent model prediction for concomitant reduction of both motors.

The axon length data summarized above are based on in vitro cultures of adult neurons that were axotomized upon excision from the ganglia. To test whether the length increases are replicable in vivo in neurons in the normal elongating phase of outgrowth without any injury, we first verified the reduction in dynein levels in Loa heterozygote axons within the sciatic nerve by immunoelectron microscopy. Loa heterozygote axons revealed approximately 30% reduction in the levels of Dync1h1-positive puncta as compared to wild-type axons (Figures 3G and 3H). We then examined axon lengths in forepaws of mouse embryos by whole mount immunostaining. The dorsal surfaces were imaged for quantification of total outgrowth of the neurofilament-positive processes. Loa heterozygote embryos had over 50% more forepaw innervation than wild-type littermates at E11 (Figures 3I and 3J) and approximately 30% more at E12 (Figures 3K and 3L). It is interesting that there were no apparent differences between the genotypes by E13 (data not shown), indicating that the role of dynein in axon length control is most prominent during the elongating phase of growth in early embryonic development and is likely compensated by other mechanisms after axons reach their targets.

**DISCUSSION**

We have used modeling approaches to examine ways in which molecular motors might enable length sensing over cellular dimensions that are beyond the effective range of diffusion-based mechanisms. Mechanisms based on either quantity or frequency readouts of signal could be formulated in silico; however, the experimental results clearly rule out a quantity-based mechanism and support frequency-encoded signaling as a basis for cell-length sensing. This is most strikingly revealed in the length increase phenotypes for dynein or kinesin alone and for both together. Since these motors transport in opposite directions in axons, reduction in motor levels should lead to opposite effects in a quantity-based mechanism, while the frequency-based model predicts these counterintuitive results. Although direct confirmation of this mechanism will eventually require identification of the signals and feedback processes involved, the work presented here clearly shows that modulating levels of microtubule motors influences cell length, and sets the stage for future work to elucidate the mechanistic details of the system.

Negative feedback loops with a time delay generate oscillating signals, enabling robust encoding of temporal information in biological systems (Mengel et al., 2010; Paszek et al., 2010). Our findings extend this concept by showing that if the time delay in such a feedback loop is provided by motor-dependent transport, such systems can also encode spatial information. The exponentially decaying signal frequency observed in our model should suffice to account for lengths of most nonneuronal cell types and for axon lengths required during initial embryonic development and innervation of targets. Once targets are innervated, neurons switch to a distinct mode of stretch-induced interstitial growth (Smith, 2009), concomitantly with transition to scales requiring length-sensing mechanisms with less marked frequency decay. This might be achieved by utilizing wave-type signaling (Muñoz-Garcia and Kholodenko, 2010) instead of motors. The motor-dependent mechanism proposed here might therefore explain the slow pace of axon regeneration in adult neurons, which could be constrained in their growth rates by an intrinsic length-sensing mechanism evolved for the embryonic scale.

(E) Immunostaining for endogenous DYNC1H1 in DRG neurons treated with control or anti-DynHC1 siRNAs. Scale bar, 60 μm.
(F) Quantification of the immunostaining over six independent experiments reveals partial downregulation of DYNC1H1 (**p < 0.0005).
(G) Immunostaining for endogenous KIF5B in DRG neurons treated with control or anti-KIF5B siRNAs. Scale bar, 60 μm.
(H) Quantification of the immunostaining over six independent experiments reveals partial downregulation of KIF5B (**p < 0.0005).
(I and J) Immunostaining for endogenous Dynein HC1 in DRG neurons treated with control or anti-KIF5B siRNAs does not show any difference in endogenous levels of Dynein HC1 after siKIF5B treatment. See also Figure S2.
Figure 3. Increased Process Length in Sensory Neurons from a Dynein Heavy Chain 1 Mutant Mouse

(A) Immunostaining of endogenous DYNC1H1 in cultured DRG neurons from wild-type and Loa/+ heterozygous mice. Scale bar, 100 μm.

(B) Reduced levels of DYNC1H1 in processes of Loa/+ neurons.
An intriguing future question will be the identity of the signaling molecules in motor-dependent length sensing. Frequency-dependent modulation of transcription factor nuclear import was recently shown to coordinate the regulation of gene expression in yeast (Cai et al., 2008). Transcription factors can be retrogradely transported in axons by importins linked to dynein (Ben-Yaakov et al., 2012); hence, frequency encoding of dynein-transported signals might feed directly into frequency modulation of nuclear translocation. Perturbations of such mechanisms in the embryo might manifest in disease onset later in life, as indeed has been described for dynein (Hafezparast et al., 2003; Weeden et al., 2011).

**EXPERIMENTAL PROCEDURES**

**Modeling and Simulations**

Movement of signals on kinesin and dynein molecular motors was simulated based on published motor velocity distributions (Deinhardt et al., 2006; Seitz and Surrey, 2006), as previously described (Kam et al., 2009). Models represented MTS as moving units with a spatial location and an assigned velocity. At the beginning of the simulation, batches of anterograde MTS are generated in the soma for every time step. Retrograde MTS batches are generated once anterograde signal above a designated threshold reaches the axon tip, and, in turn, anterograde MTS production is repressed once retrograde signal above a designated threshold reaches the cell soma. MTS are removed from the simulation at the time step following their arrival at the soma (for retrograde MTS) or at the tip (for anterograde MTS). For further details on modeling, see Movie S1, Figure S1, and...
Extended Experimental Procedures. A wide range of model configurations were examined, including a range of 20–400 MTS per batch and activation or inhibition thresholds between 10 and 100 accumulated MTS. Axons elongate at each time step throughout the simulation at a fixed rate of 4 μm/hr. All simulation scripts were written in MATLAB, and simulation executions were performed on the Weizmann Wiccopt cluster.

DRG Neuron Cultures and siRNA Screen
The study was conducted in accordance with the guidelines of the Weizmann Institutional Animal Care and Use Committee. Wild-type C57BL/6 and Loo heterozygote mice were bred with C57BL/6 YFP+6 mice (Feng et al., 2000). Adult mouse DRG cultures were transfected with siGenome siRNAs using DharmaFect 4 (Dharmacon), replated 24 hr later, and imaged 72 hr after transfection. Images were captured at 10× magnification on an ImageXpress Micro (Molecular Devices), followed by determination of morphological parameters by MetaXpress2 (Molecular Devices) and WISNeuromath (Weizmann Institute) software. The parameters reported include total outgrowth, defined as the sum of lengths of all processes and branches per cell, and maximal process length, defined as the sum of length of the longest process of a cell including all its branches. When neurite growth extended beyond the maximal field of view compatible with automated analysis, montage images were analyzed manually in random sequence by an observer blind to the details of the experiment. This parameter is reported as the longest axon, defined as the length of the longest process extending from the cell body without secondary branching. Statistical analyses were carried out using Student’s t test and a one-way analysis of variance (ANOVA).

Electron Microscopy
DRG neurons were grown on sapphire disks and fixed 48 hr after plating using high-pressure freezing in a Bal-Tec HPM10, followed by freeze substitution, washing, embedding, and ultrathin sectioning (70–90 nm). Sciatic nerves were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, washed, cut to 1 mm segments and impregnated by 2.3 M sucrose before 120 kV on a Tecnai 12 (FEI) TEM were performed on the Weizmann Wiccopt cluster.

Fibroblast Cultures and siRNA Treatment
NIH 3T3 cells grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum were transfected with siGenome using DharmaFect 1. MEFs were isolated from E14 mouse embryos, and passage 2 MEFs were collected by trophic factor-mediated coupling of protein degradation to protein synthesis. J. Cell Biol. 142, 1313–1324.

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EXTENDED EXPERIMENTAL PROCEDURES

Model Construction and Simulation Runs
Models were constructed as previously described (Kam et al., 2009), representing motor transported signals (MTS) as moving units in a computer simulation. Each such MTS has a location in space, and it can move according to a velocity assigned to it on the basis of known velocity distributions for molecular motors (see below). Number of MTS, mode of generation and thresholds for signaling activation or inhibition were varied as described below. All simulation scripts were written in MATLAB, and simulation executions were performed on the Wiccopt cluster at the Weizmann Institute’s computing center to allow parallel executions of simulations which varied in initial parameter settings. The cluster nodes consist of machines with: 2 quadcore xeon CPU’s, 1 quadcore xeon CPU, 2 dualcore AMD opteron, and 1 dualcore AMD opteron.

Motor Velocity Data Fit
Movement of kinesin and dynein molecular motors was simulated based on experimental velocity measurements from the literature (Deinhardt et al., 2006; Seitz and Surrey, 2006). In order to produce a velocity distribution function, a data fit procedure was applied for the datasets used in this work. For this purpose, we used a built-in Matlab script (fminsearch) based on the Nelder-Mead method. Since our model does not account for zero velocities, we introduced a slight modification to the Gaussian function, thus requiring the velocity distribution function to intersect with (0,0). The curve fit function used was of the form:

$$Fit = a_1 X - e^{\frac{(X - a_2)^2}{a_3}}$$

Thus, for the velocity X = 0, the function yields zero occurrences.

Goodness-of-fit was assessed by calculating the root mean square deviation (RMSD) between the observed data points and the values predicted by the calculated function:

$$RMSD = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (FitValue_i - ObservedValue_i)^2}$$

Values in the above function are given in terms of percentage-of-occurrence of given motor velocities. This measurement provides an estimate for the average distance between a given data point and the calculated curve.

Fit Results
For the dynein data (Deinhardt et al., 2006) the following results were obtained:

$$Fit = 0.2940X + e^{\frac{(X + 0.6657)^2}{1.7231}}$$

$$RMSD = 0.61\%.$$

For the kinesin data (Seitz and Surrey, 2006) the following results were obtained:

$$Fit = 53.9881X + e^{\frac{(X - 0.5263)^2}{0.1829}}$$

$$RMSD = 2.44\%.$$

The Gradient-Based Model
In these simulations, MTS are generated at the axon tip at every time step and are carried by dynein toward the cell body. At every time step a certain proportion of the MTS are removed from the system regardless of their position along the axon (thus representing signal loss). Simulations were executed for various combinations of MTS levels and rate of signal loss (% of MTS removed at every time step). The representative results in the figure are for simulations in which the signal loss rate was set to 20%, and the growth arrest threshold at 100 MTS. The blue, red and green curves depict the change in retrograde signal levels at the cell body over time for signal generation levels of 5,000, 1,000, and 500 MTS per batch, respectively.

The Frequency-Based Model
Batches of MTS are generated at every time step in the simulation of the algorithm detailed in Model Parameters below. At the beginning of the simulation batches of anterograde MTS are generated in the cell soma. Retrograde MTS batches are generated once
anterograde signal above a designated threshold reaches the axon tip, and in turn anterograde MTS production is repressed once retrograde signal above a designated threshold reaches the cell center. MTS are removed from the simulation at the time step following their arrival at the soma (for retrograde MTS) or at the tip (for anterograde MTS). Axons elongate at each time step throughout the simulation at a fixed rate of 4 μm/hour. Model configurations examined included a range of MTS per batch of between 20-400 (including configurations of different numbers of MTS per batch for anterograde versus retrograde signals), and activation or inhibition thresholds of between 10 and 100 MTS. The representative results shown in Figure 1 are for 100 MTS per batch for both anterograde and retrograde signals, with feedback loop thresholds set at 10 MTS. In Figure 1E, high and low MTS levels are 400 and 30 units per batch, respectively. The normalized-frequency threshold for growth arrest was set to 0.02.

**Model Parameters**

Numbers in parentheses are ranges used in simulations:

- **AxonLength** – axon length during the simulation (initially set to 50 μm).
- **GrowthRate** – axon elongation during time step (set to a value corresponding to 4 μm/hr in reality).
- **noAntParticles** – the user-defined number of anterograde signal particles in each batch (10 - 500).
- **noRetParticles** – the user-defined number of retrograde signal particles in each batch (10 - 400).
- **AnterogradeThreshold** – the user-defined number of anterograde signal particles required to activate the retrograde signal (5 - 80).
- **RetrogradeThreshold** – the user-defined number of retrograde signal particles required to inhibit the anterograde signal (5 - 80).
- **AntParticlesLocations** – stores the location of each anterograde signal particle in terms of distance from the cell body (initially set to zero).
- **RetParticlesLocations** – the location of each retrograde signal particle in terms of distance from the cell body (initially set to AxonLength).
- **AntParticlesVelocities** – stores the velocities associated with each anterograde signaling particle based on an experimentally measured distribution of kinesin velocities.
- **RetParticlesVelocities** – stores the velocities associated with each retrograde signaling particle based on an experimentally measured distribution of dynein velocities.
- **AntCount** – amount of anterograde signal particles accumulated at the axon tip.
- **RetCount** – amount of retrograde signal particles arriving at the cell body within time step.
- **t** – the number of time steps defined for the simulation. Each time step in the simulation represents 1 min in reality. Simulations were run up to 1000 hr of axon growth.

**Algorithm**

While Time < t:

1. If RetCount < RetrogradeThreshold
   1.1 Produce a new batch of anterograde signal
   1.1.1 Initiate new batch’s AntParticlesLocations
   1.1.2 Initiate new batch’s AntParticlesVelocities (= Randomly assign velocities to new batch particles based on velocity distribution)

2. Promote all anterograde signal particles based on their assigned velocities

3. Update AntCount based on the number of anterograde signal particles that arrived at axon tip within current time step

4. If AntCount ≥ AnterogradeThreshold
   4.1 Produce a new batch of retrograde signal
   4.1.1 Initiate new batch’s RetParticlesLocations
   4.1.2 Initiate new batch’s RetParticlesVelocities (= Randomly assign velocities to new batch particles based on velocity distribution)

   4.1.3 AntCount = 0 (remove all anterograde signal particles from axon tip)

5. Promote all retrograde signal particles based on their assigned velocities

6. RetCount = number of retrograde signal particles that arrived at cell body within current time step

7. AxonLength = AxonLength + GrowthRate

8. Time = Time + 1.

In order to understand how the frequency of the signal changes with time, we used the Matlab Spectrogram function (www.mathworks.co.kr/access/helpdesk/help/toolbox/signal/spectrogram.html), which computes the power spectral density (PSD) for each signal segment. Running a spectrogram analysis for the signal yields a series of harmonics (i.e., integer multiples of the fundamental frequency) that decay in a non-linear fashion. The color map represents the PSD values, with high values depicted at dark red. The dominant signal frequency is extracted from the output produced by the spectrogram function and described as a function of
time by scanning the PSD matrix and selecting the frequency with the highest PSD value at each time point (after eliminating the zero frequency).

Animals
The study was conducted in accordance with the guidelines of the Weizmann Institutional Animal Care and Use Committee (IACUC). Wild-type C57BL/6 and Loa heterozygote mice were bred with C57BL/6 YFP16 (Feng et al., 2000) mice and maintained at the Veterinary Resources Department of the Weizmann Institute. Mouse colonies were housed in standard cages (up to 6 mice per cage) on a 12 hr light/12 hr dark cycle with food and water ad libitum before experimental procedures. Adult (2-4 months old) animals were sacrificed by CO₂ inhalation.

Antibodies and Imaging
Rabbit anti-dynein heavy chain (DynHC R-325) was from Santa-Cruz (sc-9115); mouse anti-NFH clone N52 and mouse anti-tubulin β3 (T2200) were from Sigma. Rabbit anti-GFP was from Abcam (ab6556); rhodamine phalloidin was from Molecular Probes (Invitrogen R415). For immuno-EM we used goat anti-rabbit IgG – 6 nm (#25104) and 10 nm (#25109) gold EM grade from Electron Microscopy Science (EMS). Fluorescent secondary antibodies were from Jackson ImmunoResearch. Cultured DRG neurons were fixed with 3% paraformaldehyde and stained with anti-NFH for process length determination. Neuronal images were acquired at X10 magnification on an ImageXpress Micro (Molecular Devices) automated microscopy system. Microtubules were visualized by anti-tubulin β3 with rhodamine-phalloidin staining using an Olympus FV1000 confocal laser-scanning microscope at X60 magnification with oil-immersion Olympus UPLSAPO objective (NA 1.35) and analyzed by FV10-ASW2.0 software.

DRG Neuron Cultures and siRNA Screen
Adult C57BL/6 YFP16 mouse DRG were dissociated with 100 U of papain followed by 1 mg/ml collagenase-II and 1.2 mg/ml dispase. Ganglia were then triturated in HBSS, 10 mM glucose, and 5 mM HEPES (pH 7.35). Neurons were recovered through percoll, plated on laminin, and grown in F12 medium. Mouse siGenome smart pools (Dharmacon) were used for transfection of neuronal cultures two hours after plating, using DharmaFect 4 as the transfection reagent. 24 hr after siRNA transfection the neurons were replated in new Poly-L-Lysine and Laminin treated glass bottom 96 well plates at the same density (80,000-100,000 cells per well) and imaged 72 hr after transfection. Images were captured at X10 magnification on ImageXpress Micro. Process length, branch numbers and cell body area were determined using a MetaXpress2 software package (Molecular Devices). Statistical analyses were carried out using Student’s t test and One Way ANOVA.

Electron Microscopy
DRG neurons were grown on sapphire disks and fixed 48 hr after plating using high pressure freezing (HPF) in a Bal-Tec HPM10. Frozen samples were transferred to a Leica AFS and freeze substituted in acetone, containing 0.1% glutaraldehyde and 0.1% uranyl-acetate at –90 °C for 72 hr. Samples were then washed in alcohol and embedded in HM20 resin at –30 °C. Ultrathin sections (70–90 nm) were prepared on a Leica UCT ultramicrotome and collected on nickel grids coated with 0.3% Formvar. For immunostaining, grids were first reacted with anti-dynein HC1, followed by anti-GFP when required for double-labeling, and secondary anti-rabbit IgG with different sizes of gold particles as indicated above. The grids were then stained in uranyl acetate and lead citrate and analyzed under 120 kV on a Tecnai 12 (FEI) Transmission Electron Microscope with a EAGLE (FEI) CCD camera using TIA software. Dynein HC1 was quantified by superimposing grids on the images and counting numbers of gold particles per grid sector in cytoplasmic regions, excluding mitochondria. Right sciatic nerves of 10-week old male wild-type and Loa/+ mice were isolated at the same time and fixed by 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M cacodylate buffer for 2 hr at RT and then at 4 °C overnight. The nerves were then washed in 0.1M cacodylate buffer, cut to 1 mm fragments and impregnated by 2.3 M sucrose in the same buffer overnight at RT. Nerve fragments were then rapidly frozen in liquid nitrogen, and ultrathin sections (70–90 nm) were prepared at ~120 °C on a Leica EM FC6 ultramicrotome with cryochamber. Sections were collected on nickel grids coated with 0.3% Formvar. For immunostaining, grids with sections were reacted with rabbit anti-dynein HC1 antibody followed by secondary anti-rabbit IgG with 10 nm gold particles as indicated above. The grids were then embedded in methylcellulose with 0.2% uranyl acetate and analyzed under 120 kV on a Tecnai 12 (FEI) Transmission Electron Microscope with the EAGLE (FEI) CCD camera using TIA software. Dynein HC1 was quantified by superimposing grids on the images and counting numbers of gold particles per grid sector in cytoplasmic regions, excluding mitochondria.

Kinetic Analysis of Axonal Transport
DRG neurons from adult C57BL/6 YFP16 wild-type and C57BL/6 YFP16/ Loa heterozygote mice were isolated as previously described. Neurons were seeded on 35 mm glass bottom dishes (MatTek Corporation) at a density of 80,000 neurons per coverslip and maintained in a humidified 5% CO₂ incubator at 37 °C for 3-4 d. Mitochondrial labeling was by incubation with MitoTracker® Red CMXRos Molecular Probes (M7512) 50 nM for 30 min before imaging. Cells were washed, and imaged by time-lapse low-light microscopy on a DeltaVision system. Images were acquired every 5 s over a total of up to 150 frames per movie at X60 magnification with oil-immersion Olympus objective (NA 1.42), using FITC or Cy3 filters for YFP carriers or mitochondria, respectively. Only moving
carriers that could be tracked for at least three time points were considered. The distance covered by a carrier between two consecutive frames (5 s), was used to determine its instantaneous speed using Tracker 3.0 (www.cabrillo.edu/~dbrown/tracker/) to follow YFP puncta or mitochondria.

**Fibroblast Cultures and siRNA Treatment**

NIH 3T3 cells were grown in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma) at 37°C in 5% CO₂. One day prior to transfection the cells were collected, diluted at the appropriate concentration (6 X10⁴ per well from 6 well plate) and grown in DMEM medium supplemented with 10% FBS without antibiotics. Mouse siGenome smart pools (Dharmacon) were used for transfection, using DharmaFect 1 as the transfection reagent. Medium was changed 24 hr after transfection. 72 hr after transfection the cells were collected and processed for flow cytometry or imaging analyses. For imaging, cells were diluted to a concentration of 4X10⁴ cells per well in 96 well glass bottom plates, cultured for a further 24 hr and then fixed and stained with DAPI and Rhodamin-Phalloidin. For flow cytometry cells were resuspended in 0.5 ml PBS and analyzed using fluorescence-activated cell sorting on an LSRII cell analyzer with FCS-Express software.

Mouse embryonic fibroblasts (MEF) were isolated from E14 mouse embryos, expanded and then replated every three days. Each embryo was genotyped and grown in a separate dish. MEF were grown in DMEM medium with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂. Passage 2 MEF were collected, diluted to 4X10⁴ cells per well in a 96 well glass bottom plate, grown for another 24 hr and then fixed and stained with DAPI (Biotium) and Rhodamin-Phalloidin.

Images of 3T3 or MEF cultures were captured at X10 and X40 magnification on an ImageXpress Micro (Molecular Devices). Nucleus and cell body area were determined using a MetaXpress2 software package (Molecular Devices). Statistical analyses were carried out using Student’s t test and One Way ANOVA.

**SUPPLEMENTAL REFERENCES**

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**Figure S1. Modeling, Related to Figure 1**

(A and B) Kinesin and dynein velocity distributions used for the simulations (Deinhardt et al., 2006; Seitz and Surrey, 2006).

(C and D) A single positive feedback loop model generates a non-informative retrograde signal.

(E and F) A similar model incorporating two positive feedback steps likewise generates a non-informative retrograde signal.

(G) Dominant frequency extraction in signals obtained from the composite negative feedback loop model of Figures 1C and 1D. For further details, see Extended Experimental Procedures.
Figure S2. Partial Downregulation of Dynein Heavy Chain 1 Does Not Affect Cytoskeleton Integrity or Growth Cone Morphology, Related to Figure 2

(A) Immunostaining for endogenous dynein heavy chain 1 (Dync1h1) in DRG neurons treated with control or anti-Dync1h1 siRNAs. Scale bar, 60 μm.

(B) Quantification of the immunostainings over three independent experiments revealed partial downregulation of dynein heavy chain 1 (**p < 0.0005).

(C) Immunostaining for tubulin β3 and phallolidin-rhodamine staining for F actin did not reveal any cytoskeletal abnormalities or aberrant growth cone morphology after siRNA treatment by this protocol. Scale bar, 5 μm.
Figure S3. The Loa Mutation Does Not Affect Kinetics of Retrograde or Anterograde Transport in Heterozygous sensory Neurons, Related to Figure 3

(A) Electron micrographs showing co-localization of Dyn1h1 and YFP near microtubules in axons of cultured DRG neurons isolated from the Thy1-YFP+/+ mouse. Dyn1h1, 10 nm gold; YFP, 6 nm gold.

(B and C) (B) Kinetic analysis of YFP retrograde transport or (C) of mitochondria anterograde transport in Thy1-YFP+/+ and Thy1-YFP+/-Loa adult DRG neurons did not reveal any significant difference in velocity distributions.
Figure S4. FACS Analyses of 3T3 Cell Size, Related to Figure 4

(A) FACS analyses of 3T3 cells treated with the indicated siRNAs. The plots show forward scatter (FSC-A), a measure of cell size, versus side scatter (SSC-A), a measure of granularity.

(B) Dyn1h1 siRNA treatment causes an increase in cell size as shown by the right shift in population distribution in comparison to controls.