The light-sensitive dimerizer zapalog reveals distinct modes of immobilization for axonal mitochondria

Amos Gutnick1,2, Matthew R. Banghart2,3, Emma R. West2 and Thomas L. Schwarz1,2*

Controlling cellular processes with light can help elucidate their underlying mechanisms. Here we present zapalog, a small-molecule dimerizer that undergoes photolysis when exposed to blue light. Zapalog dimerizes any two proteins tagged with the FKBP and DHFR domains until exposure to light causes its photolysis. Dimerization can be repeatedly restored with uncleaved zapalog. We implement this method to investigate mitochondrial motility and positioning in cultured neurons. Using zapalog, we tether mitochondria to constitutively active kinesin motors, forcing them down the axon towards microtubule (+) ends until their instantaneous release via blue light, which results in full restoration of their endogenous motility. We find that one-third of stationary mitochondria cannot be pulled away from their position and that these firmly anchored mitochondria preferentially localize to VGLUT1-positive presynapses. Furthermore, inhibition of actin polymerization with latrunculin A reduces this firmly anchored pool. On release from exogenous motors, mitochondria are preferentially recaptured at presynapses.

Mitochondria are cells' primary source of ATP and their motility and localization are fundamental to cellular function. This is particularly true in neurons whose branches can be >1 m in length and whose survival and function depend on the presence of healthy mitochondria at key points throughout the cell. Much has been learned from traditional tools, such as knockouts, mutations and RNA-mediated interference, but these methods cause chronic changes that can produce lethality or compensatory changes1–5. Moreover, they act slowly and therefore are poor probes of rapid or transient events. Our understanding of this dynamic process can benefit from the ability to interact directly with discrete components. What determines which mitochondria move? What mechanisms underlie mitochondrial arrest? If mitochondria could be dragged away from their normal positions, would their motility profile change in response? Tools that can directly (and temporarily) control the movement and position of mitochondria could reveal insights about such fundamental processes1.

Many such cell biological tools have been developed to provide direct control of proteins. Chemical inducers of dimerization (CIDs), small cell-permeable molecules such as the widely used ‘rapalogs’, are designed to interact with specific protein targets and quickly induce a physical interaction between two tagged proteins6. Both chemical and genetically encoded dimersizers have been valuable for the study of axonal transport of mitochondria and other organelles7–12.

Here we present a CID of potential value to cell biologists. ‘Zapalog’ is a photocleavable small-molecule heterodimerizer that can be used to repeatedly initiate, and instantaneously terminate, a physical interaction between two target proteins. We have used zapalog to probe the molecular underpinnings of mitochondrial motility and positioning in the axon by temporarily forcing their motility through the addition of constitutively active kinesins. Through these manipulations we identified and characterized a population of mitochondria restrained by actin-dependent anchoring.

Results

Design and synthesis of zapalog. Several parameters guided the design of zapalog. To allow heterodimerization of engineered proteins, we needed a CID with two orthogonal ligand moieties that exhibit high specificity and affinity for their binding domains and lack alternative endogenous binding partners in mammalian cells at useful concentrations. The CID must be membrane permeable and the linker must be long enough to allow both binding domains to be simultaneously engaged without steric interference. The photocleavable linker must respond to a wavelength that is neither cytotoxic nor interferes with imaging common fluorophores. The compound needs to be synthesized on the milligram scale.

The chosen starting point was the heterodimerizer tri- methoprim-synthetic ligand of FK506-binding protein (TMP-SLF), a cell-permeable CID effective at low micromolar concentrations13. The SLF portion binds the FK506-binding protein (FKBP) domain of one chimaeric partner; the TMP portion binds the Escherichia coli dihydrofolate reductase (DHFR) domain of another (Fig. 1a). To render dimerization reversible with light, the alkyl linker between TMP and SLF was replaced with a dialkoxynitrobenzyl (DANB) moiety (Fig. 1b). DANB is closely related to the commonly used dimethoxynitrobenzyl chromophore and is similarly susceptible to photolysis by 405 nm light14 (Supplementary Fig. 1a). This wavelength is short enough to avoid photolysis during imaging of green or cyan fluorophores but less toxic and more spatially precise than near-ultraviolet or ultraviolet light15–17. Neither TMP nor SLF has endogenous protein targets and they exhibit sub-micromolar affinities for their binding domains, which are relatively compact (DHFR = 158 amino acids; FKBP = 107 amino acids).

Zapalog is photosensitive, cell-permeable, non-toxic and effective as a non-covalent dimerizer. We commissioned the synthesis of zapalog (detailed in Methods, Supplementary Fig. 2 and in

1Kirby Neurobiology Center, Boston Children’s Hospital, Boston, MA, USA. 2Department of Neurobiology, Harvard Medical School, Boston, MA, USA. 3Present address: Division of Biological Sciences, Neurobiology Section, University of California San Diego, San Diego, CA, USA.

*e-mail: thomas.schwarz@childrens.harvard.edu
Protocol Exchange\(^{16}\)) and obtained 10 mg of thin-layer chromatography (TLC)-purified material. With 405 nm light, zapalog in solution underwent the expected photocleavage shown schematically in Fig. 1b (Supplementary Fig. 1a). Zapalog was not affected by 458 nm light, making it suitable for use with cyan fluorescent protein imaging (Supplementary Fig. 1b).

To assess, in living COS7 cells, zapalog's ability to function as a heterodimerizer and undergo photocleavage, we modified a fluorophore translocation assay previously established for rapalogs\(^{17}\): we tagged the surface of mitochondria with Tom20–mCherry–FKBP and co-expressed cytoplasmic yellow fluorescent protein (YFP)–DHFR–Myc (Fig. 1c(i),d,e(ii)). We used a spinning-disc confocal microscope for live imaging of cytoplasmic YFP and mitochondrial mCherry at 2 frames s\(^{-1}\). Addition of zapalog to the media induced dimerization of the constructs and YFP rapidly cleared from the cytoplasm and increased on mitochondria (Fig. 1c(i) and Supplementary Video 1). We quantified the progression of YFP translocation by measuring cytoplasmic and mitochondrial YFP levels in the entirety of each cell (Fig. 1c(ii); see Methods) and plotted the time from 10% to 90% YFP translocation as a function of zapalog concentration (Fig. 1c(iii)). Zapalog exhibited equivalent on-rate kinetics to previously reported CIDs and a half-maximum concentration \((EC_{50})\) of ~100 nm\(^{-1}\). The time course of binding is likely to be faster than indicated because the measured time course for YFP recruitment relies mainly on zapalog diffusion in the bath and into the cell, and fluorophore diffusion to the mitochondria. To test the efficiency and speed of reversing the YFP translocation with exposure to 405 nm light, we repeated YFP translocation onto mitochondria as above, but removed zapalog from the media after 10 min. YFP recruitment to mitochondria persisted after zapalog was washed out, evidence of zapalog retention in the cell. We used a laser scanning confocal microscope to sequentially flash 405 nm light separately onto each cell. As shown in Fig. 1e(i,ii) and Supplementary Video 2, exposure of each cell to a single 500 ms flash of blue light proved sufficient to fully dissociate YFP localization from mitochondria in <1 s.

Zapalog can induce multiple rounds of dimerization. We hypothesized that zapalog could induce multiple rounds of dimerization: if a focused light beam photocleaved a fraction of the total pool of zapalog in the cell, the locally cleaved ligands could potentially be replaced by means of competitive binding of uncleaved molecules from the surrounding area. The resulting re-dimerization after the photolysis would open the possibility for multiple cycles of cleavage and re-dimerization, potentially on individual mitochondria. As mitochondria form a highly fused network, we first fragmented the network in HeLa cells with a 6 h incubation in media after 10 min. YFP recruitment to mitochondria persisted after zapalog was washed out, evidence of zapalog retention in the cell. We used a laser scanning confocal microscope to sequentially flash 405 nm light separately onto each cell. As shown in Fig. 1e(i,ii) and Supplementary Video 2, exposure of each cell to a single 500 ms flash of blue light proved sufficient to fully dissociate YFP localization from mitochondria in <1 s.

The mitochondrial motor complex retains its activity after release from zapalog-induced movement. We tested the effect of temporarily forced mobilization on endogenous mitochondrial motility. Previous studies used rapalogs to attach motors to mitochondria and thereby overpower the endogenous motors\(^{10}\). However, dimerization induced by rapalogs is irreversible and therefore rapalogs cannot be used to determine what happened to the endogenous motor complex when it was superseded. We quantified axonal mitochondrial motility for 5 min before zapalog addition, 5 min after 2μM zapalog addition and 5 min after zapalog was completely photocleaved (Fig. 3 and Supplementary Video 5). After photocleavage, the parameters of mitochondrial motility return to their initial values (Fig. 3c,d). With Pearson’s chi-squared tests, we compared motility before zapalog addition and after its photolysis; the variability within each parameter (that is, ‘percentage retrograde/stationary/anterograde’) originates from axon-to-axon differences \((P < 0.005)\) and not as a consequence of zapalog-induced disruption \((P > 0.1)\). Thus, the mitochondrial motility profile of an axon is inherently robust, and able to re-establish itself even after a prolonged (5 min) zapalog-induced disruption. As mentioned previously, an exception to this robustness is axons with mitochondrial pile-ups; the pile-ups did not resolve readily after photolysis, probably because the induced proximity had caused a hyperfused

Zapalog-induced mitochondrial motility reveals distinctions among stationary axonal mitochondria. To probe the mechanisms of mitochondrial positioning and movement in axons, we asked whether zapalog could tether a kinesin to mitochondria with sufficient affinity to drag mitochondria towards the (+) ends of microtubules (that is, away from the soma). We cultured embryonic day (E)18 rat hippocampal neurons in spot-grove cultures (see Methods), and co-transfected them with Tom20–mCherry–FKBP and Kif1a(1–489)–DHFR–Myc, a truncated, constitutively active version of Kif1a that includes only the motor and necklinker domains\(^{18}\). Before addition of zapalog, ~30% of mitochondria were motile and ~70% stationary, consistent with previous reports\(^{1}\). Within 1 min of 2μM zapalog addition, the majority of mitochondria \((60.3\% \pm 12.2\%)\) begin to move rapidly anterogradely at an average speed of 2.5 μm s\(^{-1}\) (Fig. 3). Immunocytochemistry with anti-Myc confirmed that zapalog recruits the tagged motors to all mitochondria (Supplementary Fig. 3a). The percentage of retrograde moving mitochondria decreased from 13.99% to 3.52%, suggesting that the exogenous kinesin overcomes the dynein motors that had been moving mitochondria retrograde. Despite zapalog-induced recruitment of the kinesin to all mitochondria, 36% of axonal mitochondria remained stationary, seemingly resisting the zapalog-induced pull. The inability of the activated motors to mobilize one-third of the axon’s mitochondria prompts us to expand on the widely used classification of mitochondria as either ‘motile’ or ‘stationary’—rather this assay subdivides mitochondria into ‘motile’, ‘movable’ or ‘immovable’ under the conditions of the assay, a distinction that implies different mechanisms or degrees of mitochondrial arrest (Supplementary Fig. 3b and Supplementary Video 5).

One necessary consideration in analysing motility after zapalog addition is that the ‘wave’ of anterograde-moving mitochondria pile up at growth cones (Supplementary Fig. 3c and Supplementary Video 6) and are gradually depleted elsewhere (Supplementary Fig. 3d and Supplementary Video 7). In some cases, mitochondria piled up at bends or constrictions in mid-axons; such blocked axons appeared unhealthy, fragmented within 10 min and were eliminated from further analysis.
Fig. 1 | Zapalog, a photocleavable heterodimerizer, can be used to reversibly translocate cytosolic YFP to mitochondria. a, A schematic illustration of zapalog function. Two proteins of interest are tagged with DHFR and FKBP domains (1). Addition of zapalog induces dimerization of the tagged proteins (2). Exposure to 405 nm light photocleaves zapalog, causing rapid dissociation of the dimer (3). Addition of uncleaved zapalog outcompetes photolysed zapalog moieties, re-establishing dimerization (4). b, The chemical structure of zapalog before and after photolysis of the DANB moiety by 405 nm light. c, (i) Time-lapse imaging demonstrates full translocation of YFP–DHFR–Myc onto mitochondria in a COS7 cell, within ~1 min after the addition of 10 µM zapalog to the medium. (ii) Quantification of multiple experiments as in (i) using 10 µM zapalog. The ratio of mitochondrial to cytoplasmic YFP–DHFR–Myc was derived from automated image analyses of the datasets and normalized, and average YFP intensity was calculated for each time point (n = 5 cells/3 independent repeats, centre value = mean, error bars represent s.e.m.; source data are available in Supplementary Table 1). (iii) From assays of YFP translocation to mitochondria, quantified as in (ii), a dose–response curve was generated by plotting the time from 10% to 90% of full YFP–DHFR–Myc translocation for each concentration of zapalog administered (n = 98 cells, 6 independent repeats, centre value = mean, error bars represent s.d., source data are available in Supplementary Table 1). d, A schematic illustration of zapalog-induced translocation of YFP. Zapalog attaches cytoplasmic YFP–DHFR–Myc to FKBP domains tethered to mitochondrial outer membranes. Exposure to 405 nm light photocleaves zapalog, releasing the YFP–DHFR–Myc back to the cytoplasm. e, (i) Before the series of images shown, YFP–DHFR–Myc translocation to mitochondria was induced by zapalog, as in c(i). After wash-out of free zapalog from HeLa cells, the YFP reporter was released from the mitochondria sequentially in each of the 3 cells by exposing the circled region to a brief (500 ms) pulse of 405 nm light. (ii) Quantification of the data shown in (i) in e, demonstrating that photolysis of zapalog is rapid, complete and spatially localizable. TM, transmembrane. Scale bars, 5 µm.
mass to form that cannot be easily mobilized (Supplementary Fig. 3c). Similarly, if prolonged exposure depletes most of the motile mitochondria proximal to the imaged region, anterograde movement into that region will be depressed (Supplementary Fig. 3d).

Anchored mitochondria co-localize with VGLUT1. We were surprised to find that consistently ~20% of mitochondria remain firmly in place even while hundreds of mitochondria are pulled past them by zapalog-enabled exogenous motors. We wished to

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**Fig. 2 | Zapalog can induce multiple rounds of dimerization.** a, (i) Bottom: a HeLa cell expressing mitochondrial Tom20-mCherry-FKBP and YFP-DHFR-Myc incubated with 10μM CCCP for 6 h to fragment mitochondria, and then 2μM zapalog for 5 min, which has caused YFP-DHFR-Myc to localize to outer mitochondrial membranes. Top: a blown-up image of the area defined in the bottom image with a yellow square within which two mitochondrion-containing 2μm × 2μm ROIs (dashed white squares) were defined and imaged at high temporal resolution. In the time series to the right, one of the ROIs (+) was periodically illuminated with a 405 nm laser (~300 nW) for 500 ms; the other (−) was not. Each flash of blue light causes photolysis of zapalog and immediate reversal of the dimerization in ROI (+) but not (−). Each instance of YFP-DHFR-Myc removal from a mitochondrion is followed by rapid re-dimerization (~30 s) due to influx of unlysed zapalog from outside the ROI, which outcompetes the lysed zapalog fragments bound to FKBP and DHFR.

(ii) A schematic illustration of repeated fluorophore translocation mediated by zapalog. (iii) Quantification of the normalized YFP signal within discrete mitochondrion-containing ROIs (2μm × 2μm) that were illuminated by a 405 nm laser, and nearby ROIs that were not (n = 7 cells/3 independent repeats, centre value = mean, error bars represent s.e.m., source data are available in Supplementary Table 1). b, (i) Bottom: a COS7 cell expressing peroxisomal PEX3-mRFP-FKBP and YFP-DHFR-Myc incubated with 2μM zapalog for 5 min, which has caused YFP-DHFR-Myc to localize to peroxisomal membranes. Top: two peroxisome-containing 6μm × 6μm ROIs were defined and imaged at high temporal resolution. In the time series to the right, one of the ROIs (+) was illuminated with a 405 nm laser (10%) for 500 ms every 40 s; the other (−) was not. Each flash of blue light causes photolysis of zapalog and immediate reversal of the dimerization in ROI (+) but not (−). Each instance of YFP-DHFR-Myc removal from peroxisomes is followed by rapid re-dimerization (~30 s) due to influx of unlysed zapalog from outside the ROI, which outcompetes the lysed zapalog fragments bound to FKBP and DHFR.

(ii) A schematic illustration of repeated fluorophore translocation mediated by zapalog. (iii) Quantification of normalized YFP signal within peroxisome-containing ROIs (5μm × 5μm) that were illuminated by a 405 nm laser, and nearby ROIs that were not (n = 4 cells/2 independent repeats, centre value = mean, error bars represent s.e.m., source data are available in Supplementary Table 1).
further characterize this pool of mitochondria that were immovable in the assay. Previous studies have pointed to several mechanisms promoting arrest of mitochondria specifically at presynaptic loci\(^1\). Likely presynaptic loci can be identified in axons as stable puncta of the presynaptic marker VGLUT\(^1\). We repeated the forced mobilization assay in neurons co-transfected with...
movable in latrunculin A (Fig. 5c). Our data strongly suggest that the data was too great to determine whether they were also more dria that did not co-localize with VGLUT1 puncta, the variance of the control (Fig. 5c). For the smaller pool of stationary mitochon-

log led to a significantly smaller proportion of mitochondria in mitochondrial movement25 (Fig. 5b). However, addition of zapa -dria was found, consistent with other reports that actin can restrain sites (Fig. 5a). A slight increase in the number of motile mitochon-

anchoring role of the actin cytoskeleton, we pre-treated neurons with latrunculin A, a blocker of actin polymerization, in a proto-
col that eliminated all detectable actin filaments without disrupting axonal tubulin or VGLUT1 (Supplementary Fig. 4). Before addition of zapalog, a blocker of actin polymerization, in a proto-
treatment (that is, previously occupied places) versus the length of axon that had held stationary mitochondria before zapalog treatment caused most of the movable mitochondria to be transported to distal axonal regions and therefore most of the movement after release was necessarily retrograde. We marked each event of mitochondrial capture, which we defined as a motile mitochondrion that stops for at least 5 min. We normalized the number of capture events to the length of axon that had held stationary mitochondria before zapalog treatment (that is, previously occupied places) versus the length of axon that had not and observed a clear bias towards previously mitochondria-occupied places (Fig. 6b,c) and presynaptic loci (Fig. 6d). As most presynaptic sites had retained an anchored mitochondrion, it was possible that contact with a stationary mito-

Capture of mitochondria at VGLUT1-positive loci. What happens to mitochondria that were pulled away from their normal positions after they are released from the zapalog-tethered motors? Specifically, in an axon segment in which every movable mitochondrion has been moved away, do returning mitochondria stop at random places or are they prone to capture at sites that previously held a stationary mitochondrion, such as presynaptic loci? To address this question, we followed mitochondria at high temporal resolution during zapalog-induced mobilization (which was maintained for >10 min), and on through 40 min after photolysis (Fig. 6a). The long zapalog treatment caused most of the movable mitochondria to be transported to distal axonal regions and therefore most of the movement after release was necessarily retrograde. We marked each event of mitochondrial capture, which we defined as a motile mitochondrion that stops for at least 5 min. We normalized the number of capture events to the length of axon that had held stationary mitochondria before zapalog treatment (that is, previously occupied places) versus the length of axon that had not and observed a clear bias towards previously mitochondria-occupied places (Fig. 6b,c) and presynaptic loci (Fig. 6d). As most presynaptic sites had retained an anchored mitochondrion, it was possible that contact with a stationary mito-

VGLUT1–Venus (Fig. 4a). About 60% of VGLUT1-positive sites co-localized with mitochondria before the addition of zapalog, and ~70% of the stationary mitochondria before co-localized with stable VGLUT1–Venus puncta. On zapalog-induced recruitment of the Kif1a motor, the number of stationary mitochondria occupying VGLUT1-positive loci was unchanged, but the number of stationary mitochondria that were not co-localized with VGLUT1–Venus (Fig. 4b) was reduced. Thus, the mitochondria that co-localized with stable VGLUT1 puncta were resistant to the zapalog-enabled pull. This pool of presumably presynaptic mitochondria is therefore not passively stationary, due for example to inactivation of their motors, but are strongly anchored in place, and more strongly than the extra-synaptic stationary mitochondria.

Anchoring of mitochondria involves actin. To test a potential anchoring role of the actin cytoskeleton, we pre-treated neurons with latrunculin A, a blocker of actin polymerization, in a protocol that eliminated all detectable actin filaments without disrupting axonal tubulin or VGLUT1 (Supplementary Fig. 4). Before addition of zapalog, latrunculin A had no detectable effect on the number of stationary mitochondria that co-localize with VGLUT1–Venus and stationary mitochondria that do not co-localize with VGLUT1-Venus (n = 28 axons/4 independent repeats; two-sided paired t-tests; NS (not significant), P > 0.05; ****P < 0.0001; source data are available in Supplementary Table 1).
nascent presynaptic sites in the axon versus those that are stationary but not presynaptic. Mitochondria were 7.2 times more likely to stop at the presynaptic locations than elsewhere.

**Discussion**

To date, several systems for controlling the localization and consequently the activity of cellular machinery have been developed\(^6,11,26–29\). Rapalogs (that is, rapamycin analogues) are prominent; these non-toxic, cell-permeable CIDs force a non-covalent interaction between proteins genetically tagged with the ~11 kDa domains FKBP and FKBP–rapamycin-binding (FRB). Rapalogs, however, lack spatiotemporal control and the dimerization they induce is irreversible\(^30\). This has prompted the development of additional genetically encoded, photoreactive systems such as TULIP\(^31\), PhyB-PIF\(^32\) and CRY2-CIB1\(^33\). Versatile and useful, these optogenetic methods have some limitations: dimerization can be leaky\(^28\) and reversibility is often slow (TULIP is the fastest at tens of seconds). Improved CIDs have also appeared. SLF\(^{′}–\)TMP is as effective as rapalog but the dimerization it induces can be slowly reversed (~10 min) when outcompeted by monomeric TMP and therefore can be used for repeated rounds of dimerizations\(^30\). MeNV–HaXS is photocleavable by ultraviolet light but binds covalently, making the dimerization it induces quickly reversible but not repeatable\(^34\). We developed zapalog to provide a CID that can bridge some of these

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Fig. 5 | Actin contributes to anchoring of mitochondria at synapses. **a**, Axonal kymographs from live imaging of neurons co-transfected with Kif1a(1–489)–DHFR–Myc, Tom20–mCherry–FKBP and the presynaptic marker VGLUT1–Venus, incubated for 6 h with either dimethylsulfoxide (DMSO) or 2.5 μM latrunculin A, and imaged before (1) and after (2) addition of 1 μM zapalog. **b**, Quantification of the percentage of mitochondria that are motile before addition of zapalog in neurons incubated in DMSO versus neurons incubated with latrunculin A. **c**, Quantification of the percentage of mitochondria that are anchored (that is, remain stationary even after addition of zapalog), subdivided into two groups: those that co-localize with VGLUT1–Venus and those that do not co-localize with VGLUT1–Venus. \(n = 28\) axons each/4 independent repeats; box-and-whisker plots display the statistical median (centre value), upper and lower quartiles (boxes), maximum values (whiskers) and outliers (dots); Kolmogorov–Smirnov non-parametric tests; NS, \(P > 0.05\); \(*P \leq 0.05\); ****\(P \leq 0.0001\); source data are available in Supplementary Table 1.
gaps and facilitate the manipulation of cargo transport in axons. Derived from TMP–SLF, zapalog can dimerize any two proteins tagged with the FKBP and DHFR domains. Unlike TMP–SLF, zapalog undergoes photolysis by blue light, instantaneously reversing the dimerization. Since binding is not covalent, re-dimerization is then possible by means of competition with new, non-photocleaved zapalog molecules, making zapalog a CID that is both repeatable and photocleavable.

Zapalog can be incorporated into many experimental paradigms already set up for rapalog, thereby endowing them with precise spatiotemporal control and repeatability. A protein of interest can be forced to repeatedly interact with its target or, conversely, be sequestered away from target sites and then released instantaneously within the confines of a subcellular ROI. The active domain of a protein of interest can in theory be coupled to its localization domain, recreating the protein’s functional form while rendering it vulnerable to inactivation with light.

We used zapalog to investigate what determines the positioning and movement of axonal mitochondria. Zapalog allowed us to discriminate different classes of stationary mitochondria and to document axonal hotspots for mitochondrial capture and resilient anchoring that are principally at presynaptic specializations.

Zapalog can tether Kif1a motor domains to mitochondria with sufficient affinity to produce highly processive anterograde movement at 2.5 &mu;m s^-1. Truncated, constitutively active motors move faster than their full-length counterparts, and the observed velocity is consistent with the known velocity of the Kif1a motor and, as expected, faster than the endogenous mitochondrial velocity mediated by the slower Kif5c. Most mitochondria could be dragged from their pre-existing locations by tethering them to the constitutively active Kif1a motors. Removing these motors restored their endogenous motility profile.

Throughout this study, we divided the mitochondrial motility assay into three phases: before zapalog addition; in the presence of zapalog; and after zapalog photolysis.

Fig. 6 | Mitochondria tend to be captured at axonal hotspots that are VGLUT1-positive. a, Axonal kymographs from live imaging of neurons co-transfected with Kif1a(1–489)–DHFR–Myc, Tom20–mCherry–FKBP and the presynaptic marker VGLUT1–Venus. Axonal mitochondria were imaged: before zapalog addition (1), during zapalog-induced mitochondrial mobilization (2) and following photolysis as they returned to the imaged region (3). Mitochondrial capture events were defined as any event wherein a motile mitochondrion becomes stationary for at least 5 min. b, Quantification of the number of capture events that coincided with a space that contained a stationary mitochondrion before zapalog addition versus capture events at all other loci along the segment (normalized to micrometres of mitochondria-occupied axon and micrometres of remaining axon). c, Quantification of the number of capture events that coincided with anchored mitochondria versus capture events at all other loci along the segment (normalized to micrometres of anchored mitochondria and micrometres of remaining axon). d, Quantification of the number of capture events that coincided with presynaptic loci (defined as stationary VGLUT1–Venus in phase 1) versus capture events at an equal number of randomly generated loci (see Methods) (n = 11 axons/3 independent repeats; box-and-whisker plots display the statistical median (centre value), upper and lower quartiles (boxes), maximum values (whiskers) and outliers (dots); two-sided paired t-tests; ***P ≤ 0.001, ****P ≤ 0.0001, source data are available in Supplementary Table 1).
of active zapalog: following zapalog photolysis. Ideally one might wish to track an individual mitochondrion through all three phases and determine, for example, whether a retrogradely moving mitochondrion immediately returned to retrograde movement on being freed from the tethered Kif1a. However, our ability to continuously track an individual mitochondrion through all three phases was limited. A mitochondrion that cannot be resolved for even one frame can no longer be identified as the same mitochondrion. To reliably follow any single mitochondrion from one time point to the next, we must balance high spatiotemporal resolution with a field of view large enough to ensure that target mitochondria do not exit the field. The average axon segment imaged was 440 μm. When tethered to Kif1a and moving 2.5 μm s\(^{-1}\), a mitochondrion in the centre of the field would exit the field in 88 s; this limited our ability to track an individual mitochondrion through all three phases, especially for mitochondria that were motile before zapalog addition. Nevertheless, we have successfully recorded many instances of mitochondria that are initially stationary (in phase 1), mobilized by zapalog (in phase 2) and then immediately stationary on zapalog photolysis (in phase 3). We have recorded only a few instances of mitochondria that are initially moving retrograde (in phase 1), dragged anterograde by zapalog (in phase 2) and then immediately revert to retrograde motility on zapalog photolysis (in phase 3; see Supplementary Fig. 3b). Even these few recorded instances carry some significance, as they indicate that when a mitochondrion is moving retrograde, dragging it in the opposite direction does not necessarily reset its endogenous motor–adapter complex, but merely overpowers it, a manifestation of the molecular tug-of-war previously demonstrated in vitro\(^{23}\). Given the technical limitations in following individual mitochondria, we opted instead to characterize mitochondrial populations during each of the three experimental phases and found that, at the population level, the original balance of anterograde and retrograde motility was restored in the wake of the forced anterograde movement.

Our observations required expansion of the standard motile versus stationary categorization of mitochondria into three categories: motile, immotile but movable, and immovable under our experimental conditions. Mitochondrial motility is mediated by motor–adapter complexes that include the motors kinesin-1 and dynein/dynactin and the adapter proteins milton/TRAK1/2 and Miro/RhoT1/2\(^{21}\). Expression of fluorescently tagged kinesin-1 previously indicated that all axonal mitochondria normally possess this motor\(^{25}\), regardless of their direction or stationary state. Motility can be terminated by inhibition of a motor protein\(^{37}\); disassociation of the complex (‘motor shedding’)\(^{38}\); non-processive, anchoring interactions with microtubules via syntaphilin\(^{12}\); and anchoring interactions with myosin and actin microfilaments\(^{25,40}\). By using zapalog to pull mitochondria, we are able to distinguish between mitochondria tethered to Kif1a and moving 2.5 μm s\(^{-1}\), a mitochondrion in the population of immovable mitochondria are not only stationary, but strongly resistant to the pull of active motors. Long-term restraint of synaptic mitochondria may be an evolutionary adaptation that guarantees adequate ATP production and Ca\(^{2+}\) buffering for the high energy demands of synapses and does so more robustly than would be achieved by transient arrest, such as the response to elevated cytosolic Ca\(^{2+}\)\(^{\text{(refs.}^{5,44-47}\text{)}}\).

Cell biological studies often rely on methods that slowly change gene expression or protein activity. Integration of photochemistry into the tool kit of pharmacology and genetics has enabled direct and spatiotemporally precise manipulation of molecules within living cells. By directly and reversibly interfering with axonal transport of mitochondria, zapalog yielded insights into the mechanisms underlying mitochondrial localization. Zapalog, as a repeatable and instantaneously photocleavable dimerizer, should prove to be useful for other inquiries in molecular cell biology.

### Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41556-019-0317-2.

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### References

1. Misgeld, T. & Schwarz, T. L. Mitostasis in neurons: maintaining mitochondria in an extended cellular architecture. *Neuron* **96**, 651–666 (2017).
2. Schwarz, T. L. Mitochondrial trafficking in neurons. *Cold Spring Harbor Perspect. Biol.* **5**, 1–15 (2013).
3. Sheng, Z.-H. & Cai, Q. Mitochondrial transport in neurons: impact on synaptic homeostasis and neurodegeneration. *Nat. Rev. Neurosci.* **13**, 77–93 (2012).
4. Saxton, W. M. & Hollenbeck, P. J. The axonal transport of mitochondria. *J. Cell Sci.* **125**, 2095–2104 (2012).
5. Misgeld, T., Kerschensteiner, M., Bareyre, F. M., Burgess, R. W. & Lichtman, J. W. Imaging axonal transport of mitochondria in vivo. *Nat. Methods* **4**, 559–561 (2007).
6. Voss, S., Klewer, L. & Wu, Y.-W. Chemically induced dimerization: reversible and spatiotemporal control of protein function in cells. *Curr. Opin. Chem. Biol.* **28**, 194–201 (2015).
7. Wilson, M. H. & Holzbaur, E. L. F. Nesprins anchor kinesin-1 motors to the nucleus to drive nuclear distribution in muscle cells. *Development* **142**, 214–228 (2015).
8. Jenkins, B., Decker, H., Bentley, M., Luisi, J. & Banker, G. A novel split kinesin assay identifies motor proteins that interact with distinct vesicle populations. *J. Cell Biol.* **198**, 749–761 (2012).
9. del Castillo, U., Winding, M., Lu, W. & Gelfand, V. I. Interplay between kinesin-1 and cortical dynein during axonal outgrowth and microtubule organization in *Drosophila* neurons. *Elife* **4**, e01609 (2015).
10. Komatsu, T. et al. Organelle-specific, rapid induction of molecular activities and membrane tethering. *Nat. Methods* **7**, 206–208 (2010).
11. Tischer, D. & Weiner, O. D. Illuminating cell signalling with optogenetic tools. Nat. Rev. Mol. Cell Biol. 15, 551–558 (2014).
12. van Bergeijk, P., Adrjan, M., Hoogenraad, C. C. & Kapitein, L. C. Optogenetic control of organelle transport and positioning. Nature 518, 111–114 (2015).
13. Czlapinska, J. L. et al. Conditional glycosylation in eukaryotic cells using a biochemical inducer of dimerization. J. Am. Chem. Soc. 130, 13186–13187 (2008).
14. Lester, H. A. & Neronne, J. M. Physiological and pharmacological manipulations with light flashes. Annu. Rev. Biophys. Bioeng. 11, 15175 (1982).
15. Banghart, M. R., He, X. J. & Sabatini, B. L. A caged enkephalin optimized for simultaneously probing mu and delta opioid receptors. ACS Chem. Neurosci. 9, 684–690 (2018).
16. Trigo, F. F., Corrie, J. E. & Ogden, D. Laser photolysis of caged compounds at 405 nm: photochemical advantages, localisation, phototoxicity and methods for calibration. J. Neurosci. Methods 180, 9–21 (2009).
17. Lemke, E. A., Summerer, D., Geierstanger, B. H., Brittain, S. M. & Schultz, P. G. Mitofusins induced by Parkin. J. Biol. Chem. 283, 893–906 (2008).
18. Chang, D. T. W., Honick, A. S. & Reynolds, I. J. Mitochondrial motility and docking of axonal mitochondria occurs locally in distal neuronal axons and requires PIN1 and Parkin. J. Cell Biol. 206, 655–670 (2014).
19. Lewis, T. L. & Polleux, F. Terminal axon branching is regulated by the KIF1A in living cultured neurons. J. Biol. Chem. 283, 2624–2629 (2008).
20. Liu, P. et al. Evidence that myosin activity opposes microtubule-based axonal transport of mitochondria. J. Cell Biol. 167, 661–672 (2004).
21. Khamo, J. S., Krishnamurthy, V. V., Sharum, S. R., Mondal, P. & Zhang, K. Damaged mitochondria occurs locally in distal neuronal axons and requires PIN1 and Parkin. J. Cell Biol. 206, 655–670 (2014).
22. Ashraf, G., Schlehe, J. S., LaVoie, M. J. & Schwarz, T. L. Mitophagy of damaged mitochondria occurs locally in distal neuronal axons and requires PIN1 and Parkin. J. Cell Biol. 206, 655–670 (2014).
23. Chang, D. T. W., Honick, A. S. & Reynolds, I. J. Mitochondrial mitofusins induced by Parkin. J. Biol. Chem. 283, 893–906 (2008).
24. Pathak, D., Sepp, K. J. & Hollenbeck, P. J. Evidence that myosin activity opposes microtubule-based axonal transport of mitochondria. J. Cell Biol. 167, 661–672 (2004).
25. Wang, X. et al. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell 147, 893–906 (2011).
26. Yi, M., Weaver, D. & Hajnóczky, G. Control of mitochondrial motility and degradation to arrest mitochondrial motility. Cell 147, 893–906 (2011).
27. Trigo, F. F., Corrie, J. E. & Ogden, D. Laser photolysis of caged compounds at 405 nm: photochemical advantages, localisation, phototoxicity and methods for calibration. J. Neurosci. Methods 180, 9–21 (2009).
28. Ankenbruck, N., Courtney, T., Naro, Y. & Deiters, A. Optochemical control of biological processes in cells and animals. Angew. Chem. Int. Ed. 57, 2768–2798 (2018).
29. Khamo, J. S., Krishnamurthy, V. V., Sharum, S. R., Mondal, P. & Zhang, K. Applications of optochemistry in intact cells and multicellular organisms. J. Mol. Biol. 429, 2999–3017 (2017).
30. van Bergeijk, P., Adrjan, M., Hoogenraad, C. C. & Kapitein, L. C. Right time, right place: probing the functions of organelle positioning. Trends Cell Biol. 26, 121–134 (2015).
31. Liu, P. et al. A bioorthogonal small-molecule-switch system for controlling protein function in live cells. Angew. Chem. Int. Ed. 53, 10049–10055 (2014).
32. Levskaya, A., Weiner, O. D., Lim, W. A. & Voigt, C. A. Spatiotemporal control of cell signalling using a light-switchable protein interaction. Nature 461, 997–1001 (2009).
33. Kennedy, M. J. et al. Rapid blue-light-mediated induction of protein function in living cells. Nat. Methods 7, 973–975 (2010).
34. Zimmermann, M. et al. Cell-permeant and photocleavable chemical inducer of dimerization. Angew. Chem. Int. Ed. 53, 4717–4720 (2014).
35. Lee, J.-R. et al. Characterization of the movement of the kinesin motor KIF1A in living cultured neurons. J. Biol. Chem. 278, 2624–2629 (2002).
36. Dere, N. D. et al. Tug-of-war in motor protein ensembles revealed with a programmable DNA origami scaffold. Science 338, 662–665 (2012).
37. Wang, X. & Schwarz, T. L. The mechanism of Ca2+-dependent regulation of kinesin-mediated mitochondrial motility. Cell 136, 163–174 (2009).
38. Wang, X. et al. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell 147, 893–906 (2011).
39. Yi, M., Weaver, D. & Hajnóczky, G. Control of mitochondrial motility and distribution by the calcium signal: a homeostatic circuit. J. Cell Biol. 167, 661–672 (2004).
40. Al Awabdh, S. et al. Neuronal activity mediated regulation of glutamate transporter GLT-1 surface diffusion in rat astrocytes in dissociated and slice cultures. Glia 64, 1252–1264 (2016).
41. Vale, R. D. The molecular motor toolbox for intracellular transport. Cell 112, 467–480 (2003).

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Competing interests

A US patent for zapogol has been filed by Boston Children's Hospital and approved (US10053445B2); T.L.S., M.R.B. and A.G. are listed as inventors.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41556-019-0317-2.

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Correspondence and requests for materials should be addressed to T.L.S.

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Methods

Synthesis of compound. Zapalog was prepared by Mediclon Inc. (Shanghai, China) as detailed in Supplementary Fig. 2 and in Protocol Exchange.

Preparation of B-1. TMP (500 mg, 1.72 mmol) was suspended in 40% HBF4 (7 ml) at room temperature. The mixture was then stirred at 100 °C for 30 min. Liquid chromatography–mass spectrometry (LC–MS) detected the desired product, and the reaction mixture was adjusted to pH 7 with 1 N NaOH. The precipitate was collected by filtration and dried to give 180 mg compound A. The reaction was repeated on a 5 g scale to give A 1.5 g. 1H NMR (400 MHz, d-DMSO-δ6): δ 8.11 (s, 1 H), 7.46 (s, 1H), 6.49 (s, 2H), 6.03 (s, 2H), 5.67 (s, 2H), 3.70 (s, 6H), 3.47 (s, 2H).

Preparation of B-2. To a solution of B-1 (500 mg, 3.0 mmol) in DMF (2 ml), K2CO3 (632 mg, 4.6 mmol) and ethyl 4-bromobutyrate (585 mg, 3.5 mol) were added. The reaction mixture was stirred at room temperature overnight, and then heated for 3 h at 50 °C. The solution was extracted with ethyl acetate, washed with H2O and dried over Na2SO4. The solvent was removed to give 800 mg of B-2 as a white solid in 95% yield. The reaction was repeated at a 10 g scale to give B-2 16.2 g.

Preparation of B-3. A solution of B-2 (500 mg, 1.78 mmol) in 1.5 ml acetic acid was slowly added to a solution of 65% HNO3 (10 ml) and acetic anhydride (2 ml) at 0 °C. The reaction was stirred for 3 h, and poured into ice-cold water. The precipitate was immediately collected by filtration, washed extensively with water, and dried under vacuum to give 350 mg of B-3 as a pale yellow solid in 61% yield. The reaction was repeated at a 16 g scale to give B-3 111 g.

Preparation of B-4. To a solution of B-4 (350 mg, 1.08) in 26 ml methanol at 0 °C, NaH (105 mg, 2.78 mmol) was slowly added in portions. The reaction was stirred for 3 h, and quenched by the addition of 20 ml NH4Cl (aq.). The reaction mixture was extracted with ethyl acetate, washed with H2O and dried over Na2SO4. The solvent was removed to give 280 mg of B-4 as a white solid in 79% yield. The reaction was repeated at an 11 g scale to give B-4 9.6 g.

Preparation of B-5. To a solution of B-5 (100 mg, 0.305 mmol) and KI (51 mg, 0.350 mmol) in 2 ml DMF at 0 °C, NaH (14 mg, 0.336 mmol) was slowly added, the mixture was stirred at room temperature for 10 min, and allylbromide (46 mg, 0.350 mmol) was added. The reaction was stirred for 3 h at 50 °C. TLC showed that the reaction was consumed completely. The mixture was purified by prep-TLC. 1H NMR showed that the structure was correct. The reaction was repeated at a 11 g scale to give B-5 916 mg.

Preparation of B-6. To a solution of B-6 (60 mg, 0.163 mmol) and osmium tetroxide (1 mg, 0.004 mmol) in 1 ml THF and 1 ml water, sodium periodate (140 mg, 0.653 mmol) was added at room temperature under Ar. Then the reaction was stirred at 50 °C for 2 h. TLC showed that the reaction was consumed completely. The mixture was purified by prep-TLC. 1H NMR showed that the product is not pure. The reaction was repeated at a 910 mg scale to give B-6 700 mg.

Preparation of B-7. To a solution of B-7 (100 mg, 0.291 mmol) and CBr4 (116 mg, 0.305 mmol) in 2 ml DMF at 0 °C. NaH (14 mg, 0.336 mmol) was slowly added, the mixture was stirred at room temperature for 10 min, and allylbromide (46 mg, 0.350 mmol) was added. The reaction was stirred at 70 °C overnight, water and ethyl acetate were added, and the organic layer was washed with water and brine, dried by Na2SO4, concentrated and purified by prep-TLC. 1H NMR showed that the structure was correct. The reaction was repeated at a 700 mg scale to give B-7 370 mg.

Preparation of B. A solution of B-7 (100 mg, 0.291 mmol) and CBr4 (116 mg, 0.305 mmol) in DCM, PPh3 (92 mg, 0.350 mmol) was added at 0 °C under Ar. Then the reaction was repeated at a 16 g scale to give B 16.2 g.

Testing sensitivity to 458 nm imaging light. To determine whether zapalog is photolysed by 458 nm light, a wavelength frequently used for imaging, a 20 µl solution of 1 mM zapalog dissolved in PBS was placed on a glass coverslip in an upright confocal microscope and illuminated with ~100 µW 458 nm laser irradiation through a ×10 objective for 1 min, or simply left in the dark, and then analysed by HPLC (5% acetonitrile in water with 0.1% TFA gradient to 100% over 10 min) (Supplementary Fig. 1b).

Molecular cloning. The following DNA constructs were previously published and used in this study: Tom20-mCherry–FKBP41, Pex-mRFP–FKBP41 and VGLUT1–Venus8. Kif1a(1–489)–DHFR–Myc (Addgene no. 117833) was generated by PCR and restriction cloning. The motor and neck-linker domains of rat Kif1a were amplified from a plasmid containing full-length Kif1a (provided by K. Verhey, U. Michigan) using the following primers: Kif1a(489)–XhoI (30 nucleotides): GTCAAGGCTGGACAGCTTCCTCTCCATGGC; BamHI–Kif1a (27 nucleotides): TACAGTGATCAGTGTCGGCGCTGCCTC. Afterwards, both the PCR product and pDHFR–Myc (Addgene no. 20214) were digested with BamHI and XhoI and the Kif1a motor fragment was inserted 5′ to the DHFR domain of YFP–DHFR–Myc (Addgene no. 118852) was generated by restriction cloning. Both pDHFR–Myc (Addgene no. 20214) and YFP–FRB (Addgene no. 20148) were cut with XbaI and NdeI and the YFP-encoding fragment was inserted upstream of DHFR.

Culturing COS7 and HeLa cells. COS7 and HeLa cells were cultured in DMEM, high glucose, GlutaMAX (Thermo Fisher Scientific) supplemented with 1% penicillin/streptomycin (10,000U ml−1; ThermoFisher Scientific) and 10% FBS (Atlanta Biological). DNA transfections in COS7 and HeLa cells were performed with GenJet (SignaGen Laboratories).

Culturing rat hippocampal neurons. Rat hippocampal neurons were isolated according to standard procedures and cultured either as spot-groove cultures or standard neuronal cultures as in ref. 17. Rat embryos were transferred at DIV6–DIV8 using Lipofectamine2000 (Life Technologies) and imaged 1 day later.

Spot-groove culture. To grow straight axons for live imaging of distal axon endings, an optical-quality 24-well plate (Ibidi) was coated with 20 µg ml−1 poly-l-lysine (Sigma-Aldrich) and 3.5 µg ml−1 laminin (Thermo Fisher Scientific). After coating, grooves were etched into each well using a pin rake (Tyler Research). Hippocampal neurons were obtained from E18 rat (Charles River) embryos and spotted in the centre of the grooves at 15×103 cells per well in 7 μl of medium. After allowing the cells to attach for 1 h in the incubator medium (Neurobasal, 2% B27, 100 µM l−1 penicillin–streptomycin, 1 mM l-glutamine, 100 nM NGF, 2 ng ml−1 GDNF) it was replaced with new medium. Neurons were transfected at DIV5–DIV8 using Lipofectamine2000 (Life Technologies) and imaged 1 day later.
Live-cell imaging and quantification. All live imaging experiments involving zapalogn were conducted in a dark room that was illuminated only with red-filtered lights. Zapalog was kept as a 10 mM stock in DMSO and diluted with medium before each experiment as needed.

Whole-cell fluorophore translocation analysis. COS7 cells were transfected with Tom20–mCherry–FKBP and YFP–DHFR–Myc, and imaged 24 h later. To determine the time course of zapalog-induced dimerization using different concentrations of zapalog, time-lapse datasets were acquired on an inverted Andor Revolution spinning-disc confocal microscope, equipped with a Piezo Z500CE stage (Prior Scientific) in an incubator maintained at 37 °C/5% CO₂. Images were captured using a ×40/NA 1.3 oil UPLPLN objective with excitation at 488 nm and 561 nm separately, each for 200 ms exposures once per second for 300 s. The laser power was set to <100 µW for each channel to minimize damage. Full-field photobleaching of zapalog was achieved by activation of a 405 nm channel (300 µW). To calculate fluorophore translocation accurately and without bias at any point in time, a MATLAB code was written to automatically compute the ratio of fluorescence in mitochondria versus cytoplasm: as illustrated in Supplementary Fig. 5, YFP and mCherry fluorescence were thresholded to generate binary masks of total cell area and of mitochondrial area for every time point. A series of masks representing cytoplasmic area in each time point was calculated by subtracting the mitochondrial area from total cell area. Finally, mean YFP intensity was measured within the areas of the mitochondrial and cytoplasmic masks, and then divided to achieve the final metric of mitochondrial/cytoplasmic YFP ratio. This entire process, including mask creation, was performed separately for each time point, as mitochondrial locations change rapidly in the cell. The code is available for download at https://github.com/ewest11/FTA-Fluorophore-Translocation-Analysis.

Targeted whole-cell fluorophore translocation assay. To determine the time course and efficiency of reversal of zapalog-induced dimerization through photolysis, cells were transfected as above and imaged 24 h later in a Leica SP8 scanning laser confocal with an HC PL APO ×63/1.40 oil CS2 objective, set to multichannel imaging of YFP and mCherry with pulsed white light laser emission at 514 nm and 584 nm, respectively, for a total of 1 frame s⁻¹. Zapalog (1 µM) was added in dark conditions to translocate the YFP from the cytoplasm to the outer membrane of mitochondria, and then washed out after 10 min by replacing the medium with new, pre-warmed medium. Using the SP8 fluorescence recovery after photobleaching (FRAP) protocol, an ROI of 25 µm × 25 µm was defined around each cell in the field and a selected cell was then illuminated separately with a 405 nm diode laser (0.5 s at 30 nW), followed by 20 s of imaging before repeating the process on the next selected cell.

Repeated, localized fluorophore translocation assay on non-networked mitochondria. At 18 h after HeLa cells were transfected with Tom20–mCherry–FKBP and YFP–DHFR–Myc, they were incubated for 6 h in 10 µM CCCP. To measure repeated fluorophore translocation events in a localized subcellular area, cells were then imaged in a Leica SP8 scanning laser confocal with an HCX PL APO ×100/1.4 oil CORR CS objective, set to multichannel imaging of YFP and mCherry with pulsed white light laser emission at 514 nm and 584 nm, respectively, for a total of 1 frame s⁻¹. Zapalog (1 µM) was added in dark conditions to translocate the YFP from the cytoplasm to the outer membrane of mitochondria, and then washed out after 10 min by replacing the medium with new, pre-warmed medium. Using the SP8 fluorescence recovery after photobleaching (FRAP) protocol, an ROI of 25 µm × 25 µm was defined around each cell in the field and a selected cell was then illuminated separately with a 405 nm diode laser (0.5 s at 30 nW), followed by 20 s of imaging before repeating the process on the next selected cell.

Repeate, localized fluorophore translocation assay on peroxisomes. COS7 cells were transfected with a peroxisomal outer membrane marker and zapalog receptor (PEX–mRFP–FKBP) and with cytosolic YFP–DHFR–Myc. After 24 h, the cells were imaged in a Leica SP8 scanning laser confocal with an HC PL APO ×63/1.40 OIL CS2 objective, set to multichannel imaging of YFP and mRFP with pulsed white light laser emission at 514 nm and 584 nm, respectively, for a total of 1 frame s⁻¹. Zapalog (1 µM) was added in dark conditions to translocate the YFP from the cytoplasm to the outer membrane of peroxisomes (~5 min). Then, using the SP8 FRAP protocol, an ROI of 20 × 20 pixels was defined for repeated rounds of 0.5 s illumination with the 405 nm diode laser (30 nW), followed by 74 s of imaging. Mean YFP intensities in illuminated versus nearby non-illuminated 20 × 20 pixel areas were normalized to range from 0 to 100, and the average YFP intensity was calculated for each time point.

Mitochondrial motility analysis. Time-lapse videos were acquired on an inverted Andor Revolution spinning-disc confocal microscope, equipped with a Piezo Z500CE stage (Prior Scientific) in an incubator maintained at 37 °C/5% CO₂. Images were captured using a ×40/NA 1.3 oil UPLPLN objective with excitation at 488 nm and 561 nm separately, each for 200 ms exposures once per second for 300 s (except where stated otherwise). The laser power was set to <30% for each channel to minimize damage. Full-field photobleaching of zapalog was achieved by activation of a 405 nm channel (300 µW). We imaged ~100 µm stretches of axon at least 500 µm away from the soma; this was done in the middle of the axon such that there were still large populations of mitochondria both upstream and downstream of the field, to prevent a significant skewing of the distribution. Mitochondrial motility was quantified as ‘percentage of time in motion’ using Kymolyzer, a custom ImageJ macro developed in our laboratory.

Quantifying presence of stationary mitochondria at presynaptic sites. As illustrated in Supplementary Fig. 6a, we analysed dual-channel kymographs (60 frames in each channel at 5 s intervals for a total imaging time of 5 min) of axons from neurons expressing both Tom20–mCherry–FKBP (mitochondrial marker) and VGLUT1–Venus (presynaptic marker). We marked all of the stationary mitochondrial and all of the stationary VGLUT1 particles and quantified for each axon the number of stationary mitochondria that coincided with a presynaptic location.

Generation of random locations along the axon. To control for possible mislocalization of mitochondria with the presynaptic marker, random locations were generated for each axon at a comparable density to actual presynaptic sites. The number of random locations generated for each axon equalled the number of presynaptic sites identified for that particular axon, and the algorithm for marking the random sites is illustrated in Supplementary Fig. 6b. The ImageJ macro ‘Kymolyzer’ is available upon request.

Statistics and reproducibility. Fluorophore translocation data (Figs. 1 and 2) are expressed as line-connected points (mean) with error bars (s.e.m for the assay time course, s.d for dose response) (Fig. 3, 5 and 6) or scatter plots of pairwise before-and-after data points (Fig. 4); the median is marked in each. Statistical analyses were performed with Apple Numbers and GraphPad Prism v7.0 for MacOS X. Paired t-tests were used to compare populations made up of pairs of data points representing repeated measurements of the same sample (Fig. 4). Welch’s t-tests were used to compare populations of unequal variances (Figs. 5 and 6). Pearson’s chi-squared tests of data shown in Fig. 5 were performed with JMP v14 from MacOX X. For all analyses P < 0.05 was considered significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Source data for Figs. 1–6 and Supplementary Fig. 3 are available in Supplementary Table 1. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

Code availability
The MATLAB code used to analyse the data shown in Fig. 1c(iii) is available for download at https://github.com/ewest11/FTA-Fluorophore-Translocation-Analysis. The ImageJ macro ‘Kymolyzer’ is available upon request.

References
47. Miyamoto, T. et al. Rapid and orthogonal logic gating with a gibberellin-induced dimerization system. *Nat. Chem. Biol.* 8, 465–470 (2012).
48. Kapitein, L. C. et al. Mixed microtubules steer dynemine-driven cargo transport into dendrites. *Curr. Biol.* 20, 290–299 (2010).
49. Herrzig, E. et al. In vivo imaging of intrasympatic vesicle exchange using VGLUT1 Venus knock-in mice. *J. Neurosci.* 31, 15544–15559 (2011).
50. Gornstein, E. L. & Schwarz, T. L. Neurotoxic mechanisms of paclitaxel are local to the distal axon and independent of transport defects. *Exp. Neurol.* 288, 153–166 (2017).
51. Pekkurnaz, G., Trinidad, J. C., Wang, X., Kong, D. & Schwarz, T. L. Glucose regulates mitochondrial motility via Milton modification by O-GlcNAc transferase. *Cell* 158, 54–68 (2014).

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Software and code

Policy information about availability of computer code

Data collection

- Imaging on Andor Spinning Disk Confocal utilized Metamorph (Molecular Devices, Inc.)
- Imaging on Leica SP8 Laser Scanning Confocal utilized LAS X (Leica Microsystems, Inc.)

Data analysis

- ImageJ, Kymolizer (see Pekkurnaz et al 2014), and a custom code that we have posted online at:
  - https://github.com/ewest11/FTA-Fluorophore-Translocation-Analysis

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All studies must disclose on these points even when the disclosure is negative.

### Sample size
For studying mitochondrial motility in axons, we implemented the standard sample sizes in the field, commonly used in the field to balance statistical power with time and resource considerations. Methodology for quantification of mitochondrial motility is discussed in Misgeld and Schwarz (2017).

### Data exclusions
As discussed in the manuscript, axons that exhibited mitochondrial pile-ups were excluded from the study due to the appearance of poor health and observed disintegration of blocked axons. This exclusion criterion was pre-established.

### Replication
All experiments include data from at least three replicates; all replications were successful.

### Randomization
As controls for experiments measuring capture of mitochondria we generated sets of random positions along the axon equal in number to the number of capture events recorded for each dataset.

### Blinding
No blinding was used as none of the analyses used in this study rely on subjective scoring.

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#### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☐   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data |

#### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

#### Antibodies

- **Antibodies used**
  - anti-myc antibody (9E10, Santa Cruz), anti-TUJ1 (Poly18020, BioLegend), anti-VGLUT1 (AB5905, Sigma-Aldrich)

- **Validation**
  - anti-myc: "routinely evaluated by immunoblot on a Myc-tagged recombinant protein"
  - anti-TUJ1: "This antibody is well characterized and highly reactive to neuron specific Class III β-Tubulin (βIII)"
  - anti-VGLUT1: "The antiserum has been tested on tissue sections from the rat central nervous system (CNS) using immunofluorescence histochemistry."

#### Eukaryotic cell lines

**Policy information about cell lines**

- **Cell line source(s)**
  - ATCC (COS7, HeLa)

- **Authentication**
  - Short tandem repeat authentication done by ATCC

- **Mycoplasma contamination**
  - Tested negative for mycoplasma contamination

- **Commonly misidentified lines**
  - No commonly misidentified lines were used in this study.

#### Animals and other organisms

**Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research**

- **Laboratory animals**
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|-------------------------|------------------------------------------------------------|
| Ethics oversight         | Rat procedures were approved by the Institutional Animal Care Committee at the Boston Children’s Hospital. |

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