Miro1-dependent mitochondrial positioning drives the rescaling of presynaptic \( \text{Ca}^{2+} \) signals during homeostatic plasticity

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Abstract

Mitochondrial trafficking is influenced by neuronal activity, but it remains unclear how mitochondrial positioning influences neuronal transmission and plasticity. Here, we use live cell imaging with the genetically encoded presynaptically targeted \( \text{Ca}^{2+} \) indicator, SyGCaMP5, to address whether presynaptic \( \text{Ca}^{2+} \) responses are altered by mitochondria in synaptic terminals. We find that presynaptic \( \text{Ca}^{2+} \) signals, as well as neurotransmitter release, are significantly decreased in terminals containing mitochondria. Moreover, the localisation of mitochondria at presynaptic sites can be altered during long-term activity changes, dependent on the \( \text{Ca}^{2+} \)-sensing function of the mitochondrial trafficking protein, Miro1. In addition, we find that Miro1-mediated activity-dependent synaptic repositioning of mitochondria allows neurons to homeostatically alter the strength of presynaptic \( \text{Ca}^{2+} \) signals in response to prolonged changes in neuronal activity. Our results support a model in which mitochondria are recruited to presynaptic terminals during periods of raised neuronal activity and are involved in rescaling synaptic signals during homeostatic plasticity.

Keywords homoeostatic; Miro1; mitochondria; plasticity; synapse

Introduction

Mitochondria play an important role in maintaining neuronal function due to their ability to produce the energy substrate ATP and to buffer local \( \text{Ca}^{2+} \) rises [1–3]. Presynaptic \( \text{Ca}^{2+} \) signals trigger vesicular release and their amplitude is known to influence synaptic transmission [4,5]. Previous pharmacological studies suggest that mitochondria can efficiently buffer \( \text{Ca}^{2+} \) in presynaptic terminals [6,7]. However, only a subset of presynaptic terminals within the same axon may contain mitochondria [8], and the impact of mitochondrial occupancy on presynaptic \( \text{Ca}^{2+} \) signalling and vesicular release in individual terminals within the same axon remains poorly understood.

Homeostatic plasticity plays a central role in stabilising network activity by rescaling synaptic weights and neuronal excitability in accordance with the activity level of neurons [9]. The homeostatic rescaling of the efficiency of synapses in order to avoid extreme levels of activity is dependent on changes in both presynaptic and postsynaptic function [9–11]. Interestingly, recent developments in imaging \( \text{Ca}^{2+} \) signals using genetically encoded presynaptically targeted \( \text{Ca}^{2+} \) indicators have shown that presynaptic \( \text{Ca}^{2+} \) responses undergo homeostatic plasticity [12]. It is unclear however how the reported rescaling of presynaptic \( \text{Ca}^{2+} \) signals is mediated and whether mitochondrial \( \text{Ca}^{2+} \) buffering can play a role in homeostatic changes of neuronal transmission efficiency.

Mitochondrial positioning can be regulated by neuronal activity, dependent on the mitochondrial trafficking protein Miro1 [13–15]. Miro1 is located in the outer mitochondrial membrane and contains two \( \text{Ca}^{2+} \)-sensing EF-hand domains by which it responds to local \( \text{Ca}^{2+} \) signalling to interrupt mitochondrial trafficking, thus depositing mitochondria at subcellular locations of high activity [13,14,16]. However, whether mitochondrial positioning at synapses is altered during long-term changes in neuronal activity and whether a role exists for Miro1-mediated mitochondrial trafficking in the tuning of synaptic mitochondrial occupancy remains unclear.

Here, by imaging presynaptic \( \text{Ca}^{2+} \) [17] and mitochondrial positioning in multiple terminals of the same axon, we show that mitochondrial occupancy determines presynaptic \( \text{Ca}^{2+} \) responses and can also affect vesicular release. Moreover, we demonstrate that mitochondrial localisation at presynaptic terminals is tuned by long-term changes in network activity dependent on Miro1-mediated mitochondrial trafficking. Further, we show that baseline \( \text{Ca}^{2+} \) responses and homeostatic changes in the presynaptic response are altered in the absence of Miro1-mediated \( \text{Ca}^{2+} \)-dependent positioning of mitochondria. This provides evidence for a novel mechanism by which mitochondria can alter presynaptic transmission and play a role in the tuning of synaptic signals during homeostatic plasticity.
Results and Discussion

In order to investigate a potential difference in the Ca\(^{2+}\) signals evoked in presynaptic terminals containing mitochondria compared to terminals without mitochondria, we co-transfected hippocampal cultures with the mitochondrial marker MitoDsRed and the presynaptically targeted version of the genetically encoded Ca\(^{2+}\) indicator GCaMP5 (based on SyGCaMP2 and GCaMP5 [17,18]). Labelling of SyGCaMP5-transfected neurons with the presynaptic markers SV2 and Piccolo confirmed that the indicator is presynaptically targeted (Fig EV1) and, using this approach, terminals with mitochondria could be easily distinguished from those without by merging both acquisition channels (Fig 1A).

While stimulating neurons electrically, with extracellular field electrodes at 10 Hz for 10 s (thus generating 100 action potentials (APs); see Materials and Methods), we compared the average presynaptic Ca\(^{2+}\) signal and found that, in terminals without mitochondria, the average Ca\(^{2+}\) signal during the time of stimulation (t = 20–30 s) was significantly greater (ΔF/ΔF₀ = 3.5 ± 0.4) than in terminals containing mitochondria (ΔF/ΔF₀ = 1.9 ± 0.3, n = 11 neurons, 91 terminals, P < 0.001; Fig 1B and C). Importantly, the mean stimulation ΔF/ΔF₀ is not determined by the unnormalised baseline fluorescence within each terminal (r = 0.05, P > 0.2 for n = 90 terminals; Fig EV2A). Further, there is no significant difference between the baseline fluorescence signals in those terminals occupied with a mitochondrion compared to those without (P = 0.1; Fig EV2A). In contrast, upon a very brief stimulation of 1 ms, which should only lead to initiation of a single AP (Fig 1D and E), we did not observe a significant difference in the presynaptic Ca\(^{2+}\) responses when those terminals occupied by a mitochondrion were compared to terminals in the same axon not occupied by a mitochondrion (Fig 1D and E).

Therefore, we sought to determine the threshold number of stimuli that was required to elicit a significant difference in presynaptic Ca\(^{2+}\) response in the presence of a mitochondrion. We found that a train of 10 stimuli (10 APs) was insufficient to generate a difference in Ca\(^{2+}\) response (ΔF/ΔF₀ = 0.4 ± 0.3 with and 0.4 ± 0.1 without mitochondria, n = 3 neurons, 21 terminals, P = 0.90; Fig 1F). This was verified in a separate data set obtained at maximum acquisition frame rate of ~18 frames per second (ΔF/ΔF₀ = 1.0 ± 0.2 with and 1.2 ± 0.3 without mitochondria, n = 13 neurons, 44 terminals, P = 0.68; Fig EV2B), to exclude the possibility that transient differences were missed when data were acquired at 1 frame per second. In contrast, trains of stimuli ≥ 20 were sufficient to generate a difference in presynaptic Ca\(^{2+}\) response (20 stimuli: ΔF/ΔF₀ = 0.8 ± 0.4 with and 1.8 ± 0.4 without mitochondria, n = 10 neurons, 55 terminals, *P < 0.05; 40 stimuli: ΔF/ΔF₀ = 0.9 ± 0.3 with and 2.0 ± 0.4 without mitochondria, n = 5 neurons, 25 terminals, *P < 0.05; 80 stimuli: ΔF/ΔF₀ = 1.3 ± 0.2 with and 2.2 ± 0.5 without mitochondria, n = 7 neurons, 21 terminals, *P < 0.05; Fig 1F). Next, we varied the frequency at which these stimuli were delivered, to see whether the rate of rise in presynaptic Ca\(^{2+}\) would have an impact on the ability of mitochondria to buffer this signal. The differences observed at 20 stimuli were maintained across a range of frequencies (5 Hz: ΔF/ΔF₀ = 0.9 ± 0.2 with and 1.3 ± 0.4 without mitochondria, n = 11 neurons, 58 terminals, *P < 0.05; 100 Hz: ΔF/ΔF₀ = 1.2 ± 0.3 with and 1.7 ± 0.4 without mitochondria, n = 12 neurons, 87 terminals, *P < 0.05; Fig EV2B). In contrast, no differences were observed with 10 stimuli delivered at 100 Hz (ΔF/ΔF₀ = 0.8 ± 0.2 with and 0.8 ± 0.2 without mitochondria, n = 4 neurons, 18 terminals, P = 0.60; Fig EV2B), despite the more rapid delivery of APs and thus more rapid rise in presynaptic Ca\(^{2+}\).

These findings suggest that even though single APs can generate small mitochondrial Ca\(^{2+}\) transients [19], mitochondria are more relevant as Ca\(^{2+}\) buffers after prolonged stimulation. This agrees with previous studies suggesting that mitochondria play a role after Ca\(^{2+}\) has accumulated in the presynaptic terminal [20,21] and is also supported by earlier less direct studies which demonstrate that either pharmacological inhibition of mitochondrial Ca\(^{2+}\) buffering or genetic intervention to direct mitochondria out of terminals increases presynaptic Ca\(^{2+}\) responses [6,22–25]. The threshold of 20 stimuli could correspond to depletion of the entire readily releasable pool of neurotransmitter vesicles, because this is thought to be fully released within 2 s of 20 Hz stimulation [26].

We then asked whether the observed difference in Ca\(^{2+}\) response is due to mitochondrial Ca\(^{2+}\) uptake mediated by the mitochondrial Ca\(^{2+}\) uniporter (MCU) and thus used 30 min Ru360 treatment to block its activity. In the control cultures, presynaptic terminals containing a mitochondrion had a decreased mean Ca\(^{2+}\) signal.
Figure 1.
Mitochondria tune presynaptic responses

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(ΔF/F₀ = 3.5 ± 0.4) compared to terminals without a mitochondrion (ΔF/F₀ = 4.9 ± 0.5; Fig 2A and C; n = 15 neurons, 96 terminals, ***P < 0.001). When MCU activity was blocked, the presence of mitochondria no longer affected the mean stimulation Ca²⁺ signal ΔF/F₀ (Fig 2B and D; 4.1 ± 0.5 for mitochondrially occupied terminals and 3.8 ± 0.9 for terminals without a mitochondrion, n = 13 neurons, 79 terminals, P = 0.53). Thus, mitochondria play a key role in the regulation of the size of presynaptic Ca²⁺ signals during trains of action potentials, contingent on their ability to buffer Ca²⁺ in the terminal via MCU.

Having established that mitochondrial occupancy and Ca²⁺ buffering can significantly affect presynaptic Ca²⁺ signals at individual synapses, we determined whether the presence of mitochondria impacts on transmitter release. To image vesicular release, we used the superecliptic pHluorin-based probe, VGlut1pHluorin, while imaging mitochondrial positioning with a mitochondrially targeted variant of LSS-mKate2 (Fig 3A) [27,28]. When stimulating the neurons for 20 s at 10 Hz, we observed a significant increase in the VGlut1pHluorin average signal in terminals without a mitochondrion compared to those terminals occupied by a mitochondrion within the same axon (ΔF/F₀ = 3.2 ± 0.7, terminals without mitochondria; ΔF/F₀ = 2.3 ± 0.9, terminals with mitochondria; n = 9 neurons, 66 terminals, *P < 0.05; Fig 3B and C). These findings suggest that the presence of mitochondria decreases local Ca²⁺ signals via MCU, leading to less vesicular fusion. While presynaptic Ca²⁺ signalling and vesicular release have previously been shown to be unaffected by MCU knockdown [29], in this study no separation was made between terminals containing or not a mitochondrion, which may have led to an underestimation of the mitochondrial impact on presynaptic Ca²⁺ due to the simultaneous sampling of all synapses.

Next, we sought to establish whether mitochondrial occupancy could determine activity-dependent changes in presynaptic Ca²⁺ signalling, via modulating the size of the presynaptic Ca²⁺ response. Mitochondria have been observed to stop in axons and at postsynaptic terminals in response to local activity [13,14], but whether prolonged changes in neuronal activity lead to a redistribution of mitochondria to and from synapses remains unclear. In order to investigate whether long-term changes in neuronal activity (48 h) alter the recruitment of mitochondria to presynaptic terminals, we co-transfected neurons with synaptophysin–GFP (SYN-GFP) to label presynaptic terminals and MitoRed to label mitochondria (Fig 4A)
and quantified the co-localisation of these two reporters. The result-
ing fraction of terminals with mitochondria (34.3% ± 3.6) is similar
to previous findings [8]. To decrease network activity, we treated
neurons with the sodium channel blocker tetrodotoxin (TTX)
(16.6% ± 3.1, 1 μM, 48 h), whereas an increase in activity was achieved by
applying the GABA_A receptor antagonist picrotoxin (PTX) (100
μM, 48 h) [30]. Silencing neurons with TTX led to a significant decrease
in the fraction of SYNGFP clusters containing a mitochondrion
(16.6% ± 3.1, P < 0.01), whereas conversely increasing neuronal
activity, driven by PTX treatment, increased the fraction of SYN-
GFP synapses containing mitochondria compared to control (DMSO
vehicle control 28.7% ± 3.9, PTX 50.0% ± 7.2, **P < 0.01; Fig 4A
and D) indicating that long-term activity serves to alter mitochon-
drial occupancy at the synapse. Importantly, the density of mitochon-
dria (mitochondria/μm: control 0.103 ± 0.01, TTX 0.108 ± 0.01,
DMSO 0.126 ± 0.02, PTX 0.127 ± 0.02; P > 0.05) and SYN-GFP clusters
(synapse/μm: control 0.218 ± 0.01, TTX 0.205 ± 0.01, DMSO 0.231 ±
0.01, PTX 0.218 ± 0.02; P > 0.05) throughout the axon was unaltered following both TTX and PTX
treatments (Fig EV3A and B). Therefore, variations in mitochondrial
occupancy of presynaptic terminals are due to changes in the
location of mitochondria, rather than an overall change in the
number of mitochondria or synapses in the axon, and the redistribu-
tion of mitochondria is from a local and previously available
pool. A recent paper illustrated that the frequency of short mito-
chondrial pauses at synapses is increased when neuronal cultures
are stimulated using field stimulation and that a block of neuronal
activity using TTX leads to an increase in mitochondrial velocity
in the axon [31], which may explain how we come to observe our
changes in occupancy after long-term treatment with PTX and
TTX, respectively.

To confirm that the mitochondrial impact on vesicular release
persists after PTX-induced mitochondrial redistribution, we imaged
vesicular release with VGlut1pHluorin with respect to mitochondrial
position (with LSS-mKate) following 48 h of PTX treatment. When
stimulating neurons for 20 s at 10 Hz, we again detected a significant
increase in VGlut1pHluorin signal in terminals without a mito-
chondrion; ΔF/F_0 = 2.4 ± 0.4, terminals without mitochondria; ΔF/F_0 = 3.1 ± 0.3, terminals within the same axon (|N| 2017
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Figure 3. Vesicular release is reduced in the presence of mitochondria.
A Example images of hippocampal neurons co-transfected with Mito-mKate2 and VGlut1pHluorin before and during field stimulation. The white arrow indicates a
terminal occupied with a mitochondrion, while the empty arrow indicates a terminal without a mitochondrion. Scale bar, 5 μm.
B Average traces of presynaptic terminals of hippocampal neurons transfected with VGlut1pHluorin and Mito-mKate2 and stimulated using field stimulation at 10 Hz
for 20 s (40–60 s). Terminals with a mitochondrion are represented by the red trace, and terminals without are represented by the black trace.
C Summary graph corresponds to the traces shown in panel (B). Mean stimulation fluorescence (time points 60–70 s) comparing terminals of the same axon which
contain mitochondria (right, ΔF/F_0 = 2.3 ± 0.9) compared to terminals without mitochondria (left, ΔF/F_0 = 3.2 ± 0.7; n = 9 neurons, 66 terminals, *P < 0.05, paired
t-test).

Data information: Experiments were performed in PO rat hippocampal neuronal cultures at DIV 10–12. Error bars represent SEM.
uncouple constitutive from activity-dependent mitochondrial trafficking (Fig EV4A–C). Overexpression of both Miro1 and ΔEF-Miro1 had no effect on the formation or localisation of SYN-GFP clusters (Fig EV4D and E). However, upon expression of wild-type Miro1, the fraction of SYN-GFP puncta overlapping MtDsRed was no longer decreased after TTX treatment, but PTX treatment still led to an increase in co-localisation (Fig 4B and E). Expression of ΔEF-Miro1 on the other hand suppressed any activity-dependent changes in mitochondrial occupancy upon PTX and TTX treatment (Fig 4C and F). Inactivity induced by glutamate receptor blockade (APV: 100 μM, NBQX: 10 μM, 24 h), known to initiate presynaptic homeostatic plasticity due to a compensatory increase in presynaptic release probability [32], also resulted in a change in mitochondrial presynaptic occupancy (control 0.302 ± 0.02, APV + NBQX 0.214 ± 0.02, t-test *P < 0.05; Fig EV4F). These activity-dependent changes were again inhibited following the expression of ΔEF-Miro1 (control 0.256 ± 0.03, APV + NBQX 0.259 ± 0.05; Fig EV4H).

Several studies have shown that Miro1 and its two EF-hand domains contribute to Ca\(^{2+}\)-dependent mitochondrial stopping and recruitment to areas of high Ca\(^{2+}\) load [13,14]. We hypothesise that increased expression of Miro1 makes mitochondria more likely to arrest close to presynaptic terminals in response to local Ca\(^{2+}\) as Miro1 overexpression increases mitochondrial trafficking [13,14,33] and mitochondria pass terminals more frequently. Thus, even rare Ca\(^{2+}\) responses during TTX treatment might be sufficient to allow mitochondrial arrest when Miro1 is overexpressed, and therefore, a higher percentage of terminals are always occupied by a mitochondrion. PTX on the other hand has the same effect whether Miro1 is
overexpressed or not, because during periods of high activity mitochondria are eventually repositioned to presynaptic terminals in response to the frequent Ca\(^{2+}\) signals. We therefore propose that mitochondria are arrested at terminals in a Miro1-mediated activity-dependent manner, followed by tethering of the mitochondria by a protein such as syntaphilin [25].

Long-term changes in neuronal activity in hippocampal cultures have been shown to generate homeostatic changes in presynaptic Ca\(^{2+}\) responses [12]. As mitochondria can reduce presynaptic Ca\(^{2+}\) signals and transmitter release (Fig 1) and long-term activity changes can drive mitochondrial movement into and out of terminals, we hypothesised that Miro1-mediated mitochondrial repositioning may contribute to homeostatic plasticity of the presynaptic Ca\(^{2+}\) response. As previously described, neurons were transfected with MtdsRed and SyGCaMP5 and the presynaptic Ca\(^{2+}\) response within a region of the axon was measured. TTX treatment (1 μM, 48 h) resulted in the expected increase in presynaptic Ca\(^{2+}\) signals, likely due to the fact that less terminals contain a mitochondrion (1.3 ± 0.6 for non-treated cultures, 2.5 ± 0.5 for TTX-treated cultures; n = 11 and n = 10 neurons, 69 and 62 terminals, respectively, *P < 0.05; Fig 5B). To address the impact of blocking activity-dependent mitochondrial recruitment to presynaptic terminals, we expressed the Ca\(^{2+}\)-insensitive mutant of Miro1 (ΔEF-Miro1) and determined whether this interferes with the presynaptic rescaling of Ca\(^{2+}\) signals. To facilitate live cell imaging in ΔEF-Miro1-transfected neurons, we co-transfected SyGCaMP5 with ΔEF-Miro1-IRES-MtdsRed, which allows bicistronic expression of both transgenes thus permitting us to readily identify ΔEF-Miro1 expression in MtdsRed-positive neurons co-expressing SyGCaMP5 (Fig EV5). We found that the homeostatic increase in Ca\(^{2+}\) response after TTX treatment was occluded when ΔEF-Miro1 is expressed (ΔF/F₀ = 2.3 ± 0.6 for non-treated cultures and ΔF/F₀ = 1.8 ± 0.3 for TTX-treated cultures; n = 7 cells, 33 and 32 terminals, respectively, P = 0.05; Fig 5B and C) suggesting a role for activity-dependent tuning of mitochondrial presynaptic terminal occupancy in the homeostatic scaling of presynaptic Ca\(^{2+}\) signals. This suggests that some of the homeostatic rescaling we observe under normal conditions is dependent on Miro1 function and in particular on Miro1’s Ca\(^{2+}\)-sensing ability mediated via its two EF-hand domains.

Our findings suggest that mitochondrial occupancy of a presynaptic terminal can tune local Ca\(^{2+}\) signals via MCU, to regulate vesicular fusion. The probability of release (P_{rel}) is known to vary even in terminals from the same axon [34,35]. This can arise from differences in the size of the readily releasable pool of these terminals [34,36] but may also arise from variability in Ca\(^{2+}\) dynamics and fusion probability of vesicles [37]. Mitochondrial ATP provision was also recently proposed to contribute to the variability of presynaptic strength [33], particularly during long stimulation trains. However, other studies using presynaptically targeted ATP probes showed that even long stimulation periods of 60 s do not necessarily lead to a depletion of presynaptic ATP, because activity-driven ATP generation (through glycolysis and oxidative phosphorylation) [38] and ATP diffusion [39] can serve to maintain presynaptic ATP levels. Thus, the importance of local mitochondrial ATP provision

![Figure 5](image-url)

**Figure 5.** Miro1 is involved in homeostatic changes in presynaptic Ca\(^{2+}\) signals.

A Live images of neurons co-transfected with SyGCaMP5 and MtdsRed or myc-ΔEF-Miro1-IRES-MtdsRed (ΔEF Miro) before and during 10-Hz field stimulation with and without TTX treatments. Scale bar, 10 μm.

B Average ΔF/F₀ SyGCaMP5 traces from terminals treated with TTX (red trace, ΔF/F₀ = 2.5 ± 0.5) in n = 10 neurons (62 terminals) and non-treated terminals (black trace, ΔF/F₀ = 1.3 ± 0.6) in n = 11 neurons (69 terminals) co-transfected with MtdsRed; *P < 0.05, t-test.

C Average ΔF/F₀ SyGCaMP5 traces from terminals treated with TTX (red trace, ΔF/F₀ = 1.8 ± 0.3) in n = 7 neurons (33 terminals) and non-treated terminals (black trace, ΔF/F₀ = 2.3 ± 0.6) in n = 7 neurons (32 terminals) co-transfected with myc-ΔEF-Miro1-IRES-MtdsRed, P = 0.5, t-test.

Data information: Experiments were performed in E16 mouse hippocampal neuronal cultures at DIV 10–12. Error bars represent SEM.
at terminals occupied by a mitochondrion in sustaining vesicular release may vary dependent on signalling demands (e.g. duration and frequency of AP firing) and synapse type [19,33,38,39]. Here, we show that mitochondrial occupancy of a terminal can directly impact the size of the Ca\(^{2+}\) response upon a train of APs. This is in agreement with a recent report that presynaptic mitochondria in cortical axons attenuate neurotransmitter release by enhancing Ca\(^{2+}\) clearance in an LKB1-dependent manner [40]. Thus, Ca\(^{2+}\) buffering by mitochondria may be the more important mediator of local mitochondrial influence at presynaptic terminals during synaptic transmission.

Homeostatic changes are important for regulating overall levels of activity in neuronal networks by avoiding extreme states of excitation or inhibition in the brain [9]. The synaptic rescaling during homeostatic plasticity is partly dependent on alteration of AMPA receptor levels at the postsynaptic density [9] but has also been shown to involve axonal and presynaptic components of neuronal transmission such as the positioning of the axon initial segment (AIS) and presynaptic transmitter release [10–12,41]. We now demonstrate that presynaptic mitochondrial occupancy may be another important factor during homeostatic rescaling.

Thus, we put forward a model whereby mitochondrial localisation at presynaptic terminals is tuned by neuronal activity and Miro1-mediated Ca\(^{2+}\) sensing. This can increase mitochondrial occupancy when terminals are particularly active, thus enabling the mitochondria to provide energy and buffer Ca\(^{2+}\) in those demanding conditions. Further, repositioning of mitochondria when network activity is altered on a longer timescale can contribute to the rescaling of presynaptic Ca\(^{2+}\) signals during homeostatic plasticity.

**Materials and Methods**

**Neuronal cultures and transfection**

Primary hippocampal cultures were prepared as previously described from E16 mice [42], E18 rats or P0 rats [13,43]. Following 15 min (12 min for P0 rat) treatment with 0.25% trypsin and trituration, cells were plated on poly-L-lysine-coated, round, 12-mm coverslips for fixed experiments or 25-mm coverslips for live experiments at a density of 250,000 per 3-cm well. Neurons were transfected either by Ca\(^{2+}\)-phosphate precipitation or by lipofection with Lipofectamine 2000 at DIV 7 and then imaged at DIV 10–12.

**Antibodies, DNA constructs and reagents**

MtDsRed, synaptophysin–GFP, myc-Miro1, myc-ΔEF-Miro1, VGlut1pHluorin and Mito-LSSmKate2 have been previously described [13,27,28]. The presynaptically targeted SyGCaMP5 was cloned using SyGCaMP2 (plasmid #26124 [17]) from Addgene as a target vector and inserting GCaMP5 from Addgene (plasmid #31788 [18]) via the restriction sites SalI and NotI [44]. ΔEF-Miro1-RES-MtDsRed was cloned with In-Fusion (Clontech) by inserting MtDsRed into pCAG-RES-gfp using the BstXI and NotI sites. Then, myc-ΔEF-Miro1 was added using EcoRI and SacI sites. Picrotoxin (FTX) was purchased from Sigma-Aldrich and used at 100 μM, and tetrodotoxin (TTX) was purchased from Tocris Bioscience and used at 1 μM. D(-)-2-Amino-5-phosphonopentanoic acid (APV) was purchased from Abcam and used at 100 μM. 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide (NBQX) disodium salt was purchased from Abcam and used at 10 μM. Ru360 was from Calbiochem and was used at 10 μM.

**Immunocytochemistry and fixed imaging**

After fixation using 4% PFA for 5 min, cells were washed twice and blocked in PBS solution containing 10% horse serum, 0.5% BSA and 0.2% Triton. Cells were stained with primary antibody for 1 h: anti-myc antibody obtained from 9E10 hybridoma lines and used as supernatant at 1:100, anti-SV2 (Neuromab) used at 1:200, anti-Piccolo (Synaptic Systems) used at 1:500, anti-tau (Millipore) used at 1:1,000 and anti-MAP2 (Synaptic Systems) used at 1:1,000. Cells were washed and stained with secondary antibody for 1 h: anti-mouse Alexa 405 (Jackson Dylight) antibody was used at 1:500, anti-mouse Alexa 568 (Invitrogen) was used at 1:1,000, anti-rabbit Alexa 555 (Invitrogen) was used at 1:1,000 and anti-guinea pig Alexa 647 (Invitrogen) was used at 1:1,000. Images of fixed cultures were taken on a Zeiss LSM700 confocal using a 63× oil objective (NA 1.4) and a 20× water objective (NA 1.0).

**Live imaging**

Imaging experiments were performed at 37°C while perfusing the coverslips in external solution containing 125 mM NaCl, 10 mM α-glucose, 10 mM HEPES, 5 mM KCl, 2 mM CaCl\(_2\) and 1 mM MgCl\(_2\), which was brought to a pH of 7.4 using NaOH. An inverted Zeiss Axiovert 200 microscope and a 63× oil objective (NA 1.4) coupled to a Photometrics Evolve camera were used to image frames with 30 ms exposure at either 1 frame per second or maximum frame rate (~18 frames per second) in the software Micro-Manager [45]. Using Chroma filters, coverslips were excited through a D470/40× filter and emission was split using an Opto-Split II (Cairn Research) [46] and a 565DCXR dichroic thereby collecting with HQ522/40M and HQ607/75M filters for SyGCaMP5 or VGlut1pHluorin and MtDsRed or Mito-LSSmKate2, respectively. Presynapses with and without mitochondria were discriminated on the basis of co-localisation of SyGCaMP5 or VGlut1pHluorin and MtDsRed or Mito-LSSmKate2, respectively: signal overlap for the duration of the imaging period was required for a synapse to be deemed occupied by a mitochondrion. Clear separation in signal was required to deem a synapse devoid of mitochondria. Field stimulation was achieved using a Grass S9 or S88 stimulator and a Warner Instruments stimulation bath. Individual stimulating pulses lasted for 1 ms and were set at 10 V as part of stimulation trains of variable frequencies (5–100 Hz) and durations (0.1–20 s). To reactivate neuronal firing, TTX-treated cultures were transferred to and washed in external solution for 20 min prior to imaging.

**Data analysis**

Movies were aligned using the Cairn Image Splitter plugin in ImageJ. Graphs showing ΔF/ΔF\(_0\) were plotted using Mathematica (Wolfram Research), and paired t-test was used to calculate statistical significance, whereby terminals with and without
mitochondria within the same axon were compared. Regions of interest were manually drawn, and after background subtraction, fluorescence was normalised to the first 10 frames. Mean stimulation fluorescence was calculated as an average across a plateau equating to stimulation duration. For co-localisation analysis, a region measuring $40 \times 40 \, \mu m$ was chosen at least 300 $\mu m$ from the soma. Co-localisation of SYN-GFP and MtDsRed was quantified as the fraction of SYN-GFP clusters which overlap with at least one MtDsRed-positive pixel. Images were thresholded in ImageJ and, using the Image Calculator tool, a third image was generated showing those pixels which were positive in both input channels. Using the Particle Analysis tool, the size and number of the thresholded clusters were analysed. Microsoft Excel was used to calculate the fraction of MtDsRed-positive SYN-GFP clusters. In order to quantify the density of mitochondria and SYN-GFP clusters within the axon, the whole axon was imaged using the 20x water-immersion objective. Images were stitched together in ImageJ, to reconstruct the whole axon, and the longest process was traced and straightened. The MtDsRed and SYN-GFP channels were thresholded and the number of thresholded clusters was analysed using the Particle Analysis tool within ImageJ. This was normalised to the straightened axon length. GraphPad Prism was used to perform ANOVAs and t-tests and to visualise bar charts. Error bars represent SEM.

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Author contributions
V.V, M.J.D, N.F.H and J.T.K designed experiments; V.V, M.J.D and N.F.H collected and analysed the data; N.F.H performed molecular biology experiments; and V.V, M.J.D, N.F.H and J.T.K designed experiments; V.V, M.J.D and N.F.H collected and analysed the data; N.F.H performed molecular biology experiments; and V.V, M.J.D, N.F.H and J.T.K wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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