The cross-talk of energy sensing and mitochondrial anchoring sustains synaptic efficacy by maintaining presynaptic metabolism

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Mitochondria supply ATP essential for synaptic transmission. Neurons face exceptional challenges in maintaining energy homeostasis at synapses. Regulation of mitochondrial trafficking and anchoring is critical for neurons to meet increased energy consumption during sustained synaptic activity. However, mechanisms recruiting and retaining presynaptic mitochondria in sensing synaptic ATP levels remain elusive. Here we reveal an energy signalling axis that controls presynaptic mitochondrial maintenance. Activity-induced presynaptic energy deficits can be rescued by recruiting mitochondria through the AMPK–PAK energy signalling pathway. Synaptic activity induces AMPK activation within axonal compartments and AMPK–PAK signalling triggers phosphorylation of myosin VI, which drives mitochondrial recruitment and syntaphilin-mediated anchoring on presynaptic filamentous actin. This pathway maintains presynaptic energy supply and calcium clearance during intensive synaptic activity. Disrupting this signalling cross-talk triggers local energy deficits and intracellular calcium build-up, leading to impaired synaptic efficacy during trains of stimulation and reduced recovery from synaptic depression after prolonged synaptic activity. Our study reveals a mechanistic cross-talk between energy sensing and mitochondria anchoring to maintain presynaptic metabolism, thus fine-tuning short-term synaptic plasticity and prolonged synaptic efficacy.
Activity-triggered presynaptic energy stress activates the AMPK–PAK signalling that induces myo6 phosphorylation, a process required for facilitating mitochondrial recruitment. Disrupting the AMPK–PAK signalling or interfering with the myo6–SNPH interplay reduces both presynaptic ATP availability and Ca2+ clearance capacity during intensive synaptic activity, leading to impaired synaptic efficacy. Our results suggest an energy-sensitive regulation of mitochondrial trafficking and presynaptic positioning, thus fine-tuning short-term synaptic plasticity and maintaining prolonged synaptic efficacy.

Results
Rescuing activity-induced presynaptic energy deficit by recruiting mitochondria. We first sought to address whether presynaptic mitochondria play a major role in the maintenance of energy supply during sustained synaptic activity. ATP diffusion from axonal mitochondria may support basal or short-term synaptic transmission. We hypothesized that presynaptic mitochondria may serve as local power stations that can supply ATP to sustain prolonged synaptic efficacy. To test this, we performed two live imaging experiments. First, we assessed presynaptic ATP levels using genetically encoded Förster resonance energy transfer (FRET)-based presynaptic ATP probe synaptophysin-GoATeam2 (Syn-GoAT2). Sustained synaptic activity was induced by γ-aminobutyric acid type A (GABA_A) receptor antagonist picrotoxin (PTX). Time-lapse imaging of cortical neurons at 14 d in vitro (DIV14) demonstrated that synaptic activity induced by 100 μM PTX is sufficient to reduce the relative intensity ratio of Syn-GoAT2 GFP (orange; ATP-bound) to GFP (green; ATP-free) starting at 30 min, indicating the depletion of presynaptic ATP (Fig. 1a,b). As a negative control, we also applied Syn-GoAT3, a Syn-GoAT2 (R122K/R126K) mutant that does not respond to ATP levels. In contrast to Syn-GoAT2, ATP-insensitive variant Syn-GoAT3 did not respond to synaptic activation (P = 0.0003), thus supporting our hypothesis that PTX-induced synaptic activity for 30 min is sufficient to induce presynaptic energy deficits.

Next, we investigated whether recruiting mitochondria could rescue presynaptic energy deficits during sustained synaptic activity. ATP levels at individual presynaptic boutons were analysed using Syn-GoAT2 before and after recruiting mitochondria during 30-min PTX treatment. Presynaptic boutons that failed to recruit mitochondria displayed reduced presynaptic ATP (P < 0.001; Fig. 1c,d). Such activity-induced energy deficits were reversed by recruiting mitochondria to presynaptic terminals (P < 0.001). Altogether, these live imaging data support our first conclusion: recruiting mitochondria to presynaptic terminals is able to reverse activity-induced energy deficits. Although ATP is dispersed along axons and diffused to presynaptic boutons50, local ATP supply by presynaptic mitochondria is critical to maintain energy homeostasis during sustained synaptic activity.

Synaptic activity facilitates mitochondrial recruitment via AMPK signalling. Given that sustained synaptic activity leads to presynaptic energy deficits, we next investigated whether synaptic activity induces the recruitment of axonal mitochondria to presynaptic terminals through an energy-sensing mechanism that has not been elucidated14,17,28. AMPK is a key cellular energy stress sensor composed of three subunits: one catalytic α-subunit and two regulatory β- and γ-subunits; the latter contains adenine-nucleotide-binding sites that allow the sensing of AMP, ADP and ATP. Recent bioenergetic studies suggested that high-frequency stimulation (HFS) can activate AMPK signalling that is essential for sustaining LTP29,30. These studies allow us to propose a working model: elevated energy consumption and reduced energy availability at presynaptic terminals activates AMPK signalling, thereby recruiting presynaptic mitochondria in response to enhanced synaptic activity.

We performed six lines of experiments to test this working model. First, we examined the time course of axonal AMPK activation by applying a highly sensitive AMPK activation biosensor (AMPKAR-EV) following PTX-induced synaptic activity from 0 to 40 min. Time-lapse imaging of cortical neurons at DIV14 show that the relative intensity ratio of yellow and cyan fluorescent proteins (YFP/CFP), which reflects AMPK activity, increased in the axonal compartment starting at 10 min after PTX treatment (Fig. 1e,f). These data suggest that sustaining synaptic activity for 10 min is sufficient to activate the AMPK signalling within axonal compartments. Second, we examined whether AMPK is activated by Ca2+/calmodulin-dependent protein kinase kinase beta (CaMKK2) in response to elevated Ca2+ levels during synaptic activity. Neurons were treated with PTX (100 μM) and STO-609 (10 μM), a CaMKK2-specific inhibitor that blocks Ca2+-induced AMPK activation51,52. STO-609 treatment delayed activity-induced AMPK activation for ~10 min (P < 0.05), and this delay was abolished at 30 min (Fig. 1e,f). The maximal axonal AMPK activation was not affected by blocking CaMKK2. Thus, while Ca2+-CaMKK2 plays an early role in synaptic activity, CaMKK2 is not a prerequisite for AMPK activation.

Fig. 1 | Activity-induced presynaptic energy deficit is rescued by recruiting mitochondria via AMPK signalling. a, b. Synaptic activity reduces presynaptic ATP levels. Cortical neurons were transfected with presynaptic ATP probe Syn-GoAT2 or ATP-insensitive Syn-GoAT3 at DIV7, followed by live imaging at DIV14 (45 frames, 2-min intervals, total 90 min). PTX was added (100 μM) before the start of the first frame. The OGP/GFP ratio reflects presynaptic ATP availability. Two-way ANOVA showed a main effect of Syn-GoAT2 versus Syn-GoAT3 over time (F1, 20 = 18.54, P = 0.0003). c, d. Recruiting mitochondria reverses presynaptic ATP deficits. Neurons were co-transfected with Syn-GoAT2 and the mitochondrial marker mito-BFP at DIV7, followed by live imaging at DIV14 (15 frames, 2-min intervals, total 30 min). White circles refer to presynaptic boutons. e, f. AMPK is activated within axons by synaptic activity. Neurons were transfected with AMPK activation biosensor AMPKAR-EV at DIV7, followed by live imaging at DIV14 (20 frames, 2-min intervals, total 40 min). CaMKK2 inhibitor STO-609 (10 μM) and/or PTX (100 μM) was added to the imaging chamber before the start of the first frame. The YFP/CFP ratio reflects AMPK activity, which was normalized to same time points without PTX treatment. g, h. AMPK is activated by synaptic activity. Neurons at DIV14 were treated with DMSO control (ctrl), AICAR (1 mM), PTX (100 μM) or PTX + AMPK inhibitor CC (10 μM) for 2 h. Cells lysates (5 μg) were immunoblotted with antibodies as indicated. The intensity of pACC-1 or AMPKα-T172 (pAMPKα) was calibrated with total ACC-1 or AMPKα, and normalized to DMSO. Data were collected from n = 3 independent experiments. i, Kymograph showing presynaptic mitochondrial recruitment following AMPK activation. Neurons were co-transfected with GFP–synapsin and DsRed-mito at DIV7–8, followed by dual-channel live imaging at DIV14 (360 frames, 10-sec intervals, 60 min). AICAR (1 mM) was added to the imaging chamber before the start of the first frame. White arrows point at mobile mitochondria that are captured at presynaptic terminals (yellow vertical lines). j, k. Images (j) and analyses (k) showing activity-induced presynaptic mitochondrial recruitment through AMPK signalling. Neurons were infected with lentiviruses encoding AMPK-WT or AMPK-KD, and/or treated with DMSO, PTX (100 μM), AICAR (1 mM), CC (10 μM) or STO-609 (10 μM) for 2 h at DIV14. The colocalized pixels of mitochondria (TOM20) and presynaptic terminals (SV2) are highlighted in the white/black images. Data were quantified from the total number of neurons indicated in parentheses (b, d, f and k) and presented as box plots (min, max, median and 25th and 75th percentiles) with dots as individual values (d) or the mean ± S.E.M. with dots as individual values (b, f, h and k) and analysed by two-way ANOVA (b and f) followed by Fisher’s least significant difference (f), two-sided Mann–Whitney (d) or one-way ANOVA test followed by two-sided Tukey’s multiple-comparisons test (h and k). Scale bars: 5 μm (c and i) and 10 μm (a, e and j). * P < 0.05.
role in the AMPK signalling\(^2,3\), energy sensing may play a primary role in activating AMPK during sustained synaptic activity that triggers presynaptic energy deficits.

Energetic stress or ATP depletion activates AMPK by phosphorylation on T172 within its α-subunit\(^22\). We next asked whether sustained synaptic activity induces this phosphorylation event at AMPK-T172. As a downstream indicator for AMPK activation, we also measured the phosphorylation of acetyl-CoA carboxylase-1 (ACC-1)-S79, a direct substrate of AMPK\(^34\). Immunoblotting analysis with phospho-specific antibodies demonstrated a significant increase of phospho-AMPK-T172 \((P < 0.05)\) and phospho-ACC-1-S79 \((P < 0.01)\) following PTX (100 μM, 2 h) treatment in neurons (Fig. 1g,h). This AMPK signalling activation is comparable to neurons treated with 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; 1 mM, 2 h), an analogue of AMP that stimulates AMPK activation\(^35\). PTX-induced phosphorylation of AMPK-T172 \((P < 0.05)\) and ACC-1-S79 \((P < 0.01)\) was abolished by AMPK inhibitor compound c (CC; 10 μM, 2 h). We further repeated this phosphorylation assay by treating cortical neurons with PTX for 0, 30, 60 or 120 min, followed by immunoblotting of AMPK-T172 and ACC-1-S79. PTX treatment for 30 min was sufficient to activate AMPK signalling (Extended Data Fig. 1a–c). These studies indicate

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**Figures and Table**

**Figure a** shows the OFP/GFP ratio for Syn-GoAT2 and Syn-GoAT3 over time with PTX treatment. **Figure b** displays the normalized OFP/GFP ratio over time for PTX and PTX + STO-609 treatments. **Figure c** illustrates the presynaptic markers colocalized with mito (%). **Figure d** shows similar data for Recruiting mito. **Figure e** presents the AMPKAR-EV (YFP/CFP) ratio over time for Ctrl, PTX, PTX + STO-609, and PTX + CC treatments. **Figure f** compares the normalized AMPKAR-EV (YFP/CFP) ratio for PTX and PTX + STO-609 treatments. **Figure g** illustrates the normalized AMPKAR-EV (YFP/CFP) ratio for Ctrl, AICAR, PTX, and PTX + CC treatments. **Figure h** shows the AMPKAR-EV (YFP/CFP) ratio for Ctrl, AICAR, PTX, and PTX + CC treatments. **Figure i** presents the GFP-synapsin DsRed-mito expression in different conditions. **Figure j** illustrates the presynaptic markers colocalized with mito (%). **Figure k** shows similar data for different treatments.
that neuronal AMPK signalling is activated by PTX-induced synaptic activity starting at 10 min in live imaging or 30 min by biochemical detection.

Next, we examined whether presynaptic mitochondrial capture could be facilitated by activating the AMPK signalling. Cortical neurons were transfected with GFP–synapsin (a presynaptic marker) and DsRed-mito (a mitochondrial marker) at DIV7–8, followed by dual-channel live imaging at DIV14 following the addition of AICAR. Mitochondria were recruited to presynaptic boutons as early as 20 min after AMPK activation (Fig. 1i). We further confirmed this by co-immunostaining SV2 and TOM20 in fixed cortical neurons. Neurons treated with either AICAR (1 mM, 2 h) or PTX (100 μM, 2 h) or overexpressing AMPK exhibited similar increases in the percentage of presynaptic terminals colocalized with mitochondria relative to dimethylsulfoxide control (DMSO: 48.10% ± 2.14%; AICAR: 76.39% ± 1.63%, P < 0.001; PTX: 74.35% ± 1.94%, P < 0.001; AMPK wild-type (WT): 67.08% ± 2.16%; P < 0.001; Fig. 1j,k). Conversely, co-treatment of neurons with PTX and AMPK inhibitor CC or expressing AMPK kinase-dead (KD) mutant abolished activity-induced mitochondrial recruitment (DMSO: 48.10% ± 2.14%; PTX + CC: 40.31% ± 1.51%, P = 0.0669; PTX + AMPK-KD: 48.20% ± 2.02%, P > 0.99). In addition, co-treatment of neurons with PTX and CaMKK2 inhibitor STO-609 did not affect activity-induced mitochondrial recruitment (PTX: 74.35% ± 1.94%; PTX + STO-609: 73.50% ± 1.87%, P > 0.99), supporting the notion that presynaptic mitochondrial recruitment is activated mainly through the energy-sensing pathway under sustained synaptic activity. Kinase activity of WT and KD AMPK constructs was validated in neurons by measuring phospho-ACC-1-S79 (pACC-1). Expressing AMPK-WT (P = 0.028), but not AMPK-KD (P > 0.05), activated AMPK signalling following synaptic activity (Extended Data Fig. 1d,e). AMPK-KD is an AMPKα1 mutant (K47R) that exerts a dominant negative effect by competing with endogenous AMPKα1 for binding two regulatory subunits (Fig. 1f). However, when cell lysates were used for immunoblotting (Extended Data Fig. 1d,e), such a dominant negative effect was diluted by un-transfected neurons in culture. We further demonstrated no detectable effect of PTX or AICAR on the density of presynaptic terminals nor on the mitochondria membrane potential (ΔΨm; Extended Data Fig. 2a–d). These results support our second conclusion: activity-induced presynaptic energy deficit is rescued by recruiting mitochondria through AMPK signalling.

Myo6 and SNPH are essential for capturing mitochondria on presynaptic F-actin. We hypothesized that axonal mitochondria are captured at presynaptic terminals via the MT–actin cross-talk that coordinates recruiting and anchoring by actin-based motors and anchors37,38. We first examined whether there is an interplay between actin-based motors and mitochondria anchoring protein SNPH. Myo6 drives minus-end-directed cargo transport along polarized actin tracks and is also found in synapses in hippocampal neurons39. Loss of myo6 impairs presynaptic function and LTP40. In addition, myo6 opposes MT-based axonal mitochondrial transport41. These studies raise the question of whether myo6 recruits and captures mitochondria at presynaptic F-actin through interacting with SNPH. We performed multiple lines of imaging analyses to address this issue.

First, we examined presynaptic mitochondria in cortical neurons following myo6 knockdown. Neurons were infected with lentiviruses encoding scrambled short hairpin RNA (scr-shRNA), mouse myo6-targeted shRNA (myo6-shRNA) or myo6-shRNA combined with expression of human myo6 that is resistant to mouse myo6-shRNA (Extended Data Fig. 3a–d), thus serving to rescue the knockdown phenotype. Depleting myo6 significantly reduced the percentage of presynapses colocalized with mitochondria (37.35% ± 1.89%, P < 0.001) compared to scr-shRNA control neurons (52.57% ± 2.39%). Coexpressing human myo6 rescued the phenotype (59.15% ± 2.14%, P > 0.05; Fig. 2a,b). We demonstrated no detectable effect of myo6 knockdown on the density and size of axonal mitochondria and the density of presynaptic terminals (Extended Data Fig. 3e,f). Second, we asked whether myo6-driven mitochondrial recruitment depends on SNPH by examining WT and Snph knockout (KO) cortical neurons. Overexpressing myo6 increased the percentage of presynapses colocalized with mitochondria in WT neurons (Flag: 45.77% ± 2.03%; Flag-tagged myo6: 66.34% ± 1.66%, P < 0.001; Fig. 2c,d). However, this effect was abolished in Snph KO neurons (Flag: 29.43% ± 1.73%; Flag–myo6: 29.35% ± 1.76%, P > 0.99). Consistently, overexpressing myo6 significantly reduced axonal mitochondrial motility in WT neurons (Flag: 35.91% ± 1.52%; Flag–myo6: 14.27% ± 1.41%, P < 0.001), but not in Snph KO neurons (Flag: 79.24% ± 1.54%; Flag–myo6: 79.04% ± 1.72%, P > 0.99; Fig. 2e,f). Depleting myo6 did not display an additive effect to the already decreased mitochondria capture at presynaptic terminals in Snph KO neurons (Snph KO + scr-shRNA: 27.82% ± 1.42%; Snph KO + myo6-shRNA: 28.66% ± 1.47%, P > 0.05; Extended Data Fig. 3g,h). Thus, myo6-driven recruitment...
and SNPH-mediated anchoring act synergistically to capture axonal mitochondria at presynaptic terminals.

We further examined whether F-actin participates in SNPH-mediated mitochondrial anchoring at presynaptic terminals. First, we assessed actin filaments in WT and Snph KO cortical neurons at DIV14 before and after treatment with latrunculin B (LatB; 2 μM, 3 h), a widely used reagent that depolymerizes F-actin. Using both Airyscan confocal microscopy and stimulated emission and depletion (STED) nanoscopy, we observed reduced F-actin following LatB treatment without impacting neuronal morphology, axonal MTs or presynaptic terminals (Extended Data Fig. 4a,b). We also examined Δψm following LatB treatment by loading fixable Δψm-dependent dye MitoTracker CMTMRos. LatB did not induce mitochondrial depolarization in axons, whereas treating neurons with Δψm uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 10 μM, 30 min) depolarized mitochondria (Extended Data Fig. 4c,d). We further demonstrated no detectable effect of LatB on the average density of presynaptic terminals in both live and fixed neurons, nor any effect on the density or size of axonal mitochondria (Extended Data Fig. 4e–g). Altogether, LatB treatment selectively depolymerizes actin filaments without changing presynaptic terminals or axonal mitochondria in mature neurons.

Next, we performed time-lapse imaging to examine whether F-actin is required for capturing mitochondria at presynaptic terminals. Cortical neurons were co-transfected with GFP–synapsin and DsRed-mito at DIV7–8, followed by dual-channel live imaging.
Fig. 3 | Myo6 mediates SNPH anchoring on F-actin. a, b, Representative immunoprecipitation assays showing SNPH–myo6 complex in mouse brains. An antibody against myo6 (a) or SNPH (b) reciprocally immunoprecipitated SNPH or myo6 from WT mouse brain homogenates (BH) but failed to detect myo6–SNPH complex from Snph KO mouse BH. c, Representative pull-down assay confirming myo6–SNPH complex. GST–SNPH Snph–/– brains. An antibody against myo6 (a) failed to detect myo6–SNPH complex from KO mouse BH. Representative pull-down assay confirming myo6–SNPH complex. GST–SNPH (kDa) showing the binding of myo6 to the N-terminal region (1–203) of SNPH. GFP-tagged myo6 or its truncated forms were expressed in HeK293 cells. The cell lysates were incubated with GST or GST–SNPH (1–469) at 4 °C for 3 h, followed by immunoblotting of myo6. Coomassie blue staining reflects the purity of truncated GST–SNPH. MTB, MT binding domain; KBD, kinesin binding domain.

g, h, Representative actin spin-down assay showing no direct interaction between SNPH and F-actin. Representative PLA (SNPH-actin) showing the in situ close proximity of SNPH and F-actin in the presence of myo6 or its truncated forms were expressed in HEK293 cells. The cell lysates were incubated with GST or GST–SNPH (1–469) at 4 °C for 3 h.

At DIV14 before (0–15 min time lapse) and after (15–75 min time lapse) LatB treatment (10μM). Kymograph analysis displayed a striking phenotype: following F-actin depolymerization, presynaptic mitochondria were released into axons as early as 20 min after LatB treatment (Extended Data Fig. 5a–c). After 60 min of LatB treatment, the percentage of presynapses capturing mitochondria was reduced from 36.34% ± 2.62% to 24.31% ± 2.05% (P < 0.001). By co-immunostaining SV2 and TOM20, we demonstrated that LatB...
treatment (2 μM, 3 h) in WT neurons significantly reduced the percentage of presynapses colocalized with mitochondria (36.78 ± 1.72%, P < 0.001) compared to DMSO control (50.35% ± 1.93%; Fig. 2g.h). Interestingly, deleting Snph reduced mitochondrial capture at presynaptic terminals under DMSO conditions (35.72% ± 1.90%, P < 0.001) similarly to LatB-treated WT neurons. LatB treatment in Snph KO neurons did not further reduce presynaptic mitochondria. We confirmed these light images with transmission electron microscopy (TEM) and found a similar reduction in mitochondrial positioning at presynaptic AZs following F-actin disruption (Extended Data Fig. 5d,e). Depolymerizing actin filaments or deleting Snph similarly enhanced axonal mitochondrial motility (WT-DMSO: 33.90% ± 1.57%; WT-LatB: 63.18% ± 1.82%, P < 0.001; KO-DMSO: 72.92% ± 2.28%, P < 0.001; Fig. 2i,j). LatB treatment in Snph KO neurons failed to further reduce mitochondrial capture at presynaptic terminals (P = 0.8873) and increase axonal mitochondrial motility (P = 0.9869). Thus, our live imaging analyses combined with TEM ultrastructural observations support the notion that both F-actin and SNPH are required for capturing presynaptic mitochondria.

Furthermore, we determined whether activity-induced recruitment of presynaptic mitochondria is coordinated by combined action of AMPK, myo6, F-actin and SNPH. Neurons with Snph KO, myo6 depletion or LatB treatment were incubated with AICAR (1 mM, 2 h) or PTX (100 μM, 2 h). While WT neurons exhibited a robust presynaptic mitochondrial capture following AICAR or PTX treatment (Fig. 1), this phenotype was abolished in Snph KO neurons (DMSO: 38.14% ± 2.75%; AICAR: 39.78% ± 1.63%, P < 0.001; PTX: 43.19% ± 1.86%, P = 0.8422), myo6 knockdown neurons (DMSO: 38.30% ± 2.30%; AICAR: 33.02% ± 1.62%, P = 0.8088; PTX: 40.90% ± 1.45%, P = 0.9989) and LatB-treated neurons (DMSO 35.74% ± 3.07%; AICAR: 40.00% ± 2.26%, P = 0.9642; PTX: 42.20% ± 1.56%, P = 0.5828; Fig. 2k,l). Thus, these data support our third conclusion: axonal mitochondria can be recruited to and captured at presynaptic terminals in response to sustained synaptic activity via a coordinated action of AMPK signalling, myo6 motor, F-actin and SNPH anchor protein.

Myo6–SNPH interplay switches mitochondrial anchoring from MTs to F-actin. To understand the mechanisms underlying myo6-driven recruitment and SNPH-mediated anchoring of presynaptic mitochondria, we performed four lines of biochemical experiments. First, we examined whether myo6 interacts with SNPH in mouse brains. An anti-myo6 antibody or anti-SNPH antibody reciprocally immunoprecipitated SNPH or myo6 from WT, but not Snph KO, mouse brain homogenates (Fig. 3a,b). Second, the myo6-SNPH complex was confirmed by a pull-down assay: glutathione S-transferase (GST)-tagged SNPH (1–469), but not GST, pulled down myo6 from mouse brain homogenates (Fig. 3c). Third, endogenous myo6 was pulled down by GST-tagged SNPH domains (1–469) or (1–203) domain, but not by truncated SNPH lacking its N-terminal region, suggesting that SNPH interacts with myo6 through its N-terminal domain (Fig. 3d,e). Furthermore, we generated two truncated myo6 mutants: myo6-(1–980) that contains the motor domain but lacks the cargo-binding domain (CBD) and myo6-(980–1286) that retains only the CBD45 (Fig. 3f). Pull-down analysis demonstrated that myo6-CBD is sufficient to bind to SNPH (Fig. 3g).

To address whether the myo6–SNPH interplay mediates cytoskeletal switching of mitochondrial anchoring from MTs to actin filaments, we performed actin spin-down assays46 to determine the direct interaction of SNPH with actin filaments. Purified GST–SNPH (1–469), a C-terminal tail-deleted SNPH with loss of mitochondrial targeting, or actinin, an actin-binding protein serving as a positive control46, was incubated for 30 min in the absence or presence of F-actin. While the majority of actinin was spun down with F-actin in the pellet (Fig. 3h), GST–SNPH (1–469) mainly remained in the supernatant after ultracentrifugation, suggesting no direct interaction between SNPH and F-actin. We next examined whether myo6 mediates anchoring of SNPH on actin by proximity ligation assay (PLA), which utilizes specific antibodies for in situ detection of SNPH and actin based on their close proximity to each other46. PLA signals were readily detected in cortical neurons. However, depleting myo6 with myo6–shRNA reduced PLA signals (P < 0.001), while deleting Snph abolished PLA signals (Fig. 3i,j)). These PLA data suggest that close in situ proximity of SNPH and actin in neurons is mediated by actin-based myo6. As controls, knockdown of myo6 or deletion of SNPH did not alter global morphology of F-actin or MTs in axonal compartments (Extended Data Fig. 6a,b). We alternatively performed PLA by applying antibodies against SNPH and βIII-tubulin. Knockdown of myo6 enhanced PLA signals reflecting the close proximity of SNPH and MTs (P < 0.001) (Fig. 3k,l), thus providing evidence of close proximity of SNPH with F-actin or MTs in axons is reciprocally regulated by myo6. Altogether, these data support our fourth conclusion: myo6–SNPH interplay mediates the switch of mitochondrial anchoring platform from MTs to F-actin.

SNPH serves as an adaptor recruiting myo6 to axonal mitochondria. Myo6 distributes on the plasma membrane and the cytosol, and its association with endosomes, Golgi-derived vesicles and..
autophagosomes is mediated by specific adaptors\(^4^4,4^7\). SNPH is distributed on the surface of axonal mitochondria\(^4^4,4^8\). Identification of a SNPH–myo6 interaction raises the question as to whether SNPH serves as an adaptor that recruits myo6 to axonal mitochondria. To address this, we examined localization of myo6 in axons of mature cortical neurons at DIV14. Confocal images and line-scan analysis showed colocalization of myo6 with axonal mitochondria labelled by cytochrome c (cyto c; Fig. 4a,b). STED super-resolution imaging further deciphered myo6 submitochondrial distribution: myo6 displayed a pattern distinct from the cyto c-labelled inner mitochondrial membrane and intermembrane space (Fig. 4c). Instead, myo6 perfectly colocalized with SNPH by forming structures that surrounded the mitochondrial shape (Fig. 4d) and formed punctate structures on the OMM labelled with TOM20 (Fig. 4e). This mitochondria-targeted pattern was abolished in Snph KO cortical neurons (Fig. 4f), where myo6 was dispensed along axons. We further characterized colocalization of myo6 with axonal mitochondria using confocal microscopy. Both Pearson’s coefficient \((P < 0.001)\) and Mander’s coefficient \((P < 0.001)\) consistently showed a significant reduction in myo6 targeting to axonal mitochondria in Snph KO neurons when compared to WT neurons (Fig. 4g,h), thus suggesting SNPH as an adaptor recruiting myo6 to axonal mitochondria.

Given that myo6 interacts with SNPH via its C-terminal CBD (Fig. 3f,g), we next determined whether the CBD is required for recruiting myo6 to axonal mitochondria. We replaced three residues within two cargo-binding interface motifs, RRL-AAA or WLY, with WWY (Fig. 5a). Despite the presence of the CBD and the WLY motif in myo6-CBD WWY, myo6-WWY-WLY targeted to mitochondria more effectively than myo6-RRL-AAA in WT neurons (Fig. 5b). Moreover, SNPH co-immunopurified with myo6-WWY-WLY and not with myo6-RRL-AAA (Fig. 5c). This suggests that SNPH is recruited to mitochondria through the CBD of myo6-WWY-WLY but not myo6-RRL-AAA.
Fig. 5 | AMPK-PAK signalling facilitates presynaptic mitochondrial capture through myo6 phosphorylation. a, b. AMPK activation triggers PAK phosphorylation in neurons. Cortical neurons at DIV14 were treated with DMSO, PTX (100 μM), AICAR (1 mM) or PTX together with AMPK inhibitor CC (10 μM) for 2 h, followed by immunoblotting. Equal amounts (5 μg) of neuronal lysates were sequentially immunoblotted with phospho-PAK1/2/3 (pPAK), total PAK and GAPDH. The pPAK intensity was calibrated with total PAK and normalized to DMSO control. Data were collected from n = 3 independent experiments. 

c. Activated PAK3 phosphorylates myo6 at T405. HEK cells were co-transfected with GFP–myo6 (355–454) or GFP–myo6 (355–454)-T405A (TA) with Flag–PAK3-KD or Flag–PAK3-CA, followed by immunoblotting 24 h after transfection. Equal amounts (5 μg) of cell lysates were loaded on phospho-tag gel and sequentially immunoblotted with antibodies against GFP, Flag and GAPDH; phosphorylation status is indicated by a band shift.

d. e. Representative images (d) and analyses (e) showing the role of myo6-T405S phosphorylation in presynaptic mitochondrial capture. Cortical neurons at DIV7 were infected with lentiviruses encoding Flag-tagged myo6, myo6-T405A (phospho-dead mutant) or myo6-T405E (phospho-mimetic mutant), followed by co-immunostaining at DIV14. The colocalized pixels are highlighted in the white/black images. 

f. Kymographs (f) and analyses (g) showing reduced axonal mitochondrial motility by expressing myo6-T405S. Cortical neurons at DIV7 were co-transfected with DsRed-mito and myo6, myo6-T405A or myo6-T405E, followed by live imaging at DIV14. Reproductive images (h) and quantitative analyses (i) showing the role of PAK3 activity in capturing presynaptic mitochondria. Cortical neurons at DIV7 were infected with lentiviruses encoding Flag-tagged PAK3, PAK3-CA or PAK3-KD, followed by co-immunostaining at DIV14. Representative images (j) and quantitative analyses (k) showing that inhibiting PAK3 abolished activity- or AMPK-induced presynaptic mitochondrial recruitment. Cortical neurons at DIV14 were treated with PTX (100 μM), AICAR (1 mM) or PTX combined with the PAK inhibitor IPA3 (10 μM) for 2 h, followed by co-immunostaining of SV2 and TOM20. Data were quantified from the total number of neurons indicated within the bars (e, i and k) or under the bar graph (g) from three experiments, expressed as the mean ± s.e.m. with dots as individual values and analysed by one-way ANOVA followed by two-sided Tukey’s multiple-comparisons test (b), two-sided Kruskal–Wallis test with Dunn’s multiple-comparisons post hoc test (e, g and i) or two-sided unpaired Student’s t-test (k). Scale bars: 10 μm.
WWY-WLY, at the CBD of myo6 (ref. 49; Fig. 4i) and found that both mutants lost their binding capacity to SNPH (Fig. 4j). While myo6 and myo6-CBD mainly appeared as vesicular structures targeting axonal mitochondria, myo6-RRL-AAA and myo6-WWY-WLY mutants displayed a diffuse pattern throughout the axon (Fig. 4k,l), further supporting that myo6 is recruited onto axonal mitochondria through its interaction with SNPH. In addition, we expressed myo6-WWY-WLY and myo6-RRL-AAA in cortical neurons to confirm the role of the myo6–SNPH interaction in anchoring pre-synaptic mitochondria. While overexpressing myo6 increased the percentage of pre-synaptic terminals colocalized with mitochondria in WT neurons (Flag: 45.77% ± 2.03%; Flag–myo6: 66.34% ± 1.66%, \( P < 0.001 \)), expressing either myo6-WWY-WLY or myo6-RRL-AAA mutant failed to increase the percentage of pre-synaptic terminals colocalized with mitochondria (Fig. 4m,n). Altogether, these results support our fifth conclusion that myo6–SNPH interplay is required for recruiting myo6 to axonal mitochondria and capturing mitochondria at pre-synaptic terminals.

AMPK–PAK signalling facilitates mitochondrial capture by phosphorylation of myo6. PAKs are a family of serine/threonine kinases that play a central role in signalling cascades remodelling the cytoskeleton and scaffolding and supporting synaptic transmission23,24,50. Interestingly, group I PAKs (PAK1/2/3) have been identified as substrates of AMPK25, and PAK2 mediates cell metabolism and survival by interacting with AMPK51. PAKs phosphorylate
Cortical neurons at DIV14 were treated with DMSO, PTX (100 μM, 2 h) or AMPK activator ACAR (1 mM, 2 h) or PTX together with AMPK inhibitor CC (10 μM, 2 h). Phospho-PAK1/2/3 were significantly increased after synaptic activity (P < 0.05) or AMPK activation (P < 0.05); such elevated phospho-PAK1/2/3 levels were abolished by inhibiting AMPK with CC (Fig. 5a,b). Second, we examined whether activated PKA is able to phosphorylate myo6 at T405, the main myo6 phosphorylation site by Group I PAKs. We selected truncated myo6 mutants containing 100-residue (355–454) as phosphorylation substrate and coexpressed neurons with myo6(355–454) or phosphorydo-dead mutant myo6 (355–454)-T405A together with PAK3-T421E, a constitutively active (CA) PAK3, or PAK3-K297L, a kinase-dead (KD) mutant. Band-shift analysis with phospho-tag gels revealed detectable phosphorylation status when myo6 (355–454) was coexpressed with PAK3-CA but not with PAK3-KD (Fig. 5c). This myo6 phosphorylation was inhibited when coexpressing the myo6(355–454)-T405A mutant with PAK-CA. These results provide a mechanistic link between AMPK–PAK energy signalling and myo6-SNPH anchoring through myo6 phosphorylation at T405.

We next asked whether myo6 phosphorylation at T405 is able to facilitate mitochondrial positioning at presynaptic terminals. While expressing phospho-mimetic mutant myo6-T405E, but not phospho-dead mutant myo6-T405A, facilitated presynaptic mitochondrial recruitment (P < 0.001; Fig. 5d,e). Enhanced presynaptic capture by expressing myo6-T405E substantially reduced mitochondrial motility along axons (P < 0.001; Fig. 5f,g). These results reinforce the notion that phosphorylation at myo6-T405 is a key process required for recruiting myo6 to mitochondria in proximity (green circles). Data were quantified from the total number of neurons indicated in parentheses, expressed as mean ± S.D. 

To characterize presynaptic Ca2+ transients, we applied synaptophysin–GECO (Syn–GECO), a genetically encoded single-colour calcium indicator that links to the C-terminal tail of synaptophysin. First, we measured Ca2+ within presynaptic terminals with or without mitochondria using Syn–GECO. The peak value of F/F0, over baseline was averaged, where F0 is the average fluorescence before stimulation, and F is the fluorescence at each pixel at individual time point. Presynaptic terminals containing mitochondria had higher Ca2+ clearance capacity (P < 0.001) when compared to those without mitochondria under the same intensive activity (Fig. 6a). Second, we examined whether the AMPK–PAK–myo6 axis plays a role in accelerating presynaptic Ca2+ clearance during intensive activity by recruiting presynaptic mitochondria. Enhancing presynaptic mitochondrial recruitment, by expressing myo6 or myo6-T405E, by activating AMPK–PAK pathway with ACAR or expressing PAK3-CA, significantly reduced the average Syn–GECO peak amplitudes during 100-Hz stimulation (Flag: 3.66 ± 0.16; myo6: 2.50 ± 0.22, P = 0.0011; myo6-T405E: 1.99 ± 0.12, P < 0.001).

**Fig. 7** | AMPK–PAK–myo6 axis accelerates synaptic recovery after sustained activity. **a–d.** Activating or inhibiting AMPK–PAK–myo6 signalling modulates short-term synaptic depression. Normalized EPSC amplitude was plotted against each action potential (AP) stimulation from paired cortical neurons evoked by 20 Hz, 1 s with 20-s inter-train intervals (ITI; a and b) or 50 Hz, 200 ms (c and d). At DIV14–18, dual whole-cell patch-clamp recording was performed on paired cortical neurons treated with ACAR (1 mM) or CC (10 μM) for 2 h (a) or presynaptic neurons expressing various proteins as indicated (b–d). Two-way ANOVA revealed a main effect: F2,59 = 7.764, P = 0.0010 (a); F2,69 = 3.595, P = 0.0327 (b); F2,56 = 10.78, P = 0.0001 (c); or F2,64 = 4.294, P = 0.0178 (d). e–g. STED super-resolution images (e) and quantitative analyses (f and g) showing larger SV clusters in proximity to mitochondria. Cortical neurons at DIV14 were co-immunostained with synaptophysin and cyt c, or treated with DMSO or PTX (100 μM for 2 h) before immunostaining. The average size of SV clusters in proximity to mitochondria (orange circles) was significantly larger than SV clusters without mitochondria in proximity (green circles). Data were quantified from the total number of neurons indicated in parentheses, expressed as mean ± s.e.m. with dots as individual values and analysed by a two-sided Mann–Whitney test. Scale bar: 2 μm. h. Presynaptic ATP declines under HFS. Neurons at DIV7–9 were transfected with Syn-GoAT2 or Syn-GoAT3, followed by live imaging at DIV14. Three trains of stimulations (200 AP at 20 Hz) were delivered to neurons through a field-stimulation chamber, a 130-s rest phase was placed between each train and 20 frames of images with 2-s intervals were taken for total 40 s (5 frames during stimulations and 15 frames after stimulations). The relative ratio of OFP/GFP signal was normalized to the start of the first train. Two-way ANOVA revealed a main effect by expressing Syn-GoAT2 versus Syn-GoAT3 (F1,58 = 18.06, P = 0.0001). Data were quantified from 30 neurons under each condition. **i.** Representative EPSC curves (i) and normalized EPSC amplitudes (j) showing accelerated synaptic recovery after HFS by activating PKA–myo6 signalling. Neurons were infected at DIV5, followed by recording at DIV14–18. Recording configuration (total 132 s) consisted of a 2-s 100-Hz train, a 10-s recovery phase of four stimuli and a 120-s rest phase. The single EPSC traces for the first and last EPSCs were plotted, and four recovery EPSCs after the first HFS train were plotted illustrating the recovery period (i). The normalized EPSC amplitude was plotted for the first of each HFS and for each of the recovery EPSCs under each condition. Two-way ANOVA revealed a main effect of gene manipulation (F2,57 = 3.306, P = 0.0438) and a significant interaction between gene manipulation and stimuli (F2,78,978 = 1.765, P = 0.0089). Data were quantified from the total pairs of neurons indicated in parentheses and represented as the mean ± s.e.m.
P < 0.001; AICAR: 1.99 ± 0.16, P < 0.001; PAK3-CA: 1.83 ± 0.18, P < 0.001; Fig. 6b–d), indicating an accelerated Ca^{2+} clearance capacity. In contrast, impairing presynaptic mitochondrial anchoring, by expressing myo6-T405A, inhibiting AMPK–PAK pathway by CC treatment or expressing PAK3-KD, increased average Syn-GECO peak amplitudes (myo6-T405A: 5.48 ± 0.34, P < 0.001; CC: 5.86 ± 0.40, P < 0.001; PAK3-KD: 5.19 ± 0.31, P < 0.001) relative to control. These live imaging data suggest a critical role of the AMPK–PAK–myo6 signalling in maintaining presynaptic Ca^{2+} clearance capacity during intensive activity.

Presynaptic calcium transients play an important role in SV exocytosis and neurotransmitter release, thus modulating synaptic efficacy. Reduced presynaptic Ca^{2+} clearance contributes to elevated Ca^{2+} levels; therefore, repetitive trains of stimulation induce short-term synaptic depression^5. Given such robust impaired presynaptic Ca^{2+} clearance (Fig. 6a–d), we asked whether altering AMPK–PAK–myo6 signalling modulates basal synaptic transmission. Cortical neurons were treated with AICAR (1 mM) or CC (10 μM) for 2h, or expressing GFP-tagged myo6, myo6-CBD, myo6-T405A (phospho-dead mutant), myo6-T405E (phospho-mimetic mutant), PAK3-KD or PAK3-CA, followed by patch-clamp recordings on paired neurons. No significant difference was observed in the average amplitude of evoked excitatory postsynaptic currents (EPSCs; 0.05 Hz; Extended Data Fig. 8a–h), suggesting that modulating AMPK–PAK–myo6 signalling does not affect basal synaptic transmission. Next, we determined whether activating or inhibiting the...
AMPK–PAK–myo6 axis modulates short-term synaptic depression during repetitive trains of stimulation (20 Hz, 1 s at 20-s intervals or 50 Hz, 200 ms). Activating the AMPK–PAK–myo6 pathway, by treating neurons with AICAR, overexpressing myo6, myo6-T405E or PAK3-CA, accelerated energy-dependent synaptic transmission, triggering a fast depression of synaptic strength during the trains (Fig. 7a–d). In contrast, inhibiting the AMPK–PAK–myo6 pathway, by treating neurons with CG, expressing myo6-CBD, myo6-T405A or PAK3-KD, impaired presynaptic mitochondrial recruitment, triggering energy deficits and 

\[ \text{[Ca}^2+] \text{] build-up at presynaptic terminals during repetitive stimulations. These defects collectively contributed to slowed synaptic depression during repetitive stimulations. To further confirm whether altered synaptic depression was due to presynaptic mitochondria, we repeated these studies in WT and Snph KO neurons and demonstrated that these manipulations of the PAK–myo6 pathway have an impact on short-term synaptic depression only in WT neurons (F2, 61 = 5.319, P = 0.0074) but not in Snph KO neurons (F2, 73 = 0.08927, P = 0.9147; Extended Data Fig. 9a,b). Given that SNPH specifically anchors axonal mitochondria, these studies support the notion that these synaptic phenotypes are indeed dependent on mitochondrial anchoring per se.

AMPK–PAK–myo6 axis accelerates synaptic recovery after prolonged synaptic activity. Synaptic activity imposes large energetic demands that are met by local ATP synthesis\(^9\). Although glycolysis maintains basal ATP levels, mitochondrial oxidative phosphorylation boosts ATP to sustain intensive synaptic transmission by mobilizing, refilling and recycling SVs, thus maintaining long-term synaptic plasticity\(^5\,\text{–}^7,\text{–}^9,\text{–}^6\). We investigated whether enhanced capture of presynaptic mitochondria maintains the size of SV clusters after sustained synaptic activity. Cortical neurons at DIV14 were treated with DMSO or PTX (100 μM for 2 h). STED imaging showed that the average size of SV clusters in proximity to mitochondria was significantly larger than SV clusters without mitochondria in proximity (P = 0.0492; Fig. 7e,f). Furthermore, activity-induced presynaptic mitochondrial anchoring maintained the average size of SV clusters after sustained synaptic activity (Fig. 7g), suggesting that presynaptic mitochondria are the main local energy source driving SV mobilization and refilling to sustain the lasting synaptic efficacy\(^7\,\text{–}^9\).

To examine whether recruiting presynaptic mitochondria is critical for the maintenance of ATP availability during sustained synaptic activity, we measured presynaptic ATP during HFS. Presynaptic ATP levels were recorded during HFS profile, which consisted of three repetitive trains of stimuli: a 200-AP train (10 s, 20 Hz) and a 130-s rest phase (10-s recovery phase and 120-s rest phase). The OFP/GFP ratio of Syn-GoAT2 ATP probe was decreased after three repetitive trains of stimuli, whereas the ATP-insensitive

Fig. 8 | Schematic of a mechanistic cross-talk between energy sensing and presynaptic mitochondrial anchoring during sustained synaptic activity. Presynaptic function is driven by highly energy-dependent processes, including assembly and maintenance of synapses, generation of action potentials and ionic gradients, and trafficking, refilling and recycling of SVs. While local glycolysis provides ATP for basal synaptic activity, mitochondria supply the majority of the total ATP in synapses, thus supporting intensive synaptic transmission and maintaining long-term synaptic efficacy. Anchored presynaptic mitochondria ideally serve as local energy sources. Given that only ~33% of presynaptic AZs in hippocampi retain mitochondria, energy-sensitive regulation of mitochondrial recruitment ensures that metabolically active presynaptic terminals are adequately supplied with ATP during intensive synaptic activity. Axonal mitochondria are recruited to and anchored on presynaptic F-actin through an anchoring platform switch that is mediated by the myo6–SNPH interplay. Enhanced synaptic activity induces presynaptic energy stress, which activates the energy-sensing AMPK–PAK signalling pathway and triggers myo6 phosphorylation, a cellular process essential for facilitating myo6-driven presynaptic mitochondrial recruitment and SNPH-mediated anchoring. Such a mechanistic cross-talk enables neurons to capture mitochondria at presynaptic terminals in response to local energy stress during sustained synaptic activity, thus fine-tuning synaptic plasticity and prolonged synaptic efficacy. Image adapted with permission from ref. 2, Elsevier.
variant Syn-GoAT3 failed to display such a decline (F1, 58 = 18.06, P < 0.0001; Fig. 7h). These data support the conclusion that presynaptic energy deficits contribute to synaptic depression during repetitive stimulation.

Next, we examined whether increased mitochondrial anchoring by expressing myo6 and its phospho-mimetic mutant myo6-T405E in cortical neurons accelerates the recovery of synaptic depression after prolonged repetitive stimulation. In a duration of 132 s consisting of a 2-s 100-Hz train, a 10-s recovery phase of four stimuli and a 120-s rest phase, neurons overexpressing myo6 or myo6-T405E induced a faster synaptic recovery after HFS (Fig. 7i). Two-way ANOVA revealed a main effect of gene manipulation (F2, 57 = 3.306, P = 0.0438) and a significant interaction between gene manipulation and stimuli (F28, 798 = 1.765, P = 0.0089). These manipulations of the PAK–myo6 pathway affected synaptic recovery only in WT neurons but not in Snph KO neurons (F3, 57 = 0.5787, P = 0.6314; Extended Data Fig. 9c), confirming that accelerated synaptic recovery following HFS is through SNPH-mediated mitochondrial anchoring.

Presynaptic mitochondria play a dual role in maintaining presynaptic Ca2+ clearance and maintaining ATP availability. To assess the relative contribution of presynaptic ATP on the recovery of synaptic efficacy after prolonged synaptic activity, we examined the recovery rate from synaptic depression after HFS by inhibiting uptake of calcium into mitochondria with 10 μM RU360, a mitochondrial calcium uniporter inhibitor. Consistent with a previous report, blocking mitochondria calcium buffering with RU360 slowed the recovery rate of synaptic depression after HFS (Extended Data Fig. 10a); however, the recovery rate was accelerated when activating the myo6–SNPH axis even with loss of calcium-buffering capacity (Extended Data Fig. 10b). Therefore, these data support our conclusion that the AMPK–PAK–myo6 pathway is critical for the presynaptic ATP maintenance, thus aiding the recovery of synaptic depression after prolonged synaptic activity.

Discussion

Our study reveals that axonal mitochondria are recruited to and anchored at presynaptic terminals through a cytoskeleton switch from axonal MTs to presynaptic F-actin, a process mediated by myo6–SNPH interplay. Prolonged synaptic activity induces presynaptic energy deficit, which activates AMPK–PAK signalling and triggers myo6-driven mitochondrial recruitment and SNPH-mediated anchoring. This mechanistic cross-talk between energy sensing and mitochondrial anchoring enables neurons recruiting presynaptic mitochondria (Fig. 8). By inhibiting the AMPK–PAK–myo6–SNPH signalling, we show that prolonged synaptic activity triggers local energy deficits and [Ca2+]i build-up, leading to impaired synaptic efficacy and accelerated synaptic depression. These defects are relevant to major neurological disorders associated with synaptic dysfunction, energy deficits and mitochondrial defects.

Presynaptic function is driven by energy-dependent processes, including generation of action potentials and ionic gradients and refilling and recycling of SVs. Mitochondria supply ~93% of the total ATP in synapses, thus sustaining lasting synaptic efficacy and plasticity. It was reported that mitochondria-derived ATP is dispersed in axons and diffused to non-mitochondria-containing presynaptic boutons. However, sustained synaptic activity is specific to mitochondria-containing presynaptic boutons during LTD. Presynaptic boutons with mitochondria have more docked SVs than those without mitochondria in the hippocampal CA1 area. ATP production from presynaptic mitochondria is the main local energy source driving SV mobilization from the reserve pool to sustain the lasting synaptic efficacy. These studies raise the question as to whether presynaptic mitochondria provide additional ATP to sustain SV release and recycling during prolonged synaptic activity. Given that only ~33% of AZs in hippocampi retain mitochondria, energy-sensitive regulation of mitochondrial terminal recruitment ensures that metabolically active presynaptic terminals are adequately supplied with ATP during prolonged synaptic activity.

By live imaging analyses, we examined ATP availability at individual presynaptic terminals during sustained synaptic activity. PTX-induced synaptic activity for 30 min is sufficient to deplete presynaptic ATP. Presynaptic ATP levels were also depleted during three repetitive trains of stimuli (10 s, 20 Hz); such energy deficits could be effectively reversed by recruiting mitochondria to presynaptic boutons. However, presynaptic boutons that failed to recruit mitochondria displayed energy depletion following sustained synaptic activity. Thus, neurons adapt a unique mechanism recruiting presynaptic mitochondria in response to an enhanced metabolic demand during prolonged synaptic activity.

Mirol1/2 regulate myo19 recruitment and stability and coordinate mitochondrial trafficking and distribution in mouse embryonic fibroblasts. However, cytoskeletal organization is quite unique in axons, where MTs are exclusively oriented with their plus ends towards terminals and two-tier actin filaments are differentially organized. While subplasmalemmal actin rings form periodic structures that wrap around axons, intra-axonal actin filaments enrich at presynaptic terminals and form highly dense mesh structures. This unique F-actin structure ideally serves as a platform for anchoring organelles. These studies suggest an opposing role of F-actin and MTs on axonal mitochondrial transport. Therefore, our study aimed to address two questions: (1) how are MT-based axonal mitochondria recruited to and captured on presynaptic actin filaments? and (2) does a myosin motor drive this anchoring platform switch?

We demonstrate that the MT–actin switch is critical for recruiting and retaining mitochondria at presynaptic terminals through the myo6–SNPH interplay. With STED imaging and time-lapse analysis, we revealed a striking phenotype: following F-actin disruption by LatB treatment, captured presynaptic mitochondria were quickly released into axons; this phenotype was not readily observed in Snph KO neurons. SNPH associates with F-actin through its interaction with myo6. STEED imaging further revealed a predominant colocalization of myo6 and SNPH on the surface of axonal mitochondria in WT but not in Snph KO neurons, suggesting SNPH acts as an adaptor that recruits myo6 to axonal mitochondria. We further showed that myo6 interacts with SNPH and targets axonal mitochondria through its C-terminal CBD region; replacing three residues within CBD motifs abolished its interaction with SNPH and its targeting to axonal mitochondria. Furthermore, by knocking down myo6 or expressing SNPH-binding defective myo6 mutants, we consistently showed reduced presynaptic mitochondrial capture. Conversely, overexpressing myo6 increased mitochondria captured at presynaptic terminals in WT neurons, but not in snph KO neurons. Altogether, these results support our model: the myo6–SNPH interplay mediates a cytoskeleton switch of mitochondrial anchoring platform from MTs to actin filaments, thus recruiting and capturing axonal mitochondria at presynaptic terminals.

Myo6 was reported to oppose MT-based mitochondrial transport, suggesting its role in anchoring rather than trafficking mitochondria. Consistently, myo6 anchors signalling endosomes to actin filaments. Although myo6 was recruited to damaged mitochondria through forming a complex with Parkin, this process is unlikely to be relevant to healthy mitochondria as they do not recruit Parkin. We found that a myo6–SNPH interaction is required for myo6 recruitment to axonal mitochondria. Interestingly, SNPH interacts with the CBD at the C-terminal tail of myo6, which was reported to confer cargo specificity through its interaction with various organelle adapters.

Miro as a Ca2+ sensor regulates mitochondrial transport. Two genetic studies showed that loss of Miro1 does not prevent Ca2+-dependent arresting of mitochondria motility, thus raising
questions as to whether Miro1 is the only essential factor for Ca^{2+}-triggered mitochondrial immobilization and whether Miro2 or other anchoring proteins can immobilize mitochondria by sensing Ca^{2+} signals. We previously tested this possibility and found that activating the Miro-Ca^{2+} pathway failed to arrest axonal mitochondria in Snph KO hippocampal neurons\(^6\). These findings highlight SNPH as one of essential players in recruiting axonal mitochondria to active synapses. Our current study reveals an energy-sensitive mechanism underlying mitochondrial anchoring at presynaptic terminals through the myo6–SNPH interplay. Unlike the Miro1–Ca^{2+} model, the cross-talk between energy sensing and mitochondrial anchoring enables neurons to recruit and capture presynaptic mitochondria in response to local energy stress during sustained synaptic activity. Prolonged synaptic activity consumes large amounts of ATP, and thus induces local energy stress that activates AMPK–PAK signalling and facilitates myo6-driven mitochondrial recruitment and SNPH-mediated anchoring. Ca^{2+} elevation arrests mitochondria on axonal MTs through Miro1–Ca^{2+} sensing, which may be a prerequisite step for recruiting mitochondria to presynaptic terminals where the dense actin filaments firmly restrain mitochondria.

Presynaptic energy deficits are defined as insufficient ATP supply and/or increased energy consumption. Sustained synaptic activity drives large ATP consumption resulting in an increased AMP/ATP ratio and AMPK activation, which upregulates glycolysis and mitochondrial respiration to maintain energy homeostasis. However, the majority of presynaptic terminals in CNS neurons lack anchored mitochondria\(^1\). Thus, recruiting presynaptic mitochondria is critical for the maintenance of lasting synaptic efficacy. We propose that AMPK signalling facilitates mitochondrial recruitment in parallel with the regulation of mitochondrial respiration to meet elevated metabolic needs at synapses. This is supported by findings that AMPK signalling is necessary for maintaining LTP during HFS in vivo\(^7\) and for regulating mitochondrial motility and replenishing ATP supply in distal regions during axonal growth\(^8,9\). Depleting SNPH abolishes AMPK-mediated phenotypes\(^10\), highlighting a critical role for SNPH in the AMPK signalling pathway.

We provided six lines of evidence to support our hypothesis that elevated energy consumption at presynaptic terminals activates AMPK signalling, which facilitates mitochondrial recruitment to rescue local energy deficits. First, activity-mediated presynaptic energy deficits induced the phosphorylation of AMPK-T172, a prerequisite for AMPK activation\(^11\). Second, presynaptic mitochondrial capture was facilitated by activating the AMPK signalling and abolished by inhibiting AMPK activity. Third, AMPK signalling regulates mitochondrial anchoring through the myo6–SNPH interplay.

While WT neurons exhibited robust presynaptic mitochondrial recruitment following activation of AMPK, this phenotype was abolished in neurons with Snph deletion, myo6 knockdown or disruption of actin filaments. Fourth, sustained synaptic activity triggered an AMPK–PAK signalling cascade through the phosphorylation of its downstream target, PAK\(^12\). Fifth, presynaptic mitochondrial recruitment was facilitated through myo6–SNPH phosphorylation at T405 by PAK signalling. Group I PAKs induce myo6 phosphorylation at T405 within the myo6 motor domain and myo6-T405 phosphorylation facilitates myo6 recruitment to actin-enriched regions\(^13,14\). This phosphorylation event was confirmed in our phospho-tag gels. Expressing phosphomimic myo6-T405E, but not phospho-dead myo6-T405A, increased presynaptic mitochondria capture. Furthermore, expressing PAK-CA, but not PAK3-KD, increased mitochondrial retention at presynaptic terminals. Altogether, our study reveals the cross-talk between energy sensing and mitochondrial anchoring that recruit presynaptic mitochondria by sensing local energy deficits.

Presynaptic mitochondria play a dual role in supporting synaptic transmission by buffering presynaptic Ca^{2+} and supplying ATP\(^15,16,27,72\). Ca^{2+} release from presynaptic mitochondria contributes to elevated residual calcium that induces short-term and post-tetanic potentiation at many types of synapses\(^46,70,71,74\). Disrupting the AMPK–PAK–myo6 axis triggers [Ca^{2+}]_{i} build-up at presynaptic terminals, thus suppressing synaptic depression during repetitive stimulations. Disrupting the AMPK–PAK–myo6 axis also triggers presynaptic energy deficits, thus slowing the recovery of synaptic depression after prolonged synaptic activity. Such energy deficits inhibit massive SV fusion and recycling induced by elevated presynaptic [Ca^{2+}]_{i}, thus limiting synaptic depression during repetitive trains. Our findings support this notion. First, presynaptic mitochondria play a crucial role in maintaining SV pool size. Second, presynaptic ATP is quickly depleted by HFS in a timescale of a few minutes. Therefore, impairing presynaptic mitochondrial positioning induces local energy deficits during trains of stimulation.

In summary, our study elucidates a mechanistic interplay between energy sensing and mitochondrial anchoring to fine-tune presynaptic mitochondrial positioning upon sensing activity-induced energy stress. This mechanistic cross-talk is critical for neurons to sustain prolonged synaptic efficacy. Impaired axonal energy metabolism and dysregulated AMPK signalling have been reported in major neurodegenerative diseases\(^48,75\). An ultrastructural examination revealed a fourfold reduction in presynaptic mitochondria in cortical regions from patients with Alzheimer’s disease (AD)\(^1\), which is consistent with our previous study demonstrating SNPH depletion in the late stages of an AD mouse model and brains obtained from AD patients\(^16\). Therefore, an impaired cross-talk between energy sensing and mitochondria anchoring may contribute to presynaptic energy deficits in AD brains, thus leading to synaptic dysfunction and cognition impairments. The fundamental insights revealed by our study conceptually advance the current knowledge of how neurons respond to metabolic energy stress and synaptic activity by adapting mitochondria trafficking and anchoring.

**Methods**

**Mouse lines and animal care.** The Snph\(^1/\) mouse line was generated by targeted gene replacement in embryonic stem cells as previously described\(^16\). The mice were maintained in the NINDS animal facility and housed in groups of 3–5 animals on a 12:12 h light/dark cycle (lights on at 6:00 a.m.) at 70 °F with 45% humidity and given ad libitum access to food and water. All animal procedures were performed according to National Institutes of Health (NIH) guidelines and were approved by the Animal Care and Use Committee of NINDS and the National Institute on Deafness and Other Communication Disorders.

**Primary neuron cultures.** For preparing WT and Snph\(^1/\) cortical neuron cultures, E18 embryonic mice of random sex were used. After dissection by papain ( Worthington), neurons were resuspended in plating medium (Neurobasal medium supplemented with 2% B-27, 0.5 mM GlutaMAX, 5 mM 2-mercaptoethanol (all Thermo Fisher Scientific), 10% FBS (HyClone) and 0.25 µg ml\(^{-1}\) insulin (Sigma–Aldrich)) and plated onto 12-mm coverslips (Deckgläser coated with poly-l-ornithine (Sigma–Aldrich; at a dilution of 1:3 in PBS) in a 24-well tissue culture plate. After 24 h of growing neurons in plating medium, half of the plating medium was replaced with the same amount of neuronal feeding medium (Neurobasal medium supplemented with 2% B-27, 0.5 mM GlutaMAX, and 5 mM 5-fluoro-2-deoxyuridine to inhibit glia proliferation). Neurons were fed every 3 d by aspirating half the medium and replacing it with the same amount of neuronal feeding medium. Neurons were transfected with various constructs at DIV7–9 using the calcium phosphate method and imaged at DIV14 with a Zeiss LSM 880 Airyscan confocal microscope.

**DNA constructs.** Lentiviral constructs encoding WT, truncated and point-mutated myo6, AMPK and PK3 were generated by PCR and subcloned into the pDONR221 vector by BP Clonase II to create Gateway entry clones (Thermo Fisher Scientific), which were then transferred to destination lentiviral vectors pHAGE-CMV-n-EF1G or pHAGE-CMV-n-HA-Flag by LR Clonase II (Thermo Fisher Scientific). EGF–myo6 (355–454) was generated by cloning truncated myo6 into pEGFP-C2. EGF–synapsin was generated by cloning the full-length synapsin sequence into pEGFP-C1. Syn-GaAT2 and Syn-GaAT3 were generated by cloning the full-length synapsin-encoding sequence into GoAteam2 and GoAteam3 (gifts from H. Imamura, Kyoto University). The following constructs were gifts from the laboratories: DsRed-mito (R. Youle, NINDS, NIH), GFP–myo6 (J. Kendrick-Jones, Medical Research Council Laboratory of Molecular Biology (MRC LMB) and F. Buss, University of Cambridge), AMPKAR-EV.
Live neuron imaging and analysis. Transfected neurons were transferred to prewarmed Hibernate E low fluorescence medium (BrainBits) supplemented with 2% B-27 and 0.5 mM GlutaMAX. 'Temperature was maintained at 37°C for 1 h, washed with PBS three times at RT for 5 min each, then incubated with Duolink PLA ligation solution at 37°C for 30 min, rewash with PBS three times at RT for 5 min each, then incubated with Duolink PLA amplification solution at 37°C for 100 min, washed with 1x PB buffer (0.2 M Tris, 0.1 M NaCl (pH 7.5)) two times at RT for 10 min each and PBS at RT for 5 min. To counterstain for a neuronal marker, neurons were post-fixed with 4% paraformaldehyde and 4% sucrose for 10 min at RT and processed for immunocytochemistry.

Labelling of F-actin. To label F-actin with phalloidin, cortical neurons were incubated with Alexa Fluor 594 phalloidin (Thermo Fisher Scientific) after immunocytochemistry as previously described. Cells were imaged with a Leica TCS SP8 (Leica Microsystems) in the 488–560 nm range using a 63× oil objective (NA 1.4). For dual-colour imaging, cells were imaged after incubation with Alexa Fluor 488 phalloidin (Thermo Fisher Scientific) (excitation: 488 nm, emission: 505–550 nm) and Alexa Fluor 568 phalloidin (Thermo Fisher Scientific) (excitation: 561 nm, emission: 566–610 nm).

Immunoblotting. Cortical neurons were infected with lentiviruses encoding scrambled or myo6-shRNA lentiviruses at DIV4–5. Cells were then collected at DIV12–14 and lysed with RIPA buffer supplemented with 0.1% Triton. Total protein levels were measured using the BCA protein assay (Thermo Fisher Scientific). Protein lysates were resolved on 8% to 12% Bis-Tris NuPAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% milk in TBS-T for 1 h and incubated with primary antibodies overnight at 4°C. Membranes were then incubated with HRP-conjugated secondary antibodies for 1 h. Immunoblotting was performed according to the manufacturer’s instructions. Western blot images were scanned and analysed using LUCIA (GE Healthcare) or a FACSAria III flow cytometer (BD Biosciences) and analysed using ImageJ.

Immunocytochemistry. Cortical neurons were fixed with 4% paraformaldehyde and 4% sucrose at RT for 20 min, washed three times with PBS for 5 min each, permeabilized in 0.15% Triton X-100 for 15 min and then incubated in blocking buffer (4% BSA, 0.1% Triton) for 1 h before washing with PBS. After washing with PBS three times at RT for 10 min each, samples were incubated with secondary fluorescent antibodies (Alexa 488, 546, 594 or 633 conjugated, Thermo Fisher Scientific) in blocking buffer for 1 h, washed with PBS, and mounted with Fluoro-Gel mounting medium (Electron Microscopy Sciences) before imaging.

Proximity ligation assay. The PLA was performed according to the manufacturer's instructions (Sigma-Aldrich). Briefly, transfected neurons were incubated with primary antibodies (SNPH–actin interaction: rabbit anti-SNPH, 1:250; and mouse anti-actin, 1:100, Abcam; SNPH–MT interaction: rabbit anti-SNPH, 1:100, Abcam; and mouse anti-β1-tubulin, 1:2,000, Sigma-Aldrich) diluted in Duolink antibody diluent at 4°C overnight, then washed three times with Buffer A (0.01 M Tris, 0.15 M NaCl and 0.05% Tween 20) at RT for 5 min each, incubated with Duolink KPL Probe A488 (4°C overnight), washed with PBS for 5 min each, then incubated in Duolink PLA ligation solution at 37°C for 30 min, rewash with PBS three times at RT for 5 min each, then incubated with Duolink PLA amplification solution at 37°C for 100 min, washed with 1x PB buffer (0.2 M Tris, 0.1 M NaCl (pH 7.5)) two times at RT for 10 min each and PBS at RT for 5 min. To counterstain for a neuronal marker, neurons were post-fixed with 4% paraformaldehyde and 4% sucrose for 10 min at RT and processed for immunocytochemistry.

Measurement of mitochondria membrane potential. To measure ΔΨm, cortical neurons were loaded with MitoTracker CMTMRos (100 nM, Thermo Fisher Scientific) for 30 min, and imaged with an inverted STED microscope. ΔΨm was assessed by the relative fluorescence intensity of MitoTracker Green imaging.

Immunoblotting. Cortical neurons were infected with lentiviruses encoding scrambled or myo6-shRNA lentiviruses at DIV4–5. Cells were then collected at DIV12–14 and lysed with RIPA buffer (0.01 M Tris, 0.15 M NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC buffer and 2 mM EDTA) with Complete protein inhibitor cocktail tablets (Roche). To assess the level of protein phosphorylation, cortical neurons treated with DMSO, PTX (100 μM, Sigma-Aldrich) or AICAR (1 mM, Millipore), or HEK293T cells transfected with WT and mutated myo6 and PAK3 constructs, were collected and lysed with RIPA buffer supplemented with 0.5 mM PMSF, protease inhibitor cocktail tablets and PhosSTOP (Roche). Equal amounts of proteins were loaded and analysed by 4–12% Bis-Tris NuPAGE (Thermo Fisher Scientific) or 7.5% Phos-tag gels (FUJIFILM Wako) and processed for immunoblot. Primary antibodies and their dilutions were as follows: rabbit anti-myo6 (1:1,000, Sigma-Aldrich), anti-SNPH (1:2,000), anti-Phospho-ACC-1 (579) (1:1,000, Cell Signalling), anti-ACC-1 (1:1,000, Cell Signalling), anti-Phospho-AMPK (Thr172) (1:1,000, Cell Signalling), anti-AMPK (1:1,000, Cell Signalling), anti-Phospho-PAK1/2/3 (1:1,000, Abcam), anti-PAK1/2/3 (1:1,000, Thermo Fisher) and anti-GAPDH (1:5,000, Millipore); mouse anti-Flag (1:2,000, Sigma-Aldrich), anti-Tubulin (1:5,000, Sigma-Aldrich) and anti-GFP (1:500, Santa Cruz). Secondary antibodies were used as follows: Mouse IgG HRP linked (1:5,000, Healthcare) and rabbit IgG HRP linked (1:2,500, GE Healthcare).

GST pull-down assay. GST-tagged proteins were expressed in Escherichia coli BL21. The BL21 cells were grown to an optical density at 600 nm (OD600) of 0.6; expressed protein was isolated using iodopropyl-b-D-thiogalactopyranoside to a final concentration of 1 mM at 25°C for 4–6 h. The bacterial pellets were resuspended in lysis buffer (10 mM HEPES, 0.5 M KCl, 0.5% Triton, 0.1 mM DTT, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and cOmplete protein inhibitor cocktail tablets). After sonication and centrifugation, the crude extract was purified using Glutathione-Sepharose 4 fast flow beads (GE Healthcare). Mouse brains were homogenized in binding buffer (25 mM HEPES, 10 mM NaCl, 1% NP-40, 0.1% SDS, 5% DMSO, 2 mM EDTA) and centrifuged for 20 min at 12,000 g at 4°C. The supernatant was carefully collected. GST beads coated with GST or SNPH and its truncated mutants were mixed with the brain homogenate or HEK293T cell supernatants and incubated for 3 h with gentle agitation. The beads were then extensively washed with 1x TBS supplemented with 0.1% Triton. Next, the beads were dissolved in LDS–PAGE sample buffer (Thermo Fisher Scientific) and heated at 75°C for 10 min. Proteins were resolved by Bis-Tris NuPAGE and processed for immunoblot analysis or Coomassie blue staining.

Co-immunoprecipitation. Mouse brains were homogenized in ELL buffer (50 mM HEPES, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM DTT, 10% glycerol and cOmplete protein inhibitor cocktail tablets). The crude homogenates were centrifuged at 12,000g for 4°C for 15 min. An anti-my6 antibody (10 μL, Sigma-Aldrich) or anti-SNPH antibody (10 μL, Abcam) was then added to the supernatants and incubated on a rotator at 4°C overnight. Protein A Sepharose
CL-4B (GE Healthcare) was added to each sample, and incubation continued for an additional 2 h followed by five washes with ELB buffer. The beads were dissolved in LDS–PAGE sample buffer supplemented with 10 mM DTT and heated at 75°C for 10 min. Proteins were resolved by Bis–Tris NuPAGE and processed for immunoblot analysis.

Actin spin-down assay. GST–SNAP1 (1–469) was purified by binding to Glutathione-Septarose 4 fast flow beads, eluted with 15 mM glutathione supplemented with 4 mM DTT and 2.5% 2-mercaptoethanol, dialysed in general actin buffer and ultra centrifuged at 150,000 g for 1 h at 4°C. The purified GST-tagged SNAP1 proteins were then used for the actin spin-down assay according to the manufacturer’s instructions (Cytokeleton). Briefly, purified GST–SNAP1 (1–469) and the positive control α-actinin (supplied in the kit) were each incubated with F-actin at RT for 30 min, followed by centrifugation at 150,000 g for 90 min at 24°C. The supernatants were collected, and pellets were resuspended in SDS–PAGE sample buffer. Both supernatant and pellet samples were resolved by SDS–PAGE and Coomassie blue staining.

Lentivirus production and infection. HEK293T cells maintained in DMEM (Thermo Fisher Scientific) containing 10% FBS (Hyclone) and 0.5 mM GlutaMAX. Virus-containing medium was harvested at 48 h and 72 h after transfection and then centrifuged at 100,000 g for 1 h for an additional 2 h followed by five washes with ELB buffer. The beads were CL-4B (GE Healthcare) was added to each sample, and incubation continued for an additional 2 h followed by five washes with ELB buffer. The beads were dissolved in LDS–PAGE sample buffer supplemented with 10 mM DTT and heated at 75°C for 10 min. Proteins were resolved by Bis–Tris NuPAGE and processed for immunoblot analysis.

Electrophysiology. For EPSC recordings, cultured cortical neurons grown on coverslips to DIV14–17 were transferred to a submersion chamber on an upright microscope (BX51WI, Olympus) and perfused with normal artificial cerebrospinal fluid (125 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM Na2HPO4, 10 mM d-glucose, 2 mM MgCl2 and 2 mM CaCl2 bubbled with 95% O2 and 5% CO2). For measuring baseline EPSC amplitude, the internal solution was composed of 130 mM K gluconate, 6 mM NaCl, 20 mM HEPES, 0.2 mM EGTA, 1 mM MgCl2, 2 mM MgATP, 0.3 mM Na2GTP (pH 7.2–7.4 with KOH), 285–300 mM Osm) for measuring synaptic transmission recovery, the internal solution was composed of 46.5 mM K gluconate, 7.5 mM KCl, 9 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 0.3 mM Na2GTP (pH 7.25 with KOH, 295 mM osm). EPSCs were recorded at least 5 min later, when they appeared steadily larger in size. RU360 (10μM), AICAR (1 μM, Millipore) and CC (10μM, Millipore) perfusion was achieved by using a circulating pump. Series resistance was monitored and not compensated, and cells in which series resistance varied by 20% during a recording session were discarded. Unless otherwise indicated, all chemicals were obtained from Sigma- Aldrich.

Statistics and reproducibility. All quantifications were performed unblinded. Statistical parameters including the definitions and exact value of n (for example, number of experiments or replications, axons, organelles and neurons), deviations and P values are reported in the figures and corresponding legends. Statistical analysis was carried out using Prism 7 (GraphPad). Comparisons between two groups were performed by unpaired Student’s t-test (sample size ≥ 30), a Mann–Whitney test (sample size <30) or a Wilcoxon matched-pairs rank test (for paired samples). Comparisons among three or more groups were performed by one-way analysis of variance (ANOVA) with post hoc testing by Dunnett’s multiple-comparisons test (sample size ≥30, compared to control group) or Tukey’s multiple-comparisons test (sample size ≥30, compared to every other group), Kruskal–Wallis test (sample size <30) with Dunn’s multiple-comparisons post hoc test, or two-way ANOVA with Tukey’s multiple-comparisons post hoc test where indicated. Comparisons of biochemical studies were performed by Fisher’s least significant difference test, or one-way ANOVA with Dunnett’s multiple-comparisons post hoc test (compared to control group) or Tukey’s multiple-comparisons test (compared to every other group) were expressed as the mean ± s.e.m. Differences were considered significant with a P value < 0.05. All experiments were repeated at least three times independently.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Competing interests
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Extended Data Fig. 1 | Activation of AMPK Signaling by PTX-induced Synaptic Activity.  **a–c.** Representative immunoblots (a) and quantitative analyses (b, c) showing synaptic activity-induced AMPK signaling in a time-dependent manner. Cortical neurons at DIV14 were treated with PTX (100 μM) for 0, 30, 60 or 120 min, followed by harvesting cell lysates for analysis. Equal amounts (5 μg) of cell lysates were sequentially immunoblotted with antibodies against AMPKα-Thr172 (pAMPKα) and ACC-1-Ser79 (pACC-1), total ACC-1 and AMPKα, and GAPDH (a). The relative intensity of pACC-1 or pAMPKα was calibrated with total ACC-1 or AMPKα respectively, and normalized to the time point at 0 min (b, c). **d, e.** Validation of AMPK WT and kinase-dead (KD) constructs. Cortical neurons were infected with lentiviruses encoding Flag, Flag-AMPK-WT or Flag-AMPK-KD at DIV4-5, treated with PTX (100 μM for 2 hr) at DIV14, followed by harvesting cell lysates for analysis. Equal amounts (5 μg) of cell lysates were sequentially immunoblotted with antibodies against Flag, AMPK, pACC-1, total ACC-1 and GAPDH. The relative intensity of pACC-1 was calibrated with total ACC-1 levels, and normalized to Flag control. Note that expressing AMPK-WT (P = 0.028), but not AMPK-KD (P = 0.7636), activated AMPK signaling. Data were collected from n = 3 (b, c) or n = 4 (e) independent experiments, expressed as mean ± SEM, and analysed by one-way ANOVA with post hoc testing by Dunnett’s multiple comparisons test (b, c) or by two-sided Fisher’s LSD test (e).
Extended Data Fig. 2 | PTX and AICAR Do Not Affect Presynaptic Density and Mitochondrial Integrity. **a, b,** Representative images (a) and quantitative analyses (b) showing similar presynaptic density along axons following synaptic activation with PTX or AMPK activation with AICAR. Cortical neurons at DIV14 were treated with PTX (100 μM) or AICAR (1 mM) for 2 hr, followed by co-immunostaining of synaptophysin (SYP) and βIII-tubulin.

**c, d,** Representative images (c) and quantitative analyses (d) showing unaffected mitochondrial integrity upon synaptic activation with PTX or AMPK activation with AICAR. Cortical neurons at DIV14 were treated with PTX (100 μM) or AICAR (1 mM) for 2 hr, followed by co-labeling of total mitochondria with mitoTracker Green (20 nM for 20 min) and mitochondria membrane potential with TMRE (50 nM for 20 min). Mitochondrial integrity was assessed by integrated intensity ratio of TMRE (red) vs mitoTracker (green) within individual mitochondria and normalized to the control group. Data were quantified from the total number of neurons indicated within bars, expressed as mean ± SEM, and analyzed by one-way ANOVA with post hoc testing by Dunnett’s multiple comparisons test. Scale bars: 10 μm.
Extended Data Fig. 3 | Myo6 Knockdown Does Not Impact Density of Axonal Mitochondria and Presynaptic Terminals. a, The alignment of mouse and human myo6 sequences that are targeted by mouse myo6-shRNA. Sequence differences are marked by red color. b, Three immunoblot repeats showing effective myo6 knockdown by myo6-shRNA in mouse cortical neurons. Neurons were infected with lentiviruses encoding scramble (scr) or myo6-shRNA (myo), or combined with co-expression of human myo6 (h-myo) at DIV5 and analyzed by immunoblotting at DIV14. Note that myo6-shRNA effectively depletes myo6 expression in mouse neurons while human myo6 (h-myo) is resistant to mouse myo6-shRNA. c, d, Representative images (c) and quantitative analysis (d) showing effective knockdown of myo6 in mouse cortical neurons. Neurons were infected with lentiviruses encoding scr- or myo6-shRNA at DIV5, followed by co-immunostaining of myo6 and βIII-tubulin at DIV14. e, f, Quantitative analyses revealing no detectable effect of myo6 knockdown on the average density and size of axonal mitochondria (e) and density of presynaptic terminals (f). Cortical neurons at DIV4 were infected lentiviruses encoding with scr-shRNA or myo6-shRNA, followed by co-immunostaining of SV2 and TOM20 at DIV14. The number (per 100 μm) and size (μm²) of axonal mitochondria and the number of presynaptic boutons (per 100 μm) were quantified. g, h, Representative images (g) and quantitative analysis (h) showing no additive reduction in presynaptic mitochondria by myo6 knockdown in snph KO neurons. Neurons were infected with lentiviruses encoding scr-shRNA or myo6-shRNA at DIV4-5, followed by co-immunostaining at DIV14. The colocalized pixels of red (TOM20) and green (SV2) channels are highlighted in the white/black images. Data were quantified from the total number of neurons indicated within bars from three experiments. Data are expressed as mean ± SEM and analyzed by two-sided unpaired Student’s t-test. Scale bars: 20 μm (c) and 10 μm (g).
Extended Data Fig. 4  |  LatB Disrupts F-Actin without Impacting Density of Presynaptic Boutons and Axonal Mitochondria.  

**a.** Disruption of F-actin with LatB. Cortical neurons were treated with DMSO or LatB (2 μM) for 3 hr at DIV14, followed by phalloidin labeling of F-actin and immunostaining of MAP2.

**b.** STED images showing reduced F-actin in axons and at presynaptic terminals upon LatB treatment. Neurons at DIV14 were treated with DMSO or LatB (2 μM) for 3 hr, followed by SiR-actin labeling and immunostaining.

**c, d.** Images (c) and analyses (d) showing unchanged mitochondrial integrity following LatB treatment. Neurons at DIV14 were treated with DMSO or LatB (2 μM) for 3 hr, or CCCP (10 μM) for 30 min, followed by labeling with CMTMRos (100 nM) for 20 min and immunostaining of TOM20. Mitochondrial integrity was assessed by integrated intensity ratio of CMTMRos vs TOM20 and normalized to DMSO.

**e–g.** LatB treatment does not affect presynaptic density in live (e) and fixed neurons (f) and the density and size of axonal mitochondria (g). For live imaging, neurons were co-transfected with GFP-synapsin and DsRed-mito at DIV7-8, and dual-channel imaging was performed at DIV14. For fixed neuron imaging (f, g), neurons at DIV14 were treated with DMSO or LatB (2 μM) for 3 hr, followed by co-immunostaining. The average number of presynaptic boutons per 100 μm was quantified from the total number of neurons indicated in bars (d) or in parentheses (e–g) where n represents number of neurons and v stands for number of presynaptic terminals (f) or mitochondria (g) from three experiments. Data are presented as box plots (min, max, median, 25th and 75th percentiles) with dots as individual values (e) or bar graphs of mean ± SEM (d, f, g), and analyzed by two-sided unpaired Student’s t-test (e) or one-way ANOVA followed by Dunnett’s test (d, f, g). Scale bars: 10 μm (a, c) or 5 μm (b).
Extended Data Fig. 5 | SNP and F-actin Are Essential for Capturing Presynaptic Mitochondria. a–c, Representative kymographs (a, b) and quantitative analyses (c) showing axonal mitochondria moving out of presynaptic terminals upon F-actin disruption in live neurons. Cortical neurons were co-transfected with GFP-synapsin and DsRed-Mito at DIV7-8, followed by dual-channel live imaging in distal axons at DIV14 (450 frames with 10-sec intervals for 75 min). After 90 frames (15 min) of live imaging, LatB was added to the chamber (10 μM) and the same axons were continuously time-lapse imaged for an additional 60 min. White arrows (a) point to mitochondria being released from presynaptic terminals; black arrows (b) point to presynaptic terminals capturing mitochondria under DMSO condition (b, left panels) or releasing mitochondria upon LatB treatment (b, right panels). The percentage of presynaptic terminals capturing mitochondria was quantified before and after LatB treatment. Note that disrupting F-actin reduces the percentage of presynaptic terminals capturing mitochondria. d, e, Electron micrographs (d) and quantitative analysis (e) showing reduced mitochondrial positioning at AZs upon F-actin disruption or snph deletion. WT or snph KO cortical neurons at DIV14 were treated with DMSO or LatB (2 μM) for 3 hr, followed by TEM. Yellow area shows AZs with SV clustering; green area shows mitochondria. Data were quantified from the total number of neurons (c) or AZs (e) per condition indicated in parentheses (c, e). Data are presented as box plots (min, max, median, 25th and 75th percentiles) with dots as individual values (c) or as bar graphs of mean percentage (e), and analyzed by two-sided Wilcoxon matched-pairs rank test (c). Scale bars: 10 μm (a, b) or 500 nm (d).
Extended Data Fig. 6 | Deletion of SNPH or Knockdown of Myo6 Does Not Affect Axonal Cytoskeleton. Representative STED super-resolution images showed that deleting *snph* (**a**) or knocking down *myo6* (**b**) does not affect global structures of axonal cytoskeleton. WT or *snph* KO cortical neurons at DIV14 were immunostained by antibody against βIII-tubulin, followed by SiR-actin labeling of F-actin (**a**), or were infected with lentiviruses encoding scr-shRNA or myo6-shRNA at DIV5 and immunostained with βIII-tubulin, followed by SiR-actin labeling at DIV14 (**b**). Scale bars: 5 μm.
Extended Data Fig. 7 | Modulating PAK-myo6 Axis Does Not Affect Density of Presynaptic Terminals or Mitochondrial Integrity. Representative images (a, c) and quantitative analyses (b, d) showing no significant effect on the density of presynaptic terminals (a, b) and mitochondrial integrity (c, d) upon activation of PAK-myo6 axis by expressing PAK-CA or myo6-T405E. Cortical neurons at DIV7 were infected with lentiviruses encoding PAK3-CA or myo6-T405E, followed by co-immunostaining of synaptophysin (SYP) and βIII-tubulin (a, b) or by labeling mitochondria membrane potential with TMRE (50 nM for 20 min) along with total mitochondrial marker mitoTracker Green (20 nM for 20 min) at DIV14 (c, d). Mitochondrial integrity was assessed by the integrated intensity ratio of TMRE (red) vs mitoTracker (green) within individual mitochondria and normalized to the control group. Data were quantified from the total number of neurons indicated within bars, expressed as mean ± SEM, and analyzed by one-way ANOVA with post hoc testing by Dunnett’s multiple comparisons test. Scale bars: 10 μm.
Extended Data Fig. 8 | Modulating the AMPK-PAK-my6 Axis Has No Impact on Basal Synaptic Transmission. Representative EPSC curves (a, c, e, g) and mean amplitudes (b, d, f, h) at 0.05 Hz showed no significant differences of basal synaptic transmission following modulation of the AMPK-PAK-my6 pathway. Dual whole-cell patch-clamp recording was performed at DIV14-18 on paired cortical neurons treated with AICAR (1 mM) or CC (10 μM) for 2 hr (a, b); or presynaptic neurons expressing GFP, GFP-tagged myo6 or myo6-CBD (c, d); myo6-T405A or myo6-T405E (e, f); PAK3-KD or PAK3-CA (g, h). Data were quantified from the total pairs of neurons indicated in bars, represented as mean ± SEM, and analyzed by one-way ANOVA (b: F2, 72 = 1.764, P = 0.178; d: F2, 104 = 0.060, P = 0.941) with post hoc testing by Dunnett’s multiple comparisons test or two-sided unpaired Student’s t-test (f: P = 0.1162; h: P = 0.7052).
Extended Data Fig. 9 | Disrupting SNPH-mediated Anchoring Abolishes the Role of PAK-myo6 in Maintaining Synaptic Transmission. a, b. Normalized EPSC amplitudes plotted against stimulus number showing that activation of PAK-myo6 pathway facilitates short-term synaptic depression in WT (a), but not in snph KO neurons (b). Dual whole-cell patch-clamp recordings were performed on paired WT or snph KO cortical neurons infected with lentiviruses encoding GFP, GFP-tagged myo6, myo6-T405E. High-frequency (50 Hz, 200 msec) pulse trains were delivered to presynaptic neurons. Two-way ANOVA revealed a main effect of gene manipulation in WT neurons (F2, 61 = 5.319, P = 0.0074) (a), but no main effect in snph KO neurons (F2, 73 = 0.08927, P = 0.9147) (b). Data were quantified from total pairs of neurons indicated in parentheses and expressed as mean ± SEM. (c) Activation of PAK-myo6 signaling axis in snph KO neurons failed to accelerate synaptic recovery after high-frequency stimulation. snph KO cortical neurons were infected with lentiviruses encoding GFP, GFP-tagged myo6-WT, myo6-T405E, or PAK3-CA at DIV5, following by dual whole-cell patch clamp recording at DIV14-18. Recording configuration (total 132 sec duration) consists of a 2-sec 100 Hz train, a 10-sec recovery phase of four stimuli, and a 120-sec rest phase. The normalized EPSCs amplitude is plotted for the first of each HFS and for each of the recovery EPSCs for each condition. Two-way ANOVA revealed no main effect of gene manipulation in snph KO neurons (F3, 57 = 0.5787, P = 0.6314). Data were quantified from total pairs of neurons indicated in parentheses and expressed as mean ± SEM.
Extended Data Fig. 10 | Presynaptic Mitochondria Contribute to the Recovery of Synaptic Efficacy During Prolonged Synaptic Activity. 

**a**, Normalized EPSC amplitudes showing mitochondrial capacity of calcium clearance contributes to synaptic recovery after high-frequency stimulation (HFS). Cortical neurons were used for dual whole-cell patch clamp recording at DIV14-18. The mitochondrial calcium uniporter inhibitor RU360 (10 μM) was added to the intracellular recording solution to block mitochondrial calcium buffering capacity. Recording configuration (total 132 sec duration) consisted of a 2-sec 100 Hz train, a 10-sec recovery phase of four stimuli, and a 120-sec rest phase. The normalized EPSC amplitude was plotted for the first of each HFS and for each of the recovery EPSCs under each condition. Note that the application of RU360 induced a slower synaptic recovery after HFS. Two-way ANOVA revealed a main effect of RU360 treatment (F1, 41 = 10.64, P = 0.0022) and a significant interaction between RU360 treatment and stimuli (F14, 574 = 9.457, P < 0.001).

**b**, Normalized EPSC amplitudes showing that myo6-SNP-captured presynaptic mitochondria significantly contribute to synaptic recovery after HFS even their calcium buffering capacity is blocked. Cortical neurons were infected with lentiviruses encoding GFP, GFP-tagged myo6 and myo6-T405E, followed by dual whole-cell patch clamp recording at DIV14-18. 10 μM RU360 was applied to intracellular recording solution. The same recording configuration was used as described above. Note that enhanced presynaptic mitochondria anchoring through activating myo6-PAK pathway accelerates synaptic recovery after HFS even when calcium buffering capacity is blocked. Two-way ANOVA revealed a main effect of gene manipulation (F2, 60 = 3.506, P = 0.0363) and a significant interaction between gene manipulation and stimuli (F28, 840 = 2.074, P = 0.001). Data were quantified from the total pairs of neurons indicated in parentheses, and presented as mean ± SEM.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Confocal images were acquired using Zeiss 2.1 SP3 (Zeiss). STED image acquisition was done by using build-in Leica Application Suite X (LASX) [3.15.16308] (Leica Microsystems). Acquired STED images were deconvolved using a Leica build-in Huygens Professional 17.10 [Scientific Volume Imaging]. Electrophysiology data were acquired using pCLAMP10.2 (Molecular Devices).

Data analysis
Statistical analysis was done using GraphPad Prism 7 [GraphPad Software]. Image quantification was using ImageJ [2.0.0-rc-54/1.51h] [NIH].

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data supporting the plots within this paper are available from the corresponding author upon reasonable request. Source data are provided with this paper.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

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

**Sample size**
Sample size choice was based on previous study (ref. -5, 21, 27, 23), not predetermined by a statistical method. Images are representative of n ≥ 10 cells as indicated from each figure legend from at least three repeats. All immunoblots are representative of at least three experiments. A minimal sample size of n = 3 biological replicates was used for immunoblots quantification.

**Data exclusions**
No data was excluded.

**Replication**
All quantifications were performed unblinded. Statistical parameters including the definitions and exact value of n (e.g., number of experiments or replications, number of axons, number of organelles, number of neurons, etc), deviations and p values are reported in the figures and corresponding figure legends. Statistical analysis was carried out using Prism 7 (Graphpad Software). For imaging studies, comparisons between two groups were performed by an unpaired t test (sample size ≥ 30) or a Mann-Whitney test (sample size < 30). Comparisons between three or more groups were performed by one-way analysis of variance (ANOVA) [sample size ≥ 30] or a Kruskal-Wallis multiple comparisons test (sample size < 30). Two-way ANOVA is performed as indicated to evaluate simultaneously the effect of two independent variables on a response variable. Data are expressed as mean ± SEM. Differences were considered significant with p<0.05.

**Randomization**
We used age and gender matched animals for WT and snph+/− brain homogenates. WT and snph+/− primary neuronal culture were performed from litter-mate animals for each experiment.

**Blinding**
Data collection and analysis was not performed blind. Blinding is not applicable because the investigator who set up the experiment is the same person doing analyses.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | n/a |
| □ | □ |
| Antibodies | Involved in the study |
| Eukaryotic cell lines | | ChIP-seq |
| Palaeontology | | Flow cytometry |
| Animals and other organisms | | MRI-based neuroimaging |
| Human research participants | | |
| Clinical data | | |

Antibodies

- Mouse anti-SV2 (DSHB, Cat#SV2); Rabbit anti-TOM20 (Santa Cruz, Cat# sc-1616); Rabbit anti-Myo6 (Sigma-Aldrich, Cat# M5187); Rabbit anti-SNPH (ref.-21); Rabbit anti-SNPH (Abcam, Cat#ab152605); Rabbit anti-synaptophysin (Santa Cruz, Cat# sc-9116); Mouse anti-Flag (Sigma-Aldrich, Cat# F1804); Mouse anti-β-tubulin (Sigma-Aldrich, Cat# T8578); Mouse anti-MAP2 (BD Biosciences, Cat# M56320); Mouse anti-cytochrome c (BD Biosciences, Cat# 564320); Mouse anti-Myo6 (Santa Cruz, Cat# sc-393558); Mouse anti-GFP (Santa Cruz, Cat# sc-9996); Rabbit anti-Phospho-ACC (Ser79) (Cell Signaling, Cat# 11818); Rabbit anti-ACC (Cell Signaling, Cat# 3676); Rabbit anti-Phospho-AMPA (Thr172) (Cell Signaling, Cat# 50081); Rabbit anti-AMPK (Cell Signaling, Cat# 5831); Rabbit anti-Phospho-PAK1/2/3 (Abcam, Cat# ab40795); Rabbit anti-GAPDH (Millipore, Cat# CB1001); Mouse anti-Actin (Abcam, Cat# ab8226); Mouse IgG HRP-linked (GE Healthcare, Cat# NA931); Rabbit IgG HRP-linked (GE Healthcare, Cat# NA934); Goat anti-mouse, Alexa 488 Conjugate (Thermo Fisher Scientific, Cat# 11017); Donkey anti-rabbit, Alexa 488 Conjugate (Thermo Fisher Scientific, Cat# 21206); Goat anti-mouse, Alexa 546 Conjugate (Thermo Fisher Scientific, Cat# 11038); Goat anti-rabbit, Alexa 546 Conjugate (Thermo Fisher Scientific, Cat# 11035); Goat anti-rabbit, Alexa 594 Conjugate (Thermo Fisher Scientific, Cat# 11037); Goat anti-mouse, Alexa 633 Conjugate (Thermo Fisher Scientific, Cat# 21052); |

Validation

All antibodies were previously published, and for western blots were validated by the manufacturer we purchased from.
Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | HEK293T from ATCC, Cat.#(CRL-3216) |
|---------------------|-----------------------------------|
| Authentication      | HEK293T cell line was not authenticated. |
| Mycoplasma contamination | All cell lines tested were negative for mycoplasma contamination. |
Commonly misidentified lines (See kLAC register) | No misidentified cell lines used in the study.

Animals and other organisms

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

| Laboratory animals | Used for the experiments were embryonic day 18 (E18) embryos from female mice. Mouse strain used in the study were C57BL/6J (Charles River) (Strain Code: 027) and snip-/ mice line was generated by targeted gene replacement in embryonic stem cells as previously described (ref. 21). All mice were housed in groups of 3-5 animals on a 12/12-h light/dark cycle (lights on at 6:00 a.m.) at 70°F with 45% humidity, and given ad libitum access to food and water. |
| Wild animals       | No wild animals were used in this study. |
| Field-collected samples | The study does not involve field-collected samples. |
| Ethics oversight   | All animal procedures were performed according to National Institutes of Health (NIH) guidelines and were approved by the Animal Care and Use Committee of NINDS/National Institute on Deafness and Other Communication Disorders (NIDCD). |

Note that full information on the approval of the study protocol must also be provided in the manuscript.