Control of synaptic vesicle endocytosis by an extracellular signalling molecule

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Signalling cascades control multiple aspects of presynaptic function. Synaptic vesicle endocytosis was assumed to be exempt from modulation, due to its essential role maintaining synaptic vesicle supply and thus neurotransmission. Here we show that brain-derived neurotrophic factor arrests the rephosphorylation of the endocytosis enzyme dynamin I via an inhibition of glycogen synthase kinase 3. This event results in a selective inhibition of activity-dependent bulk endocytosis during high-intensity firing. Furthermore, the continued presence of brain-derived neurotrophic factor alleviates the rundown of neurotransmission during high activity. Thus, synaptic strength can be modulated by extracellular signalling molecules via a direct inhibition of a synaptic vesicle endocytosis mode.
Neurotransmitter release from central nerve terminals is modulated by multiple signalling cascades activated by extracellular ligands. This process facilitates the fine control of synaptic output by either inhibitory/excitatory neurotransmission or retrograde neurotransmitters. In contrast, synaptic vesicle (SV) endocytosis has not been demonstrated to be modulated by extracellular signalling cascades, presumably due to its obligatory role in maintaining the available stock of SVs inside nerve terminals.

SVs are retrieved from the nerve terminal plasma membrane after exocytosis by either clathrin-mediated endocytosis (CME) or activity-dependent bulk endocytosis (ADBE). CME is the dominant SV retrieval mode during low intensity stimulation and retrieves single SVs de novo from the plasma membrane. In contrast, ADBE is triggered during high-intensity stimulation and forms endosomes directly from large plasma membrane invaginations. SVs then bud from these endosomes in a clathrin- and adaptor protein-dependent manner.

ADBE is tightly coupled to neuronal activity via the dephosphorylation of the large GTPase, dynamin I at two sites (Ser-774 and Ser-778) by the calcium-dependent phosphatase calcineurin. After stimulation terminates, dynamin I is phosphorylated, firstly on Ser-778 by the constitutively active enzyme cyclin-dependent kinase 5 (cdk5), an event which permits phosphorylation of Ser-774 by glycogen synthase kinase 3 (GSK3). The repolymerization of dynamin I by either cdk5 or GSK3 is essential for the triggering of ADBE by subsequent stimuli.

Brain-derived neurotrophic factor (BDNF) has established roles in the long-term control of neuronal migration, survival and development. In addition to its trophic actions, acute exposure to BDNF has both presynaptic and postsynaptic effects on neurotransmission. BDNF signals through the TrkB receptor to activate a number of intracellular signalling cascades, such as the mitogen-activated protein kinase (MAPK), the phospholipase Cγ and phosphatidylinositol 3-kinase (PI3K) pathways. Activation of the PI3K pathway results in a downstream inhibition of GSK3. BDNF selectively inhibits ADBE via an inhibition of dynamin I phosphorylation.

We next examined whether inhibition of PI3K could reverse the observed effects of BDNF on GSK3-dependent dynamin I Ser-774 phosphorylation. This was the case, with application of LY294002 preventing the BDNF-mediated inhibition of dynamin I Ser-774 phosphorylation (Fig. 2d), while having no effect on cdk5-dependent Ser-778 phosphorylation (Fig. 2f). Thus, GSK3-dependent phosphorylation of Ser-774 is negatively regulated by a PI3K/PDK1/Akt signalling cascade that is triggered by BDNF.

**Results**

**BDNF inhibits dynamin I phosphorylation on Ser-774.** The phosphorylation of Ser-774 on dynamin I by GSK3 is essential for the triggering of multiple cycles of ADBE. This suggests that ADBE should be negatively regulated by signalling cascades that inhibit GSK3 activity, as blocking dynamin I phosphorylation arrests this endocytosis mode. One potential signalling molecule that could inhibit ADBE is BDNF, as it can inhibit GSK3 via activation of a PI3K-dependent cascade (Fig. 1a). To test this hypothesis, we first confirmed that BDNF could control GSK3 activity in our primary neuronal culture system, cerebellar granule neurons (CGNs). GSK3 activity can be reported by the extent of phosphorylation of Ser-9 on the enzyme using phosphorylation-specific antibodies. In CGNs, Akt is activated by high-frequency stimulation and transiently phosphorylates GSK3 on Ser-9 (ref. 14). This effect was replicated in this study, with strong stimulation evoking a transient phosphorylation of both Akt and GSK3 which returned to resting levels on repolarization of the cultures (Fig. 1c,e). Application of BDNF (100 ng ml⁻¹) resulted in a hyperphosphorylation of both Akt and GSK3 in all experimental conditions (Fig. 1c,e), indicating BDNF triggers an intracellular signalling cascade that phosphorylates both Akt and GSK3.

We next determined which intracellular signalling cascade BDNF was operating through to control GSK3 activity. We first investigated the potential role of TrkB receptors as they are highly expressed in CGNs. Application of the tyrosine kinase antagonist K252a inhibited BDNF-induced phosphorylation of both Akt and GSK3, indicating a TrkB-specific effect (Supplementary Fig. S1). TrkB receptors signal via multiple intracellular signalling cascades, one of which utilizes PI3K.

In this cascade production of PI(3,4,5)P3 by PI3K activates phosphoinositide-dependent kinase 1 (PDK1) to phosphorylate and activate Akt. To determine whether BDNF signals via PI3K, the selective antagonist LY294002 (10 μM) was applied to CGNs. LY294002 abolished BDNF-induced hyperphosphorylation of both Akt and GSK3 (Fig. 1d,f) indicating BDNF exerts its effect via a PI3K-dependent cascade.

Phosphorylation of GSK3 inhibits its activity, therefore we next assessed whether BDNF could control dynamin I Ser-774 rephosphorylation, an event mediated by this enzyme (Fig. 2a). In the absence of BDNF, Ser-774 was dephosphorylated on depolarization, and then repolymerized on removal of the stimulus (Fig. 2c). This repolymerization event was abolished in the presence of BDNF (Fig. 2c). Importantly, BDNF had no effect on the repolymerization of Ser-778 on dynamin I (Fig. 2e), an event mediated by cdk5 (ref. 9). Thus, BDNF selectively inhibits the repolymerization of Ser-774 on dynamin I via inhibition of GSK3.

We next examined whether inhibition of PI3K could reverse the observed effects of BDNF on GSK3-dependent dynamin I Ser-774 repolymerization. This was the case, with application of LY294002 preventing the BDNF-mediated inhibition of dynamin I Ser-774 repolymerization (Fig. 2d), while having no effect on cdk5-dependent Ser-778 repolymerization (Fig. 2f). Thus, GSK3-dependent phosphorylation of Ser-774 is negatively regulated by a PI3K/PDK1/Akt signalling cascade that is triggered by BDNF.

**BDNF selectively inhibits ADBE.** GSK3-dependent Ser-774 repolymerization controls ADBE, therefore inhibition of this event by BDNF has implications for presynaptic function during elevated neuronal activity. Therefore, we next determined whether BDNF could negatively control ADBE. First, we examined the uptake of large (40 kDa) fluorescent dextrans, as they specifically report fluid phase uptake via ADBE due to size exclusion from SVs. Acute application of BDNF had no effect on dextran uptake evoked by a train of high-frequency action potentials (80 Hz, 10 s, Fig. 3a). However, inhibition of dynamin I Ser-774 repolymerization will not be apparent under these conditions, as dynamin I is highly phosphorylated at rest and dephosphorylated on stimulation (Fig. 2c). To reveal a potential regulatory effect of BDNF on ADBE, cultures were challenged with a priming stimulus to dephosphorylate dynamin I, rested in the continued presence of BDNF (to inhibit dynamin I repolymerization) and then dextran uptake was assessed during a second stimulation (Fig. 3b). Under these conditions, BDNF produced a robust inhibition of dextran uptake, which was prevented by the PI3K antagonist LY294002 (Fig. 3b). This inhibition was occluded by the GSK3 antagonist CT99021 (Fig. 3b) indicating BDNF was mediating its effect via this enzyme and almost certainly via inhibition of Ser-774 repolymerization. A BDNF-dependent inhibition of ADBE was also observed in primary hippocampal cultures, indicating its effect was not limited to cerebellar neurons (Supplementary Fig. S2).
To confirm that Ser-774 on dynamin I was the principal target of BDNF, we repeated the dextran uptake experiments in neurons expressing either wild-type dynamin I or a phospho-null version mutated at Ser-774. Neurons expressing the S774A mutant displayed a large inhibition of dextran uptake at S1 compared with wild-type, as previously shown (Fig. 3c). The S774A mutant inhibited dextran uptake at S2 to an almost identical extent, with the level of inhibition equal to wild-type expressing neurons in the presence of BDNF (Fig. 3d). Importantly, BDNF did not reduce dextran uptake further in S774A-expressing neurons (Fig. 3d) providing compelling evidence that BDNF inhibits ADBE via arrest of dynamin I Ser-774 phosphorylation, but only after a prior priming stimulus.

Figure 1 | BDNF inhibits GSK3 activity via a PI3K-dependent cascade. (a) Scheme illustrating that BDNF would activate a signalling cascade, which would phosphorylate and inactivate GSK3. (b) CGNs were placed in incubation medium for 10 min before stimulation with 50 mM KCl (1 min). Following stimulation CGNs were repolarized for 10 min. Samples were prepared from cultures before stimulation (basal, B), directly after KCl stimulation (K) or after 10 min repolarization (R) as indicated by arrowheads. BDNF (100 ng ml$^{-1}$) or LY294002 (LY, 10 µM) were present throughout the experiment where indicated. Lysates were separated by SDS–PAGE and probed for either (c, d) phospho-Ser-473 on Akt (PAkt Ser473) or (e, f) phospho-Ser-9 on GSK3 (PGSK3 Ser-9) on immunoblots. Quantitative analysis is shown in the graphs in (c–f). These graphs display the extent of phosphorylation of either Ser-473 on Akt (c, d) or Ser-9 on GSK3 (e, f). All values were normalized to the amount of synaptophysin (SYP) as a loading control, and expressed as a percentage of Basal control ± s.e.m. (c) n = 7, (d) n = 6, (e) n = 7, (f) n = 6; one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001 compared with basal control unless indicated.
To confirm the negative control of ADBE by BDNF, morphological studies were performed examining uptake of the fluid phase marker horse radish peroxidase (HRP). Triggering of ADBE results in the appearance of HRP-labelled endosomes, whereas CME generates HRP-labelled SVs (Fig. 4b-e). Acute application of BDNF had no effect on the number of HRP-labelled endosomes generated by a strong stimulation (Fig. 4f). However, when BDNF was present during and after a priming depolarization (S1) the number of HRP-labelled endosomes generated by a second stimulus (S2) was significantly reduced.

Figure 2 | BDNF inhibits dynamin I rephosphorylation on Ser-774 via a PI3K-dependent cascade. (a) Scheme illustrating that cdk5 phosphorylates Ser-778 on dynamin I (DynI), allowing GSK3 to phosphorylate Ser-774. Our hypothesis was that BDNF would activate Akt to phosphorylate and inactivate GSK3, thus inhibiting its ability to rephosphorylate Ser-774 on DynI. (b) CGNs were placed in incubation medium for 10 min before stimulation with 50 mM KCl (1 min). Following stimulation, CGNs were repolarized for 10 min. Samples were prepared from cultures before stimulation (basal, B), directly after KCl stimulation (K) or after 10 min repolarization (R) as indicated by arrowheads. BDNF (100 ng ml⁻¹) or LY294002 (LY, 10 μM) were present throughout the experiment where indicated. Lysates were separated by SDS–PAGE and probed for either (c, d) phospho-Ser-774 (PDynI Ser-774) or (e, f) phospho-Ser-778 (PDynI Ser-778) on dynamin I on immunoblots. Quantitative analysis is shown in the graphs in (c–f). These graphs display the extent of phosphorylation of either Ser-774 (c, d) or Ser-778 (e, f). All values were normalized to the amount of synaptophysin (SYP) as a loading control, and expressed as a percentage of Basal ± s.e.m. (c) n = 9, (d) n = 3, (e) n = 5, (f) n = 4, one-way analysis of variance (ANOVA). *P < 0.05, **P < 0.01, ***P < 0.001.
BDNF sustains neurotransmission during high-frequency stimulation in CGN nerve terminals.

BDNF sustains neurotransmission during high-frequency stimulation in CGN nerve terminals. The selective control of ADBE by BDNF suggests that it...
Figure 4 | BDNF arrests HRP-endosome formation after prior stimulation. (a) CGNs were placed in incubation medium for 10 min before stimulation with 50 mM KCl for 2 min (S1). CGNs were then repolarized for 10 min before a second stimulus with 50 mM KCl (S2). HRP (10 mg ml⁻¹) was co-applied with KCl at either S1 or S2 and CGNs were immediately fixed after stimulation as indicated by arrowheads. BDNF (100 ng ml⁻¹) was present throughout the experiment where indicated. Representative images display typical nerve terminals at S1 for either Ctrl (b) or BDNF (c) and at S2 for either Ctrl (d) or BDNF (e). White arrows indicate HRP-labelled endosomes, black arrows indicate HRP-labelled SVs. Scale bar, 150 nm for b-e. Mean number of HRP-labelled (solid bars) or empty (open bars) endosomes (f) or SVs (g) at either S1 or S2 in control (Ctrl) or BDNF-treated (BD) cells ± s.e.m. (n = 3 independent experiments for all conditions; one-way analysis of variance (ANOVA). ***P < 0.001 compared with all other conditions).

Discussion
Presynaptic control of neurotransmitter release is a well-established phenomenon and involves control of the SV-fusion event via modulation of either neuronal excitability, calcium influx or release probability. We have shown that extracellular signalling molecules can also control SV endocytosis during high-intensity stimulation, with subsequent consequences for neurotransmission. This work reveals a novel regulatory mechanism to control neurotransmission during high-intensity stimulation and to our knowledge this is the first demonstration of an extracellular signalling cascade controlling SV endocytosis.

Where possible we evoked ADDBE using high-frequency action potentials, however, the design of particular experiments necessitated the use of elevated KCl. We have extensively characterized these two stimuli in our culture system and have found that in terms of (1) the extent of SV exocytosis evoked, (2) the amount of ADDBE and CME triggered, (3) the replenishment of both the RRP and reserve pools and (4) the extent of Akt and GSK3 phosphorylation, application of 50 mM KCl is equivalent to the delivery of 800 action potentials at 80 Hz.

GSK3-dependent phosphorylation of Ser-774 on dynamin I is essential for ADDE to proceed. As GSK3 activity is negatively regulated by phosphorylation, we searched for potential candidate signalling molecules that may control ADDBE. BDNF was selected for study as CGNs express TrkB receptors and BDNF is released in an activity-dependent manner from central neurons. We show that BDNF mediates its action via a P13K-dependent intracellular signalling cascade that activates Akt to inhibit GSK3, resulting in arrest of dynamin Ser-774 rephosphorylation. We could not directly test the effect of TrkB receptor inhibition on dynamin I rephosphorylation, as K252a reduced Ser-774 rephosphorylation by ~80% in the absence of BDNF. A key point relating to the inhibition of dynamin I rephosphorylation via this cascade is that the effects of BDNF on both ADDBE and neurotransmission are only revealed after a priming stimulus. Thus, there is no acute effect of this cascade on either process, providing compelling evidence that the mechanism by which BDNF acts is a protein rephosphorylation event that occurs after stimulation terminates. In agreement, identical results on ADDBE and neurotransmission were observed using either cdk5 or GSK3 antagonists.

BDNF increases spontaneous and evoked neurotransmitter release in a number of neuronal systems. However, other studies have reported no effect on baseline transmission. The presynaptic mechanism by which BDNF increases glutamate release is TrkB-dependent and mediated by an intracellular MAPK-signalling cascade. Phosphorylation of the SV protein synapsin is essential in mediating the BDNF effect, as either synapsin I knockout or mutation of the MAPK phosphorylation site on synapsin I negated this effect. A requirement for the myosin VI motor complex has also been proposed, potentially involving an actin-dependent delivery of SVs. In our culture should modulate neurotransmission during high-frequency firing, as ADDBE is the dominant mechanism of SV retrieval under these conditions. To test this, we examined the effect of applying BDNF on neurotransmission at the parallel fibre—Purkinje cell synapse in intact cerebellar slices. Specifically, we determined the effect of BDNF on synaptic depression, where neurotransmission is progressively decreased during a high-frequency train of action potentials. This depression is exacerbated when CME is inhibited, reflecting a depletion of available SVs for release. Application of BDNF had no effect on the evoked depression in slices (600 action potentials, 40 Hz) when it was added 1 h before stimulation (Supplementary Fig. S4). In contrast, slices that had experienced a prior identical depolarizing stimulus in the presence of BDNF exhibited a profound relief of depression when challenged with a second stimulus (S2) 10 min later (Fig. 6a,b). This relief of depression at S2 was prevented by the presence of LY294002 and occluded by CT99021 (Fig. 6c–f), indicating that this effect was mediated by the same cascade that controls GSK3 rephosphorylation of Ser-774 on dynamin I. The relief of depression was not due to an increase in release probability, as paired pulse facilitation was not altered at either S1 or S2 by BDNF (Supplementary Fig. S5). Thus, BDNF enhances synaptic transmission during high-intensity firing via a negative control of both GSK3 activity and ADDBE.
BDNF has both presynaptic and postsynaptic effects, therefore to ensure any modulation of GSK3 was presynaptic, we included the GSK3 antagonist CT99021 in the patch pipette. The fact that no effect was observed on neurotransmission during high-frequency firing.

Figure 5 | BDNF has no effect on SV exocytosis. CGNs expressing sypHy were placed in incubation medium for 10 min. The sypHy fluorescence response was then monitored for 40 s before addition of bafilomycin A1 (200 nM). After 10 min, CGNs were stimulated with a train of 800 action potentials (80 Hz). Finally, cultures were exposed to 50 mM NH₄Cl. (a) Representative traces of the sypHy response in the presence (solid) or absence (clear, Ctrl) of BDNF (100 ng/ml) are displayed showing the response to bafilomycin A1, action potentials and NH₄Cl (indicated by bars). Traces are normalized to the NH₄Cl pulse. (b) Quantified data showing the extent of spontaneous (Spon, dark grey) and evoked (80 Hz, light grey) SV exocytosis in the absence (Ctrl) or presence (BDNF) of BDNF. Both values are displayed as a proportion of the total SV pool ± s.e.m., Ctrl, n = 4, BDNF, n = 5 independent experiments, Student’s t-test P = 0.17 for spontaneous and P = 0.45 for evoked.

We have shown that activation of a presynaptic signalling cascade by an extracellular signalling molecule, BDNF, results in inhibition of GSK3 activity, the arrest of both dynamin Ser-774 phosphorylation and ADBE and finally enhancement of neurotransmission during high-frequency stimulation.

A key question raised by this study is when would BDNF be released to impact on ADBE? A number of studies have shown that BDNF is released by neurons during high-intensity stimulation. Recent work using GFP-tagged BDNF in primary hippocampal cultures have suggested that the majority of BDNF that is secreted is via dendritic release, potentially feeding back to hippocampal cultures have suggested that the majority of BDNF that is secreted is via dendritic release, potentially feeding back to synaptic strength in either neurodegenerative conditions or such as epileptic seizure. In agreement epilepsy is associated with increased levels of BDNF, which may exacerbate seizures by increasing neurotransmission via its block of ADBE.

We have shown that activation of a presynaptic signalling cascade by an extracellular signalling molecule, BDNF, results in inhibition of GSK3 activity, the arrest of both dynamin Ser-774 rephosphorylation and ADBE and finally enhancement of neurotransmission during high-frequency stimulation. This suggests that different SV endocytosis modes, and ADBE in particular, may be a potential target for strategies to modify synaptic strength in either neurodegenerative conditions or disorders of neuronal excitability such as epilepsy.

Methods

Materials. FM2-10, tetramethylrhodamine-dextran, Lipofectamine 2000, penicillin/streptomycin, phosphate buffered salts, fetal calf serum and minimal essential medium, were obtained from Life Technologies (Paisley, UK). Synaptophysin antibody was from Synaptic Systems (Germany). The dynamin I phosphospecific Ser-774 and Ser-778 antibodies were from AbD Serotec (Cambridge, UK). The phospho-Akt Ser-473 and phospho-GSK3 β/α Ser-9/21 antibodies were from Cell Signalling (Herts, UK). Glutaraldehyde and osmium tetroxide were from Agar Scientific (Essex, UK). BDNF was from Cambridge Bioscience (Cambridge, UK). Bafilomycin A1 was from Acros Organics (Loughborough, UK). CT99021 was from Stratich Scientific (Suffolk, UK). Full-length rat dynamin 1α was fused to the fluorescent protein mCerulean at its C terminus, with the Ser774Ala mutation generated by site-directed mutagenesis. SypHy was generated by inserting the available SV membrane and cargo will be packaged into release ready SVs at the plasma membrane, rather than via a series of endosomal intermediates. Regardless of mechanism, it is becoming apparent that modulation of SV endocytosis modes could be a route for bidirectional manipulation of neurotransmission during high-frequency firing.

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pH-sensitive green fluorescent protein, pHlourin at the second intravesicular loop of rat synaptophysin. All other reagents were from Sigma (Poole, UK).

Primary cell culture. In all cases, animals were killed by schedule 1 procedures in accordance with UK Home Office Guidelines. Primary cultures of CGNs were prepared from the cerebella of 7-day-old Sprague–Dawley rat pups of either sex. Cultures were used between 8–12 days in vitro. For all experiments, cultures were removed from culture medium into incubation medium (in mM: 170 NaCl, 3.5 KCl, 0.4 KH2PO4, 20 TES (N-tris(hydroxy-methyl)-methyl-2-aminoethanesulfonic acid), 5 NaHCO3, 5 glucose, 1.2 Na2SO4, 1.2 MgCl2, 1.3 CaCl2, pH 7.4) for 10 min before commencing experiments.

Assays of protein phosphorylation status. After equilibration in incubation medium (10 min), cultures were rested in incubation medium for a further 10 min in the presence or absence of compounds. Cultures were then stimulated for 1 min with 50 mM KCl (50 mM NaCl removed to maintain osmolarity) and allowed to repolarize for 10 min in incubation medium. CGNs were immediately lysed in SDS sample buffer (67 mM SDS, 2 mM EGTA, 9.3% glycerol, 12% β-mercaptoethanol, bromophenol blue, 67 mM Tris, pH 7.4) for 10 min before commencing experiments.

Fluorescence imaging of dextran uptake. Uptake of tetramethylrhodamine-dextran (40kDa) was monitored in the following manner. For S1 experiments,

**Figure 6 | BDNF relieves synaptic depression at cerebellar synapses.** (a) Cerebellar slices were incubated with combinations of either BDNF (100 ng ml⁻¹), LY294002 (LY 10 μM) or CT99021 (2 μM) for 1h before being transferred to the recording chamber. Slices were challenged with 600 action potentials (40 Hz, S1) then allowed to recover for 10 min before a second challenge with an identical stimulus train. (b) Example mean EPSCs recorded at three time points during the train of 600 stimuli. The numbers above denote the sweep numbers that were averaged to obtain the trace. (c,e) Summary plots showing the dependence of the EPSC amplitude on the stimulus number at S2. The first EPSC was normalized to 1 to allow direct comparison of the different recording conditions on the rundown of the amplitudes of the EPSCs. (d,f) The mean EPSC amplitude after 200 action potentials is displayed for each experimental condition ± s.e.m. (Ctrl n = 11, BDNF n = 9, LY n = 7, BDNF/LY n = 9, CT n = 9, BDNF/CT n = 9; one-way analysis of variance (ANOVA) in (e). ***P < 0.001, **P < 0.01 compared with BDNF; in (f) ***P < 0.001, *P < 0.05 compared with Ctrl; all other conditions were not significant to each other).
CGNs were left for 10 min in incubation medium and then stimulated with a train of 800 action potentials (80 Hz, 10 s) in the presence of 50 μM tetramethylrhodamine-dextran. Action potentials were delivered using a Warner Instruments field stimulation chamber (RC-21BRFS) with parallel platinum wires (6 mm apart). Cultures were stimulated with 1 ms pulses of 100 mA for all conditions. For the S2 experiments, CGNs were stimulated with 50 mM KCl for 1 min after a 10 min rest in incubation medium. Cultures were then repolarized for 10 min and then stimulated with 800 action potentials (80 Hz) in the presence of tetramethylrhodamine dextran. BDNF or LY294002 were present for 10 min before S1 stimulation and at all points thereafter where indicated. Dextran loading was determined by the number of fluorescent puncta in a defined field of view for unstimulated neurons. Using a × 20 objective, 550 nm excitation and >575 nm emission on a Zeiss Axio-observer D1 inverted epi-fluorescence microscope. The number of dextran puncta per field was quantified using FIJI (National Institutes of Health). The images were first thresholded using the Max-Entrophy algorithm and the number of puncta (between 1–3.5 μm²) counted. The average number of dextran puncta per field for each experiment (usually 10 fields of view per experiment) were averaged for the same conditions and the value for the unstimulated background was subtracted to give the corrected dextran uptake. At least four independent experiments were performed for each experimental condition.

Monitoring of sYPHy fluorescence. CGNs were transfected with the genetic reporter sYPHy² using lipofectamine 2000 between 4–6 days in vitro. Transfected neurons were imaged after 72 h using a >40 oil immersion objective and 480 nm excitation and >525 nm emission on a Zeiss Axioscop microscope. After a 40 s period to establish a stable baseline, bafilomycin A1 (200 nM) was added to the imaging chamber. The sYPHy response was recorded and normalized to the peak sYPHy response in the presence of NH₄Cl. The peak response during both spontaneous (after 10 min) and evoked (>800 action potentials) was calculated as a function of the total SV pool (revealed by NH₄Cl addition).

Labelling of endocytosis pathways by HRP. Cells were processed for HRP labelling as described below. For S1 experiments, cultures were stimulated for 2 min with stimulation medium (50 mM KCl) supplemented with HRP (10 mg ml⁻¹) after 10 min in incubation medium. For S2 experiments CGNs were treated identically to S1 stimulated cultures (no HRP added in S1 stimulation solution) but then repolarized in incubation medium for 10 min before a second exposure to stimulation medium supplemented with HRP (10 mg ml⁻¹). Cells were fixed immediately after either 30 or 60 s or 525 nm excitation on a Zeiss Axioscop epi-fluorescence microscope. After a 40 s period to establish a stable baseline, bafilomycin A1 (200 nM) was added to the imaging chamber. The sYPHy response was recorded and normalized to the peak sYPHy response in the presence of NH₄Cl. The peak response during both spontaneous (after 10 min) and evoked (>800 action potentials) was calculated as a function of the total SV pool (revealed by NH₄Cl addition).

Hippocampal cell culture. Primary cultures of hippocampal neurons were prepared from E17.5 mice of either wild-type or S774A mutant dynamin I tagged with mCerulean using lipofectamine 2000 after 6 days in vitro and imaged after 8 days in vitro. Transfected neurons were located by viewing at 430 nm excitation (emission >525 nm). Dextran uptake was quantified as the number of dextran puncta per 100 μm of transfected neurite (usually five fields of view per experiment). Neurite length was measured using the Simple Neurite algorithm in Fiji. The unstimulated background was subtracted to give the corrected dextran uptake. At least three independent experiments were performed for each experimental condition.

Statistical analysis. Two samples were compared using a Student’s t-test, whereas sample sets greater than two were compared using a one-way ANOVA.

References

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Author contributions

M.A.C., K.J.S. and M.J. designed experimental protocols and lead the project. M.A.C., K.J.S. and M.J. wrote the manuscript. K.J.S., J.P. and E.M.P. performed all experiments.

Additional information

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