Calcineurin and huntingtin form a calcium-sensing machinery that directs neurotrophic signals to the nucleus

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When a neurotrophin binds at the presynapse, it sends survival signals all the way to the nucleus on signaling endosomes. These endosomes fuel their own journey with on-board glycolysis—but how is that journey initiated and maintained? Using microfluidic devices and mice, we find that the calcium released upon brain-derived neurotrophic factor (BDNF) binding to its receptor, tropomyosin receptor kinase B (TrkB), is sensed by calcineurin on the cytosolic face of the endosome. Calcineurin dephosphorylates huntingtin, the BDNF scaffold, which sets the endosome moving in a retrograde direction. In an in vitro reconstituted microtubule transport system, controlled calcium uncaging prompts purified vesicles to move to the microtubule minus end. We observed similar retrograde waves of TrkA- and epidermal growth factor receptor (EGFR)-bearing endosomes. Signaling endosomes in neurons thus carry not only their own fuel, but their own navigational system.

INTRODUCTION
A neuron’s survival depends on molecular traffic moving in the right direction, at the right time, over the right distance. Molecular motors move along the microtubule network carrying a variety of adaptor proteins with their specific cargoes, according to the direction the molecule needs to be transported (1). The kinesin family of motor proteins moves in an anterograde direction, bearing newly synthesized molecules that regulate presynaptic activity toward the axon terminal, while dynein motor proteins move their cargo in a retrograde direction toward the soma (2–4). Among the cargoes that must be transported retrogradely are vesicles such as lysosomes, autophagosomes, and “signaling endosomes” (2, 5, 6).

Signaling endosomes carry survival signals to the nucleus to promote and maintain neural connectivity (7–9). Target cells produce neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), or neurotrophin 3/4, which then bind their specific receptors at the presynaptic terminal. Once bound, the neurotrophin is internalized into an endosome that is then borne back to the soma by dynein (5–10). This endosome then associates with adaptor and effector proteins that induce a cascade of signals to reinforce connections within the neuronal network (11). Although the mechanism involved in receptor activation and internalization has been elucidated in the case of NGF and its receptor tropomyosin receptor kinase A (TrkA) (12), we have yet to find the signal that triggers retrograde transport of neurotrophins.

We are particularly interested in BDNF, which is the main trophic factor in the central nervous system (13). Disruptions in BDNF transport contribute to the pathogenesis of a number of neurological diseases, from Huntington’s disease (HD) and Alzheimer’s to Rett syndrome (13, 14). BDNF can be transported in either an anterograde or retrograde direction, depending on whether the protein that scaffolds it, huntingtin (HTT), links up with dynein or kinesin proteins. HTT phosphorylation at S421 by the serine/threonine kinase Akt recruits kinesin, whereas dephosphorylation by calcineurin (CaN) or the protein phosphatase PP2B causes kinesin to be released from the complex, allowing dynein to carry out retrograde transport of vesicles, including those containing BDNF and amyloid precursor protein (APP) (14–16). The machinery that carries out this retrograde transport is rather involved. HTT binds to the dynein motor protein, which walks along the microtubules, as well as to the HTT interactor HAP1 (HTT associated protein 1), which connects to the p150Glued subunit of the dynactin complex, which, in turn, binds to both microtubules and the vesicle. In this way, the vesicle is tethered to the motor protein to be transported along the microtubule network (17, 18).

As detailed as this outline of events may seem, there are still crucial questions that remain to be answered. We still do not know what signals determine HTT phosphorylation or dephosphorylation, nor do we have any idea how such signals reach HTT bound to motile vesicles. These questions become more puzzling when we consider that the initiating event—BDNF binding its receptor TrkB—takes place at the presynaptic terminal’s plasma membrane, yet it affects vesicles that are moving along the axon at velocities up to 2 to 3 μm/s. Perhaps the greatest puzzle is how this transport is maintained over the extraordinary length of the axon and the dendritic arbor (19, 20).

We previously found that vesicles, including those containing TrkB, carry their own glycolytic machinery to fuel their transport (21, 22). Because this left open the question of how a large quantity of neurotrophin-bearing endosomes receive directional signals to guide them all the way back to the soma, we conducted proteomic studies of motile vesicles (22, 23). Here, we show that one of the proteins from our mass spectrometry analysis, CaN, resides on the surface of neuronal endosomes purified from corticostriatal projections and forms part of an unexpected endogenous guidance system.

RESULTS
CaN localizes on the surface of motile vesicles
We used Thy1:p50-GFP mouse forebrains for our proteomic study (22–24), isolating vesicles that were immunopositive for p50–green
fluorescent protein (GFP), which is expressed predominantly in corticostriatal projecting neurons (fig. S1A). We identified the catalytic subunit of CaN (CaNA) in its three isoforms (α, β, and γ) and the regulatory subunit CaNB (Fig. 1A). We confirmed that, in the vesicular fraction (P3) from wild-type (WT) mouse forebrain, CaN is present with HTT and the p150 Glued subunit of dynactin (fig. S1B). Because we are interested in understanding CaN activity, we decided to focus on the catalytic subunit CaNA (hereafter referred to simply as CaN).

We first examined CaN localization in isolated cortical axons grown in microfluidic devices (described in greater detail below; see also Materials and Methods). Because CaN dephosphorylates HTT at S421, which is specifically involved in directing BDNF transport (15, 25), we were particularly interested in the proximity of CaN to HTT. We permeabilized axons to remove the cytosolic staining and found that CaN colocalizes with HTT on puncta that could correspond to vesicles, as previously reported for HTT staining in axons (fig. 1B and fig. S1C) (21). We confirmed this observation in free-cultured cortical neurons at 5 days in vitro (DIV5) using a two-dimensional stimulated emission depletion (2D-STED) super-resolution microscope (Fig. 1C). To determine CaN’s location more precisely, we purified vesicle fractions and analyzed them by LC-MS/MS, liquid chromatography–tandem mass spectrometry; LC-MS/MS

**Fig. 1. CaN is enriched on the surface of vesicles, colocalizes with HTT on Rab5+ endosomes, and interacts with HTT in vivo.** (A) Mass spectrometry analysis of vesicles purified from Thy1:p50-GFP mouse brains shows CaNA ranks highly among vesicle-associated proteins. LC-MS/MS, liquid chromatography–tandem mass spectrometry; KIF5C, kinesin family member 5C. (i) Airyscan microscopy of isolated axons identified by the postfixation incubation with Alexa Fluor 488 conjugate of wheat germ agglutinin (WGA). Scale bar, 2 µm. (ii) Line scan analysis (left) and (iii) quantification of colocalization (right). The graph shows the endogenous colocalization of CaN with agglutinin (WGA). Scale bar, 2 µm. (B) KIF5C, kinesin family member 5C. (i) Airyscan microscopy of isolated axons identified by the postfixation incubation with Alexa Fluor 488 conjugate of wheat germ agglutinin (WGA). Scale bar, 2 µm. (ii) Line scan analysis (left) and (iii) quantification of colocalization (right). The graph shows the endogenous colocalization of CaN with agglutinin (WGA). Scale bar, 2 µm. (D) Immunogold labeling of vesicles isolated from mouse brain shows the presence of both CaN (5-nm gold particles) and HTT (15-nm gold particles) on vesicles with a higher percentage of colocalization in large vesicles (diameter > 150 nm) compared to small vesicles (< 60 nm). EM, electron microscopy. Mann-Whitney test, *P < 0.05; nCTRL = 188 and nCaN = 209. Scale bars, 50 nm. (E) Vesicular fractions (P3) were incubated with increasing concentrations of Proteinase K (PK) to digest proteins present on the surface of vesicles; only BDNF remains, meaning that it is within the vesicle. The predicted molecular weights (MWs) are indicated on the left, in gray. αSyn, α-synuclein.
CaN and HTT colocalize with TrkB and Rab5

When we purified endosomes from Thy1:p50-GFP mouse forebrains, we found enrichment for Rab5, TrkB, HTT, and CaN (Fig. 2A). This suggested that we might be witnessing the first step of retrograde neurotrophin signaling, which involves Rab5-positive early endosomes (27, 28). Incubating the final pellet containing the endosomal fraction with a Rab5 antibody coimmunoprecipitated HTT and CaN (Fig. 2B), and super-resolution STED microscopy revealed that CaN localizes on Rab5 endosomes in cortical neurons (Fig. 2C). Airyscan high-resolution confocal microscopy revealed colocalization of endogenous CaN, endogenous HTT, Rab5–yellow fluorescent protein (YFP), and endogenous Rab5 on roughly one-third of the vesicles (Fig. 2D and fig. S2A).

Furthermore, endogenous HTT colocalized with endogenous Rab5 (fig. S2B) and endogenous TrkB (fig. S2C). TrkB-mCherry vesicles colocalized with endogenous HTT and CaN (fig. S2D). BDNF treatment increased the percentage of TrkB-mCherry vesicles that contain CaN and are immunopositive for Rab5 (Fig. 2E, F, and S2D), as confirmed by STED microscopy (Fig. 2G). Last, anti-Rab5 and anti-TrkB antibodies coimmunoprecipitated endogenous TrkB and endogenous Rab5, respectively (Fig. 2H). These data suggest that these vesicles are signaling early endosomes.

Synaptic BDNF and TrkB retrograde signaling in reconstituted corticostriatal circuits

To study signaling early endosomes in a physiologically relevant system, we took advantage of microfluidic devices to reconstitute mature corticostriatal circuits (29, 30). The devices consist of a presynaptic and a postsynaptic compartment containing cortical and striatal neurons, respectively, and a middle synaptic compartment that receives axons from cortical neurons and dendrites originating from striatal neurons (Fig. 3A). The three compartments are connected by 3-μm-wide microchannels that are 500 μm long for axons and 75 μm long for dendrites. Because the axonal channels are 500 μm long, only axons from the cortex can reach the synaptic compartment. Conversely, a laminin gradient from the cortical chamber to the striatal chamber (in the context of steady poly-d-lysine concentration) limits the number of striatal axons that can reach the synaptic chamber. We verified the preferential orientation of the corticostriatal circuit from pre- to postsynaptic compartments by infecting striatal neurons with a GFP-encoding lentivirus. We stained all the compartments with antibodies recognizing GDP, along with a dendritic [microtubule-associated protein 2 (MAP2)] and an axonal [phosphorylated neurofilament H (NFH)] marker (fig. S3A). Around 20% of the neurites entering the synaptic compartment of the striatal compartment (GFP+) were also SMI31+, indicating that around 80% of the axons in the synaptic compartments are cortical axons (fig. S3A). By DIV10, the circuit achieves functional maturity, as defined by kinetics of neurite outgrowth, synapse formation and function, axonal transport, and neural activity (29, 30). By this time point, cortical neurons have established functional excitatory connections to striatal neurons. All experiments in the microfluidic devices were therefore performed at DIV10 to DIV12.

We first transduced cortical presynaptic neurons with a lentivirus encoding Trk-B-mCherry and then used high-resolution spinning confocal microscopy to image Trk-B-mCherry particles in the distal part of the axons (movie S1). We generated kymographs from video recorded at DIV10 to DIV12 to trace the movement of vesicles before and after adding BDNF (Fig. 3A). At time zero (T0), before any treatment, TrkB-containing vesicles moved in both anterograde and retrograde directions (Fig. 3B), but the net flux (the number of anterograde minus the number of retrograde vesicles) was positive, indicating that TrkB-containing vesicles were predominantly moving from the soma to the presynapse (Fig. 3C). Next, we added BDNF (50 ng/ml) to the synaptic compartment and recorded the movement of presynaptic TrkB-containing vesicles for 30 s every 2.5 min. TrkB-containing vesicles adopted a net anterograde to net retrograde flow from 2.5 to 7.5 min after BDNF addition, after which they reverted to net anterograde flow (Fig. 3, B to D). Since we observed no substantial reduction in the number of anterograde-moving vesicles, this change in net flux was due to an increase in the number of TrkB-containing vesicles moving in the retrograde direction (Fig. 3D and fig. S3B). There was no change in the velocity of vesicles moving in either direction (average velocity, 1.53 μm/s; fig. S3C).

To track the internalization of the TrkB receptors, we used BDNF labeled with quantum dots (QDs), which couple to endocytosed neurotrophins and enable the experimenter to follow the fate of the organelles over time (31). QDs seem to modify the kinetics of endocytosis, however: whereas retrograde motion begins within 2.5 min after BDNF application, most of the QDs remained in the synaptic compartment, exhibiting a sort of oscillatory motion, within the first 5 min after BDNF addition (Fig. 3E and movie S2). Similar movements were previously observed with the endocytosis of tetanus neurotoxin fragments (27). It took about 75 min after QD-BDNF infusion for the QDs to start traveling along the axons (movie S3), in line with previous studies that recorded trafficking of internalized QD-BDNF (or QD-NGF) 1 to 3 hours, rather than minutes, after infusion (31–33). Despite the delay, this experiment confirmed the endocytosis of TrkB/BDNF.

We next asked whether these TrkB/BDNF-carrying endosomes mature or remain early Rab5+ endosomes (34). In the case of retrograde neurotrophin signaling, the early Rab5-positive signaling endosomes transition to Rab7-binding late endosomes (34). As others have reported (27), within 5 min of administering BDNF the fraction of vesicles positive for both TrkB-mCherry and Rab5–YFP increased (Fig. 3F). The greater number of retrograde-moving TrkB+ vesicles seems to reflect an increase in the number of TrkB+Rab5+ vesicles, since there was no change in the number of TrkB–Rab5+ vesicles (fig. S3D). We also observed an increase in the number of TrkB+Rab5+ vesicles at the endogenous level (fig. S4A). Since retrograde traffic needs to be carried all the way to the soma to induce survival signals, and Rab7 is required after the Rab5-dependent early step to induce long-range transport toward the soma (27), we asked whether the retrograde-moving TrkB vesicles...
Fig. 2. CaN and HTT are present on TrkB signaling endosomes. (A) Western blot of Thy1:p50-GFP brain fractions after successive centrifugations. The light membrane–enriched fraction (P3) contains endosomes, as shown by the presence of Rab5, HTT, CaN, and TrkB. (B) Immunoprecipitation (IP) of the endogenous Rab5 (αRab5) in endosomes purified from Thy1:p50-GFP mouse forebrains. Immunoglobulin G (IgG) served as a negative control. (C) 2D-STED microscopy of neurites shows the colocalization of Rab5 and CaN immunostaining on the same endosome. Scale bars, 1 µm. (D) (i) High-resolution microscopy of axons in a microfluidic chamber shows CaN and HTT present on Rab5-positive endosomes (n = 35 axons from two independent experiments). Scale bar, 2 µm. (ii) Line scan analysis and (iii) graph representing the percentage of triple colocalization. (E) Isolated axons were immunolabeled with antibody targeting overexpressed TrkB-mCherry (α mCherry), Rab5-YFP (α GFP), and endogenous CaN (α CaN). Graph at right shows that triple colocalization increased after 5 min of BDNF treatment. Mann-Whitney test, *P < 0.05; n = 50 to 54 from three independent experiments. Scale bars, 2 µm. (F) The plot profile of gray value intensity shows TrkB-mCherry, Rab5-YFP, and CaN peaks coincide, indicating colocalization in isolated axons. (G) 2D-STED microscopy confirmed CaN and TrkB-mCherry colocalization on vesicles. Scale bars, 1 µm. (H) Extracts of purified endosomes were incubated with Rab5 or TrkB antibodies (αRab5 or αTrkB) or immunoglobulin as control (IgG). CaN is enriched in Rab5- or TrkB-associated endosomes immunoisolated from the light membrane fractions of Thy1:p50-GFP mouse forebrains.
**Fig. 3.** BDNF induces a wave of TrkB signaling endosomes. (A) Corticostriatal connections form in microfluidic chambers in vitro. Kymographs, which show vesicle movement in both directions, were acquired at DIV10 to DIV12 when the neurons reach a mature stage, as shown by staining with synaptophysin I (SypI) and postsynaptic density 95 (PSD95). Scale bars, 20 μm (for kymograph) and 2 μm (for SypI/PSD95). LV, lentiviral vector. (B) Kymographs show movement of TrkB-mCherry vesicles before and 5 min after BDNF infusion (50 ng/ml). (C) BDNF treatment led vesicles to change their direction of movement from anterograde to retrograde. Kruskal-Wallis test, Dunn's multiple comparisons test, *P < 0.05 and **P < 0.01; n = 30 to 40 axons from five independent experiments. (D) Analysis of axonal TrkB-mCherry vesicle transport from T0 and every 2.5 min thereafter. Each dot represents the average number of vesicles observed for each 100 μm of a given axon (n = 30 to 40 axons from five independent experiments). Left: Number of TrkB-containing anterograde vesicles remained fairly steady over time. Right: Number of retrograde vesicles peaked at T5. Kruskal-Wallis test, Dunn's multiple comparisons test, *P < 0.05 and **P < 0.01. (E) After adding BDNF labeled with QDs (QDs-BDNF) to the synaptic compartment (top), we recorded the movement of TrkB-GFP and QDs-BDNF with dual acquisition at 5 and 75 min (bottom). Scale bars, 10 μm. (F) Distal TrkB-mCherry endosomes colocalized with Rab5-YFP, which is present on early endosomes; BDNF treatment in the synaptic compartment increased colocalization of TrkB endosomes with Rab5-positive vesicles. Unpaired two-tailed Student's t test, **P < 0.01; n = 30 to 32 axons from three independent experiments. Scale bars, 2 μm.
also carry Rab7. There was increased colocalization of TrkB-mCherry with Rab7-YFP, indicating the formation of late endosomes (fig. S4B). To investigate whether such a transition from early to late endosomes occurs in axons, we compared the kinetics of Rab5-YFP and Rab7-YFP colocalization with TrkB-mCherry after BDNF treatment. As we observed for Rab5 (Fig. 2F and fig. S4A), Rab5/TrkB colocalization increased after 5-min incubation, but it decreased after 15 min (fig. S4C). In contrast, Rab7 colocalization with TrkB was apparent after 5-min BDNF incubation (fig. S4B) but even more marked after 15 min (fig. S4C). This endosomal maturation indicates that BDNF treatment triggers a sustained response, with a wave of TrkB signaling endosomes transitioning from early to late endosomes, all moving in the retrograde direction. We will refer to this response as the TrkB retrograde wave.

The TrkB retrograde wave requires activation of both TrkB and CaN

TrkB activation is necessary for retrograde transport of TrkB-signaling endosomes. To determine whether the retrograde wave also requires TrkB activation, we took advantage of the fact that BDNF binding leads to TrkB phosphorylation at several sites, including Y816 (35). We calculated the ratio of Y816-phosphorylated TrkB to Rab5+ vesicles before and 5 min after BDNF treatment. The percentage of Rab5+ vesicles positive for phospho-TrkB (pTrkB) antibody increased after BDNF treatment (Fig. 4A). After 5 min of BDNF stimulation, CaN colocalized with active TrkB on Rab7+ endosomes, and the level of colocalization was even higher after 15 min of BDNF stimulation (fig. S4D). CaN is therefore present on endosomes bearing the active TrkB receptor during endosome maturation and transport to the soma. Conversely, administration of the Trk receptor inhibitor K252a (36) to the synaptic compartment 30 min before addition of BDNF prevented the retrograde wave (Fig. 4B, graph on far right), without affecting the number or velocity of anterograde TrkB-containing vesicles (fig. S5, A and B). Thus, TrkB activation and phosphorylation are required for the retrograde wave.

Because CaN activation is required for the endocytosis of TrkA-signaling endosomes (12), and BDNF trafficking is regulated by CaN (37), we hypothesized that CaN-mediated phosphatase activation triggers the retrograde wave. We therefore inhibited CaN pharmacologically by adding FK506 in the synaptic compartment 30 min before recording TrkB retrograde trafficking in response to BDNF infusion at the synapse. FK506 increased anterograde velocity (fig. S5C), as previously shown (37), but abolished the retrograde wave by reducing the number of retrograde vesicles (Fig. 4C) and increasing the number of anterograde vesicles (fig. S5D).

We next reduced CaN expression by 70% with a GFP-tagged lentivirus expressing short hairpin-mediated RNA against the α and β CaN isoforms (fig. S5E) and measured the effect on TrkB trafficking in GFP-immunopositive axons (fig. S5, E and F). CaN knockdown inhibited the TrkB retrograde wave by reducing the number of retrograde vesicles and increasing the number of anterograde-moving vesicles without affecting their velocity (Fig. 4D and fig. S5, G and H). We conclude that BDNF induces retrograde trafficking of TrkB vesicles through the activity of the phosphatase CaN.

TrkB retrograde signaling requires HTT dephosphorylation in mice

We were now in a position to return to the question of how CaN would influence retrograde TrkB vesicle transport, as well as why CaN would outnumber, and colocalize with, HTT. Previous studies have shown that HTT dephosphorylation at S421 promotes retrograde transport of BDNF (15), and CaN dephosphorylates HTT at S421. CaN’s proximity to HTT on the surface of Rab5+ vesicles therefore suggested to us that it might be maintaining HTT dephosphorylation, which would fate the complex to move in the retrograde direction. HTT dephosphorylation liberates kinesin-1 from the molecular motor complex, leaving the dynein/dynactin complex associated with vesicles (15). The limitation of these previous experiments is that they overexpressed short HTT fragments in neurons that were randomly cultured (i.e., not integrated into a mature network), so we were unable to discriminate axons from dendrites (15).

To determine whether endogenous HTT S421 phosphorylation influences TrkB transport specifically in axons in response to synaptic BDNF, we took advantage of two lines of homozygous knock-in mice: one in which HTT S421 is replaced by an aspartic acid (HttS421D/S421D or HTT-SD), mimicking constitutive phosphorylation, and one in which S421 is converted to an alanine, which is unphosphorylatable (HttS421A/S421A or HTT-SA) (16, 38). To measure kinesin-1 recruitment to vesicles, we performed subcellular fractionation of HTT-SD and HTT-SA mouse brains. CaN protein levels were similar regardless of HTT phosphorylation status, but much more kinesin-1 associated with vesicles in HTT-SD than in HTT-SA mouse brains (Fig. 5A). These data indicate that chronic HTT phosphorylation (S421D mutation) should prevent the retrograde transport of TrkB endosomes. We therefore isolated HTT-SD cortical neurons from homozygous mice and plated them in the pre- and postsynaptic compartments of our microfluidic device. BDNF application at the synapse in this context did not lead to a TrkB retrograde wave and had little effect on the number or velocity of vesicles (Fig. 5B and fig. S6, A to C). This suggests that, in axons, there is a steady-state level of highly phosphorylated HTT promoting anterograde trafficking of TrkB vesicles to the presynapse under the secretory pathway (39).

To investigate the physiological consequences of HTT phosphorylation status in vivo, we reproduced the increase in BDNF at the synapse using stereotactic injections of an adeno-associated virus (AAV) encoding BDNF-mCherry together with the fluorescent retrograde tracer cholera toxin B (CTB) (488) in the dorsolateral striatum (DLS). Mice were unilaterally injected in the DLS at 21 days of age (P21) and euthanized 2 weeks after injection (Fig. 6A). Because retrograde axonal transport of TrkB signaling endosomes carries extracellular signal–regulated kinase (ERK) survival signals to the nucleus (40, 41), we immunostained brain slices with p-ERK antibody. The injected hemisphere in WT mice had an increased number of p-ERK–positive cells in the sensory motor cortex, but the control hemisphere did not (Fig. 6B). There was no such change in the HTT-SD mice (Fig. 6B). Therefore, HTT-S421 regulates retrograde transport of TrkB to mediate neuronal survival. These results are consistent with previous findings that deletion of TrkB results in cortical layer thinning (42).

The transport of activated neurotrophin receptors does not stop at the soma but, as shown for TrkA, continues on to the dendritic terminals of the sympathetic neurons where the receptors localize within the postsynaptic density (PSD) clusters to promote synaptic maintenance (11). We therefore took advantage of our HTT-SD mice to investigate whether a fraction of activated TrkB (p-TrkB, recognizing Y816 phosphorylation) endocytosed at the presynapse of the corticostriatal synapse could, after having reached the soma,
**Fig. 4.** The retrograde wave requires the activation of both TrkB and CaN. (A) Axons were immunoisolated to analyze colocalization of TrkB-mCherry, Rab5-YFP, and p-TrkB before and after 5 min of BDNF (50 ng/ml) treatment. Mann-Whitney test, *P < 0.05; n = 70 to 80 axons from three independent experiments. Scale bars, 2 µm. (B) Inhibiting TrkB with K252a prevented the retrograde wave of TrkB-mCherry endosomes. Two-way analysis of variance (ANOVA), Sidak’s multiple comparisons test, *P < 0.05; n = 30 to 38 axons from three independent experiments. The graph represents the movement of TrkB-mCherry vesicles after BDNF infusion in control conditions (dark gray bar) and in the presence of the TrkB inhibitor K252a at 10 µM, added 30 min before BDNF infusion (light gray bar). (C) Inhibiting CaN with FK506 (1 µM added 30 min before BDNF) also prevented the retrograde wave. Two-way ANOVA, Sidak’s multiple comparisons test, **P < 0.01 and *P < 0.05; n = 25 to 38 axons from three independent experiments. (D) Depleting CaN with lentiviral infection of shCaNAα+β (light gray) blocks TrkB retrograde wave compared to infection with shLuc (dark gray). Two-way ANOVA, Sidak’s multiple comparisons test, **P < 0.01, n = 30 to 42 axons from three independent experiments.
travel up to the dendritic terminals. Again, we euthanized the mice 2 weeks after injection and observed an increase in the number of adjacent p-TrkB and PSD95 puncta in the injected versus control hemisphere in WT mice but not HTT-SD mice (Fig. 6C). These data indicate that increasing BDNF at the corticostriatal synapse increases active TrkB in the dendrites of cortical neurons and that HTT phosphorylation enables retrograde transport of distal axon–derived TrkB signaling endosomes for the maintenance of dendritic arborization.

TrkB-induced calcium release is necessary for BDNF to induce retrograde transport of endosomes

If CaN activation and HTT dephosphorylation induce the retrograde transport of endosomes in vitro and in vivo, the next obvious question is what activates CaN. Having found that BDNF binding to TrkB is necessary for inducing the TrkB wave (Fig. 3C), we postulated that Y816 of TrkB, known to activate phospholipase C-γ (PLCγ) and subsequently release calcium, could be the trigger (35).

We used lentiviral approaches to express TrkB-mCherry with a Y816F mutation in presynaptic cortical neurons and analyzed their trafficking in response to BDNF infusion at the corticostriatal synapse (movie S4). Blocking phosphorylation at TrkB Y816 abolished the retrograde wave (Fig. 7A) without much affecting anterograde transport, except for reducing velocity at T10 (fig. S7, A and B). To determine whether BDNF leads to an increase in calcium specifically at the cortical presynapses, we transduced cortical neurons with the GFP calcium sensor GCaMP6f, which is ultrasensitive and shows fast kinetics (43), along with either TrkB-mCherry or the TrkB-Y816F mutant. We recorded both calcium release activity and TrkB trafficking in the double-infected neurons and found that the frequency of calcium spikes increased with BDNF treatment, in correlation with the TrkB retrograde wave (Fig. 7B). BDNF induced a significant increase in calcium events in TrkB-expressing neurons while having no effect in neurons transduced with the TrkB-Y816F mutant, which cannot be phosphorylated and thus cannot activate PLCγ. Calcium events were completely abolished by the Y816F mutation, similar to nontransduced neurons (Fig. 7, C and D, and movies S5 and S6). The Y816F mutation had no obvious influence on the localization of the receptor at the synapse (Fig. 7E) or on its expression levels in cortical neurons (fig. S7C).
These data further confirm the requirement for receptor activation and subsequent phosphorylation of this tyrosine (Fig. 4).

To determine whether the observed increase in the levels of intracellular calcium directs the transport of endosomes, we incubated cortical neurons expressing TrkB-mCherry with the cell-permeant Ca$^{2+}$ chelator 1,2-bisPAPTA-AM [(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester]. Ca$^{2+}$ chelation prevented BDNF infusion from inducing the retrograde transport of

__Fig. 6. In vivo dendritic relocation of TrkB$^+$ endosomes, synaptic maintenance, and enhancement of survival signals require HTT phosphorylation. (A) Representative photo of the injection performed in the DLS. CTB and AAV-BDNF mCherry were unilaterally injected at p21, and, after 2 weeks, brains were sliced and immunostaining was performed using p-TrkB/PSD95 or p-ERK. (B) The number of p-ERK–positive cells in the cortex of WT and HTT-SD mutant mice was counted in the injected hemisphere and divided by the number of counted positive cells in the noninjected control hemisphere (CT). There were more positively marked cells in WT mice in the hemisphere that received the BDNF injection, but no changes were observed in either hemisphere in the HTT-SD mice. Two-way ANOVA, Sidak’s multiple comparisons test, $** P < 0.01$; $n = 3$ to 4 slices per brain and $n = 3$ brain per genotype. ns, not significant. Scale bars, 20 µm. (C) The number of adjacent p-TrkB/PSD95 spots in cortical dendrites is expressed as a ratio between the injected and the control hemisphere. The number of p-TrkB (Y816) puncta in close proximity to PSD95 protein reveals increased colocalization in WT (HTT-WT) but not HTT-SD mice. DAPI, 4′,6-diamidino-2-phenylindole. Unpaired two-tailed Student’s t test, $^* P < 0.05$; $n = 3$ slices per brain and $n = 3$ brains per genotype. Scale bars, 5 and 1 µm (for inset).
Fig. 7. Calcium-mediated TrkB activation is required to initiate the retrograde wave. (A) Overexpression of the TrkB-mCherry WT or mutant Y816F in rat cortical neurons shows that lack of phosphorylation at Y816 blocks the retrograde transport of TrkB-mCherry endosomes upon BDNF induction (blue) compared to the WT situation (gray). Two-way ANOVA, Sidak’s multiple comparisons test, **P < 0.01; n = 26 to 38. (B) The GCaMP6f signal rose after addition of BDNF (50 ng/ml). Friedman nonparametric test followed by Dunn’s multiple comparisons test, ***P < 0.001 and ****P < 0.0001; n = 46 axons from four independent experiments. (C and D) Calcium events were not detectable at T0 in WT or Y816-overexpressing axons. BDNF treatment triggered a significant increase in calcium events from T2.5 until T10 in axons expressing TrkB-WT (dark gray) but not TrkB-Y816F (blue). Neurons expressing only endogenous TrkB and to which only GCaMP6f was added served as controls (light gray). Kruskal-Wallis test, Dunn’s multiple comparisons test, ***P < 0.001 and **P < 0.01; n = 45 to 55 axons for WT and n = 68 for Y816F axons from at least three independent experiments. (E) There was no difference between WT and mutant (Y816F) axons in TrkB-mCherry and Syph1 colocalization. Mann-Whitney test, P = 0.1472; n = 56 to 63 neurites). Scale bars, 2 µm. (F) Analysis of vesicle movement in neurons treated with 100 µM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) in the synaptic chamber. Two-way ANOVA, Sidak’s multiple comparisons test, *P < 0.05 and **P < 0.01; n = 20 to 37 axons from four independent experiments. Calcium chelation prevented the BDNF-mediated TrkB-mCherry retrograde wave.
Trk vesicles. Thus, calcium is necessary for directing TrkB-containing organelles toward the soma (Fig. 7F and fig. S7, D and E). Together, these results indicate that the local increase in calcium concentration at the presynapse somehow triggers the retrograde wave of TrkB-signaling endosomes.

Vesicular calcium sensing determines the direction of vesicular transport
How would these changes in calcium concentration be transduced into a signal for transport? CaN’s position on the vesicle surface suggested to us that it might provide an endogenous calcium-sensing mechanism, similar to the way vesicles harness the glycolytic machinery to provide their own adenosine 5’-triphosphate (ATP) (21). To test this possibility, we prepared motile vesicle fractions as described previously (Fig. 1) (22) and incubated them with buffers mimicking no, low, and high calcium content (Fig. 8A) before measuring the level of phosphorylated HTT at S421 (pS421). The higher the calcium concentration, the less pS421 (Fig. 8, A and B), indicating that CaN is active on the vesicular surface and that vesicles can sense a change of calcium concentration in the environment.

We next tested whether the calcium-sensing capacity of vesicles is sufficient to change the direction of transport. We prepared rhodamine-labeled microtubules and were able to use the higher concentration of red-marked tubulin at the seed (minus) end to distinguish it from the plus end. We then seeded these polarity-marked microtubules in a flow chamber and added motile vesicles purified from Thy1:p50-GFP transgenic mouse brains as previously described (22, 24). Using the same approach that allowed us to find that motile vesicles carry their own glycolytic machinery (22), we labeled the motile vesicle–enriched fraction with a green lipophilic DiO (see Materials and Methods), loaded it in the chamber, and analyzed vesicle movement on microtubules by total internal reflection fluorescence (TIRF) microscopy (Fig. 8, A and C). Specifically, we isolated p50-GFP motile vesicles from Thy1:p50-GFP mice and Thy1:p50-GFP; HTT-SD mice, incubated them for 30 min with the photosensitive calcium chelator nitrophenyl (NP)-EGTA, and analyzed their movement on microtubules (Fig. 8, C and D). We detected no difference in protein levels in vesicles from either mouse line (fig. S8A). We then flashed selected microtubules with a 405-nm laser to increase calcium concentration and analyzed the movement of vesicles before and after ultraviolet (UV) photolysis (movie S7). The UV-induced calcium release increased the number of vesicles moving retrogradely, but not in mice in which S421 cannot be dephosphorylated (S421D) HTT (Fig. 8D). There was no change in velocity in either direction (fig. S8B).

In sum, vesicles have the capacity to sense changes in calcium concentration in their immediate environment and to change their directionality in a manner that involves HTT dephosphorylation. Our findings indicate that this mechanism is the first step in allowing signaling endosomes to start their long-distance retrograde journey within axons.

The retrograde wave occurs for TrkA and epidermal growth factor receptor as well
To investigate whether the observed mechanism can be generalized to other signaling endosomes, we investigated axonal retrograde trafficking of TrkA (movie S8), the receptor for NGF. We first expressed TrkA-GFP in a mature corticostriatal circuit and treated the microchambers with NGF (50 ng/ml), a concentration that activates TrkA receptors (44). NGF led to a rapid but transient change in the direction of TrkA transport, similar to what we observed for TrkB (Fig. 8E and fig. S8C).

As TrkA and TrkB both belong to the family of neurotrophin receptors (7–9), we wanted to test a rather different growth factor. We therefore expressed GFP-tagged epidermal growth factor receptor (EGFR) in our mature corticostriatal circuit and incubated the synaptic compartment with EGF (2.5 ng/ml), a concentration that leads to EGFR internalization (45). Although EGFR-GFP is more cytosolic than TrkA-GFP or TrkB-mCherry and is less often seen within vesicles (movie S9), EGF still triggered a retrograde wave of EGFR similar to those observed for TrkA and TrkB (Fig. 8F and fig. S8D). We propose that the retrograde wave is a common mechanism among endosomes for mediating signals over long distances in axons (Fig. 9).

DISCUSSION
Several years ago, we found that motile vesicles fuel their own transport by using HTT to harness the glycolytic machinery to the vesicle (21, 22). We now find that endosomal vesicles have their own endogenous calcium-sensing machinery, which enables them to “choose” the direction of transport through a rather intricate series of steps: BDNF binds to TrkB, leading to its phosphorylation at Y816 (35). PLCγ1 then docks at this phosphorylated site, up-regulating Ca2+ levels (35) and triggering CaN-mediated dephosphorylation of HTT S421 (this study). HTT dephosphorylation liberates kinesin from the vesicle (15), leaving only dynein [(15) and this study], with the result that the vesicle moves toward the soma (this study; Fig. 9). These observations illustrate the complexity of retrograde neurotrophin signaling and suggest that the long-range trafficking of signaling endosomes requires a high level of coordination between dynein activators and adaptors.

One of the challenges in investigating these mechanisms is the technical difficulty of capturing trafficking at sufficiently high spatial and temporal resolution in a mature neuronal circuit. Although recent studies have shown that microfluidic devices are useful for dissecting mechanisms related to endosomal signaling (10, 11), distal axons were not connected functionally to postsynaptic neurons. Therefore, to better mimic neurophysiology, we designed our microfluidic devices to include a synaptic compartment and reconstitute corticostriatal circuits (29, 30). This enabled us to apply BDNF and various drugs to localized domains such as synapses, record the responses in real time in a functional circuit, and detect quantal events of retrogradely moving signaling endosomes with high resolution. Previous strategies to detect signaling endosomes moving retrogradely have used neurotrophic factors coupled to quantum dots (QDs) (31–33) or tetanus toxin fragments (Hc2T) (27). The strength of these approaches is that they reveal the endosomes that have internalized the QD- or Hc2T-coupled trophic factor. In the current study, the average velocity of endosomes (1.53 μm/s) is comparable to what has been published for QD-NGF–containing endosomes (mean = 1.21 μm/s) (32) or for QD-BDNF–containing endosomes (mean = 1.42 μm/s) (33) and slightly higher than that for tetanus neurotoxin–positive endosomes (0.2 to 1.2 μm/s) (27). The endosomal response to BDNF application, however, is much more rapid than what can be recorded with QDs: Retrograde trafficking increased as early as 2.5 min after BDNF application, whereas QD trafficking was apparent only 75 min after incubation. This suggests that although the coupling of a QD to NGF is useful to follow a single event, it does not capture the first
Fig. 8. CaN-HTT on the vesicular surface transduces changes in local Ca\(^{2+}\) concentration into retrograde transport. (A) Microtubules were polymerized in vitro and their polarity marked by different concentrations of rhodamine-labeled tubulin at the minus (−) and plus (+) ends. Motile vesicles purified from offspring of p50-GFP or p50-GFP × HTT-SD mice were added to microtubules seeded in flow chambers and their movement analyzed by total internal reflection fluorescence (TIRF) microscopy. (B) Purified vesicles were treated with increasing concentrations of CaCl\(_2\) and Western blot membranes were incubated with the antibody recognizing HTT S421 phosphorylation. One-way ANOVA, Dunnett’s multiple comparisons test, *P < 0.05; n = 3. (C) Rhodamine-labeled microtubules were polymerized in vitro with lower concentrations of red tubulin during microtubule elongation to orient them. The labeled microtubules were then attached to a glass slide covered with anti-tubulin antibodies, incubated with the purified “motile” vesicle fraction in the presence of the calcium caging compound NP-EGTA, and lastly ultraviolet (UV)-flashed to release calcium. (D) Calcium uncaging increased the number of vesicles moving retrogradely in p50-GFP WT but not in p50-GFP × HTT-SD mouse brain. Two-way ANOVA, Sidak’s multiple comparisons test, *P < 0.05; n = 24 to 26 from five independent experiments. Scale bar, 5 \(\mu\)m. (E and F) Axonal TrkA-GFP and epidermal growth factor receptor (EGFR)–GFP trafficking in cortical neurons from 0 to 12.5 min after either (E) NGF (50 ng/ml) or (F) EGF (2.5 ng/ml) was added to the synaptic chamber. Transient retrograde waves were observed, similar to that seen for TrkB-GFP in response to its ligand BDNF. Kruskal-Wallis test, Dunn’s multiple comparisons test and one-way ANOVA, Holm-Sidak’s multiple comparisons test, *P < 0.05, **P < 0.01; n = 20 to 36 axons from three independent experiments.
endocytic step. Previous studies have also reported QD-BDNF and QD-NGF in axons only after a period of 1 to 3 hours, and the QDs were usually assessed only after 3 hours (31–33). These differences in the kinetics of BDNF and QD-BDNF in TrkB internalization and retrograde trafficking could be due to steric differences, as observed for QDs during synaptic vesicle recycling (46).

The early time window within which we analyzed endosomal trafficking in our study could influence the number of signaling endosomes measured upon BDNF application. Our results support the notion that administration of a trophic factor at a given synapse induces the retrograde trafficking of a limited number of signaling endosomes. Counting the number of visualized TrkB retrograde-moving vesicle between T2.5 and T7.5 and subtracting the number of TrkB-positive vesicles observed before BDNF application (T0) led us to estimate that between 3.5 and 5.6, TrkB-positive signaling endosomes were formed from 5 to 8 min. While we cannot be sure that these correspond to de novo signaling endosomes, this number was lower than a prior observation of up to 10 QD-NGF–positive endosomes in 1 min (32). This difference could be due to the different time windows (i.e., measuring endosomal trafficking 2.5 min after BDNF application versus 3 hours). From a neurobiological perspective, it is conceivable that the neurotrophic response begins immediately upon receptor binding and continues to build over hours (or even days) to provide ongoing support for synapse formation and maintenance.

The discovery that a neurotrophin has co-opted a calcium-sensing machinery to ensure its own transport to the nucleus raised the possibility of similar mechanisms for other neurotrophins. In support of the previous observation that endocytosis of NGF-activated TrkA requires CaN activation (12), we found that NGF induces a retrograde wave of TrkA signaling endosomes. Moving a bit farther afield, we also found that EGFR induces a retrograde wave of EGF signaling endosomes. Our results align with just-published work showing that EGFR activation by EGF binding triggers calcium release, which causes APPL1 to be quickly liberated from preexisting endosomes; APPL1 then binds to EGFR, and dynein transports the APPL1-EGF–bearing endosomes to the perinuclear region within minutes (47). There seem to be specific subpopulations of endosomes with dedicated factors that regulate sorting of activated receptors to specific endosomal compartments, as has been shown for endophilins (48). It is possible that the same HTT-CaN mechanism could be at play with NGF-TrkA and EGF-EGFR, since HTT regulates the activity of both TrkA and EGFR (49–51). The CaN-HTT complex might also extend to LC3-positive TrkB-containing autophagosomes (52), TrkB-SIPA1L2 amphisomes at boutons (53), or the retrograde transport of NGF/TrkA within multivesicular bodies (54)—but it is equally plausible that there are analogous complexes that serve a similar role to HTT/CaN in the transport of specific endosomes. Similar mechanisms, using messengers other than calcium, could take place in other cell types and regulate the retrograde...
trafficking of antigenic proteins to the endoplasmic reticulum (ER) in dendritic cells, Shiga toxin to the cytosol, and the HIV1 envelope protein to the trans-Golgi network (55).

The question of whether there are analogous complexes becomes particularly important in the context of disease pathology. What happens when Htt is dysfunctional, as in the case of HD? BDNF retrograde transport and TrkB internalization, trafficking, and signaling to the soma of striatal neurons are all perturbed in HD (56, 57). The current study suggests that impaired retrograde routing contributes to the dysfunction and degeneration of the cortical layers that are a hallmark of HD (58, 59). However, it is likely that the brain attempts to compensate for these defects, at least early in development. It is possible that it co-opt other protein complexes and coactivators that participate in endosomal retrograde transport, such as snapin, which recruits dynemin to endosomes (41), or Hook1, a dynemin coactivator that promotes the movement of subpopulations of Rab5- and Rab7-positive endosomes from the distal axon to the soma (10). It will be interesting to examine the functioning of the BDNF-TrkB-CaN-HTT pathway in HD and other diseases involving impaired neurotrophin signaling. These include Rett syndrome, in which dynemin- and Htt-dependent trafficking of BDNF is disrupted (16). Dysregulation of endosomal signaling could also be relevant for Alzheimer’s disease, since β-amyloid oligomers reduce the velocity of BDNF-signaling endosomes moving in the retrograde direction (60). Moreover, elevated expression levels of APP and of its β- C-terminal fragment impair retrograde axonal transport of NGF, leading to degeneration of cholinergic neurons (61). Defects in neurotrophin signaling have also been reported for an early-onset familial form of amyotrophic lateral sclerosis that is due to a mutation in the Rab5 guanine nucleotide exchange factor, Alsn, which causes mislocalization of TrkB in neurons (62).

This phenomenon could also be relevant to the field of cancer. Ligand-activated EGFR and its subsequent internalization/transport trigger intracellular signaling pathways necessary for growth and tissue homeostasis, and their dysregulation is frequently associated with cancers (63). In line with the role of EGFR and its receptor, the additional role of neurotrophin receptors in nonneuronal cells and their association with cancers (64) underscores the importance of understanding the mechanisms by which signaling endosomes navigate trophic signals within the cell. Gaining deeper mechanistic insights into the pathogenesis of these diseases could open new possibilities for treatment.

MATERIALS AND METHODS

Animals

Animals were maintained with access to food and water ad libitum and kept at a constant temperature (19° to 22°C) and humidity (40 to 50%) on a 12:12-hour light/dark cycle. All experimental procedures were performed in an authorized establishment (Grenoble Institut des Neurosciences, U1216, license #B3851610008) in strict accordance with the recommendations of the European Community (86/609/EEC) and the French National Committee (2010/63) for care and use of laboratory animals under the supervision of authorized investigators (permission #91-448 to S. Humbert). All the mice and use of laboratory animals was on a C57/BL6J background. Homozygous Htt⁰⁴²¹D/S⁰⁴²¹D knock-in mice (referred to as HTT-SD), in which the S421 is mutated to aspartic acid; WT or Htt⁰⁴²¹D/S⁰⁴²¹D female mice were crossed with WT or Htt⁰⁴²¹D/S⁰⁴²¹D females. For the p50-GFP x Htt⁰⁴²¹D/S⁰⁴²¹D strain, p50-GFP transgenic mice expressing the p50 dynamin subunit of dynamin fused to GFP under the Thy1 neuronal promoter were crossed with heterozygote Htt⁰⁴²¹D/+ or homozygote Htt⁰⁴²¹D/S⁰⁴²¹D mice.

Neuronal culture

Primary cortical and striatal neurons were prepared as previously described (56). Briefly, cortices were dissected from E17.5 (embryonic day 17.5) rat embryos and E15.5 mice embryos and digested with a solution of papain and cysteine. This was followed by two incubations with trypsin inhibitor solutions (Sigma-Aldrich, #T9253) and mechanical dissociation. The primary cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. All the experiments related to microfluidics were performed at DIV10 to DIV12.

Microfluidic fabrication

Fabrication of the polydimethylsiloxane microfluidic devices with synaptic compartment has been previously described (29). For details, see (65). The coating gradient was prepared as follows. The presynaptic and synaptic parts of the device were filled with poly-D-lysine (0.5 mg/ml), while the postsynaptic part was filled with a mix of poly-D-lysine (0.5 mg/ml) and laminin (10 μg/ml). The device was incubated overnight at 4°C. The devices were then washed out three times with growth medium (Neurobasal medium supplemented with 2% B27, 2 mM GlutaMAX, and 1% penicillin/streptomycin) and placed at 37°C before the neurons were plated.

Stereotaxic injections

Stereotaxic injections into the DLS were performed on WT and Htt⁰⁴²¹D/S⁰⁴²¹D mice at P21. We used both female and male mice from the same litter. First, we anesthetized the mice with isoflurane (1.5 to 2.0%). We then made a midline incision in the skin between the bregma and interaural line and drilled a 1-mm hole in the skull (coordinates: X = −34, Y = 2.4, Z = −2). At this point, we used a capillary connected to a nanoinjector to inject a 500-nl mix of CTB and BDNF mCherry AAV at a speed of 100 nl/min. We then sutured the incision, kept the mouse warm under a heat lamp to allow undisturbed recovery, and provided it booster food.

We waited 2 weeks after surgery before using AAV-injected mice for experiments to allow full expression of viral-mediated gene expression. At P35, we injected the animals with 100 μl of Dolethal and then perfused them transcardially with cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) at a speed of 3.5 ml/min. We removed the brains, fixed them overnight in 4% PFA, and cryoprotected them in 30% sucrose in PBS before embedding them in OCT (optimal cutting temperature compound). We used a cryostat to generate coronal sections (30 μm).

Constructs, plasmids, and lentivirus

The neurons were infected by lentivirus or AAV for 24 hours, only in the cortical compartment, at an early stage (DIV0 to DIV2). The lentiviruses were washed once with growing medium. The following plasmids, AAV, and lentiviruses were used for the study: pWPXLD-Synl-TrkB-mCherry and pWPXLD-Synl-TrkB-GFP originating from p-PE-GFP-N1-TrkB (Rosalind Segal) was cloned in pmCherry-N1 vector (Clontech, #632523); pWPXLD-Synl-TrKA-GFP originating from p-PE-GFP-N1-TrKA (Reiji Kuruvilla); pWPXLD-Synl-Rab5-YFP from pCMV6-Rab5 (OriGene, SC322309); pWPXLD-Synl-EGFR-GFP
from pBABE EGFR WT (Addgene, #11011); AAV5-GCaMP6f (University of Pennsylvania, AV-5-PV2822); and pPWXLd-Syn1-TrkB-mCherry mutated at Y816 to phenylalanine [5′-CCGTCCTTCTCCTGGACATCCATGGGATTCAC-3′ (sense) and 5′-CCAGGAAGACGGGGCACGCTTGGCCAAGT-3′ (antisense)]. The pPWXLd expressed the synapsin I promoter instead of human elongation factor-1 alpha (EF1α) promoter. The oligos to silence CaN were annealed and cloned into pSIN/H1 promoter [for shCaNaκ, 5′-TATGCCCCCAAGATATGATCGAACGAGGAGTATTTCA CGTTTAACAA GATTAAACGT GAAATACTCTGGGGGCA A-3′ (antisense); for shCaNB, 5′-TATGCCCCGG GTTGAATTGGT CTTAAT CTTGTTAAAC GTGAAATACTCTGGGGGCA A-3′ (antisense)].

Reagents
Human BDNF (50 ng/ml) (PeproTech, 450-20), human NGF-β (50 ng/ml) (Sigma-Aldrich, N1408), and human EGF (2.5 ng/ml) (Sigma-Aldrich, E9644) were resuspended in PBS containing bovine serum albumin (BSA), diluted in growth medium (see the “Microfluidic fabrication” section), and added to the synaptic chamber before BDNF treatment. Additional treatments were added as follows. K252a (Sigma-Aldrich, K1639) was used at 10 μM for 15 min inside the synaptic chamber before BDNF treatment.

Immunostaining
All the compartments of the microchambers were fixed at DIV10 to DIV12 with 4% PFA and 4% sucrose in PBS during 20 min at room temperature (RT). Then, the fixation buffer was rinsed three times with 1× PBS and blocked for 1 hour at RT with 1% BSA + 2% normal goat serum + 0.1% Triton X-100. Only the compartment of interest was incubated with primary antibodies at 4°C overnight, and then the secondary antibodies were added at RT for 1 hour. The secondary antibodies were washed three times, and the immunofluorescences were maintained in 1× PBS for a maximum of 1 week at 4°C. The following primary antibodies were used in the blocking solution: HTT (Cell Signaling Technology, #5656; 1:200), PSD95 (Millipore, #MAB1598; 1:1,000; mouse), synaptophysin (Syp) (Abcam, #AB14692; 1:200; rabbit), mCherry (Thermo Fisher Scientific, #16D7; 1:500; rabbit), GFP (Millipore, #AB16901; 1:500; chicken), CaN (Sigma-Aldrich, #C1956; 1:200; mouse), PSD95 (Millipore, #AB1695/07-1491; 1:500; rabbit), mCherry (Thermo Fisher Scientific, #16D7; 1:200; rat), mCherry (Novus, #NPB2-25158; 1:500; chicken), Rab5 (Abcam, #AB218624; 1:1000; rabbit), p-TrkB–Y816 (Sigma-Aldrich, #ABN1381; 1:1000; rabbit), TrkB (Upstate, #07-225 and BD Transduction Laboratories, #610101; 1:1000; rabbit), wheat germ agglutinin (WGA) (Thermo Fisher Scientific, #11570806; 1:1000), MAP2 (Abcam, #AB5392; 1:1000; chicken), GFP (Molecular Probes, #A11122; 1:500; rabbit), GFP (Abcam, #13970; 1:1000; chicken), and SMI31 (Biologic, #801601; 1:1000; mouse). For 2D-STEM microscopy, we used the Abberior kit containing the secondary antibodies (STAR RED anti-mouse or -rabbit, STAR ORANGE anti-mouse or -rabbit) and mounted coverslips with the Abberior mount solid.

QDs preparation, incubation, and acquisition
QDs were prepared as described previously (66). Briefly, streptavidin QDs565 (100 nM; Thermo Fisher Scientific, #Q10131MP) and biotinylated BDNF (1 μM; Thermo Fisher Scientific, #21330) were incubated for 2 min at RT in PBS containing 1% BSA. Neurons at DIV10 to DIV12 were rinsed in warm Dulbecco’s PBS and then starved for 2 hours with warm Phenol-free FluoroBrite Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, #1896701) supplemented with 1% GlutaMAX, 2 mM Hepes, and ascorbic acid (50 ng/ml). The medium was refreshed each 30 min. At the last incubation, the medium in the synaptic chamber was substituted by Neurobasal supplemented with 1% of GlutaMAX and QDs-BDNF for 5 min. The excess of QDs was removed, and neurons were kept in warm FluoroBrite during the acquisition. Cotrafficking of TrkB-GFP and QD-BDNF in the distal axonal compartment was analyzed 5, 10, 15 min and 1 hour after incubation with QD-BDNF.

Immunohistochemistry

PDS/p-TrkB
Brain slices were unmasked with L.A.B solutions (Polysciences, #24310) for 20 min at RT. After sections were blocked for 2 hours at RT in 5% normal goat serum and 0.1% Triton X-100 in PBS, they were incubated overnight at 4°C with PDS95 (Millipore, #MAB1598; 1:400) and p-TrkB (Millipore, #ABN1381; 1:500) antibodies. After three washes, sections were incubated at RT for 2 hours with appropriate fluorescent secondary antibodies. After three washes, sections were mounted on slides in Dako.

p-ERK 3, 3’-diaminobenzidine (DAB) staining
Slices were incubated in 3% H2O2 to block endogenous peroxidase and were then unmasked with L.A.B solutions (Polysciences, #24310) for 20 min at RT. Non-specific sites were blocked for 3 hours in 5% normal donkey serum, 1% BSA, and 0.1% Triton X-100 at RT. Slices were then incubated with anti-p-ERK (Cell Signaling Technology, #4370; 1:200) for 48 hours at 4°C, followed by 3-hour incubation with biotinylated donkey anti-rabbit (Millipore #ABN1381; 1:500) and the ABC kit (VECTORSTAIN, Vector Labs). Staining was revealed using a Nickel-DAB solution. Sections were then dehydrated, delipidated, and mounted on slides with Permount before microscopy.

Vesicle purification and Western blot analysis
Vesicles were prepared as previously described (22). Briefly, mouse forebrains were dissected and homogenized on ice with a glass potter in vesicle lysis buffer [10 mM Heps-KOH, 175 mM l-aspartic acid, 65 mM taurine, 85 mM betaine, 25 mM glycine, 6.5 mM MgCl2, 5 mM EGTA, 0.5 mM D-glucose, 1.5 mM CaCl2, and 20 mM dithiothreitol (DTT) (pH 7.2), with protease inhibitors]. The material was passed through a 25-gauge needle with a 1-ml syringe and then centrifuged at 1200 rpm so that we could collect the total fraction (T). The supernatant was centrifuged at 3000 rpm for 10 min to obtain supernatant 1 (S1; cytosol without nuclei). S1 was processed with additional centrifugation at 12,000 rcf for 40 min to yield P2 (which contains Golgi and ER) and S2 (which is the cytosol without the ER, Golgi, and nuclei). Last, the supernatant was ultracentrifuged at 100,000g to separate the vesicular fractions (P3) from the cytosolic ones (S3). Lysates were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer’s protocols (Bio-Rad). After incubation with 5% BSA in tris-buffered...
saline with Tween 20 (TBST) [10 mM tris (pH 8.0), 150 mM NaCl, and 0.5% Tween 20] for 45 min, the membrane was incubated with antibodies against HTT (Millipore, #MAB2166; 1:500), TrkB (Upstate, #07225 and BD Transduction Laboratories, #610101, 1:1000), CaNA (Cell Signaling Technology, #2314; 1:1000), p150Glu (BD Biosciences, #612708; 1:1000), and tubulin (Sigma-Aldrich, #F2168; 1:10,000) at 4°C for 12 hours. Membranes were washed three times for 10 min and incubated with a 1:10,000 dilution of horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies for 2 hours. Blots were washed with TBST three times and developed with the ECL system (Amersham Biosciences) or Pico/Femto according to the manufacturer’s protocols.

Proteinase K sensitivity and CaN activity assays
The Proteinase K sensitivity assay was performed as previously described (67). Fifty micrograms of vesicle fraction (P3) was added to serial dilutions of Proteinase K (Sigma-Aldrich, #P6556) to make a final concentration of 0, 0.05, 0.25, 1.25, or 6.25 μg/μl in digestion buffer (10 mM Hapes, 10 mM KCl, 2 mM EGTA, and 300 mM sucrose) in a final volume of 50 μl. Vesicles were subjected to digestion by incubation for 30 min at 37°C. To stop the reaction, we added 5 mM of phenylmethylsulfonyl fluoride. For the CaN activity assay, the P3 fractions were incubated with increasing concentrations of CaCl2 (0, 0.1, 1, and 10 μM) for 30 min at 32°C. For both assays, incubation was followed by the addition of Laemmli sample buffer and immediate incubation in 95°C for 5 min.

Endosomes purification and coimmunoprecipitation assay
The protocol was adapted from (41). Briefly, 2-month-old mouse Thy1:p50-GFP brains were treated with 1 ml of homogenization buffer [10 mM Hapes (pH 7.4), 1 mM EDTA, 0.32 M sucrose, and protease inhibitors] and passed into Potter (loose and tight). The buffer [10 mM Hepes (pH 7.4), 1 mM EDTA, 0.32 M sucrose, and 300 mM sucrose] in a final volume of 50 μl. Vesicles were subjected to digestion by incubation for 30 min at 37°C. To stop the reaction, we added 5 mM of phenylmethylsulfonyl fluoride. For the CaN activity assay, the P3 fractions were incubated with increasing concentrations of CaCl2 (0, 0.1, 1, and 10 μM) for 30 min at 32°C. For both assays, incubation was followed by the addition of Laemmli sample buffer and immediate incubation in 95°C for 5 min.

Transmission electron microscopy
For each experiment, 10 μg of vesicle fraction (P3) was adsorbed to Formvar/carbon- supported nickel grids (Gilder grids, 200-mesh Cu/P—EMS, G200-CP), washed with PBS samples, fixed in 2% glutaraldehyde, incubated with quenching solution (50 mM glycine) three times for 3 min each, and blocked 10 min at RT in blocking solution (PBS + 1% BSA). The first primary antibody for CaN (Millipore, #AB1695; 1:30 in PBS + 1% BSA) was incubated for 1 hour at RT, washed in PBS containing 0.1% BSA, and incubated with 5-nm gold anti-rabbit antibody (1:50 in PBS + 1% BSA) for 30 min at RT. The grids were then washed in PBS, and the protocol was repeated, starting from fixation in glutaraldehyde but using the second primary antibody for HTT (Cell Signaling Technology, #5656; 1:30) and 15-nm gold anti-rabbit antibody. Last, grids were fixed in 2.5% glutaraldehyde, washed again in PBS, and stained and embedded by incubation in 1.6% methylcellulose/0.5% uranyl acetate for 10 min at RT in the dark. The samples were examined using a JEOL 1200 EX transmission electron microscope (at 80 kV) equipped with a digital camera (Veleta). Images were taken at a magnification of ×120,000 (with a scale of 100 nm).

In vitro transport assay
Thy1:p50-GFP or Thy1:p50-GFP mice were crossed with HTT<sup>S421D/S421D</sup> mice. Brains of the offspring were fractionated in vesicle buffer [10 mM Hapes-KOH, 175 mM L-aspartic acid, 65 mM taurine, 85 mM betaine, 25 mM glycine, 6.5 M MgCl₂, 5 M EGTA, 0.5 mM Mg-ATP, 1.5 mM CaCl₂, and 20 mM DTT (pH 7.2), with protease inhibitor] using increasing centrifugation speeds as described above with an additional final sucrose gradient step to isolate motility vesicles (22). In vitro polymerized rhodamine-labeled microtubules were seeded in a flow chamber. Purified vesicles were diluted in motility buffer [BRB80, 0.5% Pluronic F127, 1 mM Taxol, BSA (10 mg/ml) in BRB80, 1 M DTT, glucose oxidase (0.5 mg/ml), 20 M Mg-ATP, glucose (15 mg/ml), and catalase (470 U/ml)] and labeled with the green fluorescent lipophilic carbocyanine tracer [DiOC18(3), Thermo Fisher Scientific] after a 20-min incubation with NP-EGTA at 10 μM concentration. The vesicles were then loaded in the chambers and analyzed by TIRF microscopy coupled with UV fluorescence recovery after photobleaching (FRAP) (Scale bar 5 μm) of the regions of interest (ROIs).

Live cell and confocal imaging
All the live cell images were acquired using an inverted microscope (Axio Observer, Zeiss) coupled to a spinning disk confocal system (CSU-W1-T3; Yokogawa) connected to an electron-multiplying CCD (charge-coupled device) camera (ProEM+1024, Princeton Instrument) and maintained at 37°C and 5% CO₂. Images were taken...
every 0.2 s for 30 s for TrkB-mCherry, TrkA-GFP, and EGFR-GFP trafficking and for the dual color acquisition (488 to 565) of TrkB-GFP and QDs-BDNF [with a 63× 1.46 numerical aperture (NA) oil immersion objective]. TrkB, HTT, and CaN immunostaining were visualized inside the cortical axonal microchannel, whereas for p-TrkB and PSD95, the Z-stack acquisitions were taken in the cortical layer V of brain slices. Immunostaining images were acquired with a 63× oil immersion objective (1.4 NA) using a Zeiss LSM 710 inverted confocal microscope coupled to an Airyscan detector to improve signal-to-noise ratio and resolution. For STED microscopy, we imaged with a 100× oil immersion objective (1.46 NA) using the Abberior 2D-STEDYCON upright confocal microscope. Phospho-ERK immunohistochemistry was visualized with a 20× objective (0.45 NA) using a slide scanner (AxioScan Z1, Zeiss).

**Quantification and statistical analyses**

**Electron microscopy**

HTT and CaN colocalization on purified vesicles were counted manually and compared to control conditions in which only the immunogold particles (5- and 15-nm sizes), but no antibody were used. Three grids per conditions were analyzed, and five fields per grid were counted for each condition \( (n = \text{number of counted vesicles}) \).

**Kymograph analysis**

Kymographs were generated using KymoToolBox plugin for Imagej (21) with a length of 100 μm \((x\text{ axis})\) and a total time of 30 s \((y\text{ axis})\) to extract the following kinetic parameters, as previously described (29): anterograde and retrograde velocities, number of anterograde and retrograde vesicles, linear flow rate, and directional flux. Vesicles were considered motile when their velocity was above 0.12 μm/s. Each condition was tested using two to three chambers from at least three independent cultures. In each chamber, one field containing at least four axons was analyzed to reach a minimum number of 30 axons \( (n = \text{number of axons}) \).

**Immunolabeling of isolated axons**

Immunostained vesicles in distal axons were quantified with the Jacob plugin for Imagej in case of labeling of two endogenous proteins and reported as the number of pixels colocalizing per 100 μm \((69)\). Mander’s coefficients 1 and 2 were equal, so we report only coefficient number 1. When three proteins were stained, the analysis was performed with a customized macro for Imagej developed in our laboratory. In particular, the images are enhanced using a DoG (Difference of Gaussians) filter adapted to the vesicle size. Masks are created on each channel using manual thresholding that is kept constant for each individual channel and replicates. Last, the number of particles is automatically counted for the single, dual, and triple channels and expressed as the percentage of colocalization.

**In vivo p-ERK analysis (DAB)**

We counted the number of p-ERK–immunopositive cells manually and expressed the result as a function of analyzed area in square millimeters. We analyzed three sections per mouse, from three mice, and used three areas per acquisition.

**In vivo p-TrkB/PSD95 analysis**

We used Imagej for colocalization analyses. Airyscan images were thresholded to remove nonspecific signal and define an area of interest of at least 100 μm in length around neurites. We then manually counted the number of p-TrkB spots that overlapped PSD95 spots or were juxtaposed to them (i.e., separated by no more than 2 pixels or 130 nm). Results were expressed as a function of neurite length and were normalized to 100 μm. Each condition was tested using two chambers per culture from three independent cultures. In each chamber, three fields were analyzed in which three ROIs were selected.

**Calculation of retrograde TrkB-positive signaling endosomes**

The number of TrkB-positive signaling endosomes moving in the retrograde direction per minute in a given axon was estimated as follows: \( \text{Ne} = (\text{Nr}_{t2.5-T7.5} - \text{Nr}_{t0}) \times 2 = 1.4 \text{ endosomes/min.} \) Nr corresponds to the mean number of retrograde-moving TrkB-containing vesicles at 2.5, 5, and 7.5 min, the time periods for which the number of retrograde TrkB vesicles is significantly higher than at T0. T0 corresponds to the time before BDNF application, when the TrkB-containing vesicles that are recorded correspond to TrkB vesicles moving anterogradely and retrogradely but are likely not signaling endosomes, as they do not result from endocytosis in response to BDNF. Numbers are obtained by recording trafficking of a single axon of 100 μm in length for a 30-s period (which is why, in the equation above, we multiply by 2 to get the number per minute).

**Calcium events analysis (GCaMP)**

The number of calcium events was counted manually in a given GCaMP6-positive axon for a 30-s period beginning at T0 (before BDNF application), and again every 2.5 min from T2.5-T12.5 after BDNF application. Each condition was tested using two to three chambers from at least three independent cultures. In each chamber, one field containing at least four axons was analyzed to reach a minimum number of 30 axons \( (n = \text{number of axons}) \).

**Statistical tests**

All statistical analyses were done with GraphPad Prism8 (GraphPad Software, San Diego, CA). We assessed the normality of the data with a D’Agostino-Pearson test, using a threshold of \( \alpha = 0.05 \). When \( n \) was too small to be analyzed by a normality test, such as occurs in Western blotting analyses, then we assumed normality.

Trafficking kinetics were compared using one-way analysis of variance (ANOVA), followed by Dunnett’s post hoc analysis for multiple comparisons when the data were normally distributed. If the assumption of normality was not rejected, then the groups were compared using a Kruskal-Wallis test, followed by either a Dunn’s post hoc analysis (time or genotype) or two-way ANOVA; when two variables were analyzed (time and treatment or genotyping), we followed this with a Holm-Sidak’s test.

Calcium events (GCaMP6f) in TrkB-WT–positive axons were analyzed using a Friedman nonparametric test, followed by Dunn’s post hoc analysis; when the increase in calcium was analyzed comparing the number of events in TrkB-WT– and TrkB-Y816F–infected axons, an unpaired two-tailed Student’s \( t \) test (Mann-Whitney test) was performed.

The presence of CaN and HTT on purified vesicles for electron microscopy experiments, immunofluorescence comparing treatment, and TrkB-Y816-SypI colocalization was all analyzed using an unpaired two-tailed Student’s \( t \) test (Mann-Whitney test) or Kruskal-Wallis test, followed by a Dunn’s post hoc analysis. When the colocalization was analyzed with the JACoP plugin, we chose the ROI as a portion of an isolated axon. The data for each ROI were compared to the corresponding value for random colocalization (control condition or “CT”), and the Mander’s coefficients were calculated (Mander’s coefficients 1 and 2 were equivalent in our cases, so we report only coefficient 1). The values for the control condition were obtained by multiplying the percentage of pixels...
present in each acquired fluorescent channel (e.g., green and red channels) by the area of the ROI. In this way, we evaluate the amount of random overlapping between the two channels in the ROI. The statistical test performed is a Wilcoxon matched-pairs signed-rank test. Synapse formation (p-TrkB/PSD95) and the level of protein revealed by Western blot were analyzed using unpaired two-tailed Student’s t test or one-way ANOVA, followed by Dunnnett’s multiple comparison test.

In vitro motility assays and presynaptic signaling (p-ERK) were analyzed using unpaired two-way ANOVA, followed by a Holm-Sidak’s test when comparing more than two variables (treatment and genotyping). All results are presented as means ± SEM. We took P < 0.05 to indicate a statistically significant difference. All statistical tests, n, and specific P values are listed in table S1.

SUPPLEMENTARY MATERIALS
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REFERENCES AND NOTES
1. M. M. Fu, E. L. Holzbaur, Integrated regulation of motor-driven organelle transport by scaffolding proteins. Trends Cell Biol. 24, 564–574 (2014).
2. S. Maday, A. E. Twelvetrees, A. J. Moughamian, E. L. F. Holzbaur, Axonal transport: Cargo-specific mechanisms of motility and regulation. Neuro Neurol. 84, 292–309 (2014).
3. H. D. MacGillivray, C. C. Hoogenraad, Membrane trafficking and cytoskeletal dynamics in neuronal function. Mol. Cell. Neurosci. 91, 1–2 (2018).
4. R. P. Tas, A. C hazeau, B. M. C. Cloin, M. L. A. Lambers, C. C. Hoogenraad, L. C. Kapitein, Differentiation between oppositely oriented microtubules controls polarized neuronal transport. Nature. 564, 1264–1271.e7 (2015).
5. E. E. Zahavi, R. Maimon, E. Perlson, Spatial-specific functions in retrograde neuronal signalling. Traffic. 18, 415–424 (2017).
6. D. Villarreal-Campos, G. Schiavo, O. M. Lazo, The many disguises of the signalling endosome. FEBS Lett. 592, 3615–3632 (2018).
7. E. Scott-Solomon, R. Kuruvilla, Mechanisms of neurotrophin trafficking via Trk receptors. Mol. Cell. Neurosci. 91, 25–33 (2018).
8. A. W. Harrington, D. D. Ginty, Long-distance retrograde neurotrophic factor signalling in neurons. Nat. Rev. Neurosci. 14, 177–187 (2013).
9. O. E. Tasdemir-Yilmaz, R. A. Segal, There and back again: Coordinated transcription, translation and transport in axonal survival and regeneration. Curr. Opin. Neurobiol. 62, 68–66 (2020).
10. M. A. Olencik, R. Dominguez, E. L. F. Holzbaur, Dynine activator Hook1 is required for trafficking of BDNF-signaling endosomes in neurons. J. Cell Biol. 218, 220–233 (2019).
11. K. M. Lehigh, K. M. West, D. D. Ginty, Retrogradely transported TrkA endosomes signal locally within dendrites to maintain sympathetic neuron synapses. Cell Rep. 17, 86–100 (2017).
12. D. Bodnar, M. Ascano, R. Kuruvilla, Isoform-specific phosphorylation of dynamin1 by calcium/calcineurin couples neurotrophin receptor endocytosis to axonal growth. Nature. 70, 1085–1091 (2013).
13. B. Lu, G. Nagappan, Y. Lu, BDNF and synaptic plasticity, cognitive function, and specific functions in retrograde neuronal signalling. Traffic. 18, 415–424 (2017).
14. V. Mariano, D. D. Ginty, Long-distance retrograde neurotrophic factor signalling in neurons. Nat. Rev. Neurosci. 10, 850–860 (2009).
15. T. Wang, S. Martin, T. H. Nguyen, C. B. Harper, R. S. Gormal, R. Martinez-Marmol, S. Karanamithi, E. J. Coulson, N. R. Glass, J. J. Cooper-White, B. van Swinderen, F. A. Meunier, Flux of signalling endosomes undergoing axonal transport conveys to promote survival of target-dependent neurons. Nat. Commun. 7, 12976 (2016).
16. M. S. Thion, J. R. McGuirre, C. M. Sousa, L. Fuhrmann, J. Fitamant, S. Lebourge, S. Vacher, S. T. du Montcel, I. Bieche, A. Benet, P. Mehlensch, A. Vincent-Salomon, S. Humbert, Unraveling the role of huntingtin in breast cancer metastasis. J. Natl. Cancer Inst. 107, dpv208 (2015).
17. E. E. Zahavi, J. J. A. Hummel, Y. Han, C. Bar, R. Scuici, M. Alteal, C. C. Hoogenraad, Combined kinase-1 and kinase-3 activity drives axonal trafficking of TrkB receptors in Rab6 carriers. Dev. Cell 56, 494–508.e7 (2021).
18. H. M. Heerssen, M. F. Pazyra, R. A. Segal, Dynine motors transport activated Trks to promote survival of target-dependent neurons. Nat. Neurosci. 7, 906–904 (2004).
19. B. Zhou, Q. Cai, Y. Xie, Z. H. Sheng, Snapin recruits dynine to BDNF-TrkB signaling endosomes for retrograde axonal transport and is essential for dendrite growth of cortical neurons. Cell Rep. 2, 42–51 (2012).
20. B. Xu, K. Zang, N. R. Ruff, Y. A. Zhang, S. K. McConnell, M. P. Stryker, L. F. Reichardt, Cortical degeneration in the absence of neurotrophin signaling: Dendritic retraction and neuronal loss after removal of the receptor TrkB. Neuron. 26, 233–245 (2000).
21. T. W. Chen, T. J. Wardill, Y. Sun, S. R. Pulver, S. L. Remedios, A. Basian, E. R. Schreiter, R. A. Kerr, M. B. Orger, J. Layton, L. L. Looger, K. Svoboda, D. S. Kim, Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature. 499, 295–300 (2013).
44. R. Kuruvilla, L. S. Zweifel, N. O. Glebova, B. E. Lonze, G. Valdez, H. Ye, D. D. Ginty, A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkB trafficking and retrograde signaling. Cell 118, 243–255 (2004).

45. S. Sigismund, T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P. P. Di Fiore, S. Polo, Clathrin-independent endocytosis of ubiquitinated cargos. Proc. Natl. Acad. Sci. U.S.A. 102, 2760–2765 (2005).

46. Q. Zhang, Y. Q. Cao, R. W. Tsien, Quantum dots provide an optical signal specific to full collapse of fusion vesicles. Proc. Natl. Acad. Sci. U.S.A. 104, 17843–17848 (2007).

47. H. M. York, A. Patil, U. K. Moothri, A. Kaur, A. Bhomwik, G. J. Hyde, H. Gandhi, A. Fulcher, K. Gaus, S. Arumugam, Rapid whole cell imaging reveals a calcium-APPL1-dynein nexus that regulates cohort trafficking of stimulated EGFR receptors. Commun. Biol. 4, 224 (2021).

48. K. Burk, J. D. Murdoch, S. Freytag, M. Koenig, V. Bharat, R. Markworth, S. Burkhart, A. Fischer, C. Dean, EndophilinsA regulate endosomal sorting of BDNF-TrkB to mediate survival signalling in hippocampal neurons. Sci. Rep. 7, 2149 (2017).

49. J. Rong, J. R. McGuire, Z.-H. Fang, G. Sheng, J.-Y. Shin, S.-H. Li, X.-J. Li, Regulation of intracellular trafficking of huntingtin-associated protein-1 is critical for TrkB protein levels and neurite outgrowth. J. Neurosci. 26, 6019–6030 (2006).

50. C. Moreira Sousa, J. R. McGuire, M. S. Thion, D. Gentien, P. de la Grange, N. L. Kononenko, G. A. Classen, M. Kuijpers, D. Puchkov, T. Maritzen, A. Tempes, J. C. Lievens, T. Rival, M. Iche, H. Chneiweiss, S. Birman, Expanded polyglutamine peptides disrupt EGFR receptor signaling and glumate transporter expression in Drosophila. Hum. Mol. Genet. 14, 713–724 (2005).

51. J. L. Plotkin, M. Day, J. D. Peterson, Z. Xie, G. J. Kress, I. Rafalovich, J. Kondapalli, M. Barnat, M. Capizzi, E. Aparicio, S. Boluda, D. Wennagel, R. Kacher, R. Kassem, S. Lenoir, W. W. Poon, A. J. Carlos, B. L. Aguilar, N. C. Berchtold, C. K. Kawano, V. Zograbyan, W. Xu, A. M. Weissmiller, J. A. White II, F. Fang, X. Wang, Y. Wu, M. L. Pearn, X. Zhao, T. S. Gertler, M. Flajolet, P. Greengard, M. Stavarache, M. G. Kaplitt, J. Rosinski, C. S. Chan, E. D. Gundelfinger, M. Kneussel, C. Spilker, A. Karpova, M. R. Kreutz, SIPA1L2 controls and prevents neurodegeneration. Nat. Commun. 5, 5484 (2014).

52. S. Polo, Clathrin-independent endocytosis of ubiquitinated cargos. Trends Cell Biol. 33, 634–643 (2013).

53. A. Fischer, C. Dean, EndophilinAs regulate endosomal sorting of BDNF-TrkB to mediate survival signaling in hippocampal neurons. Cell 129, 243–255 (2007).

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