Uptake and recycling of pro-BDNF for transmitter-induced secretion by cortical astrocytes

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Activity-dependent secretion of brain-derived neurotrophic factor (BDNF) is thought to enhance synaptic plasticity, but the mechanisms controlling extracellular availability and clearance of secreted BDNF are poorly understood. We show that BDNF is secreted in its precursor form (pro-BDNF) and is then cleared from the extracellular space through rapid uptake by nearby astrocytes after θ-burst stimulation in layer II/III of cortical slices, a paradigm resulting in long-term potentiation of synaptic transmission. Internalization of pro-BDNF occurs via the formation of a complex with the pan-neurotrophin receptor p75 and subsequent clathrin-dependent endocytosis. Fluorescence-tagged pro-BDNF and real-time total internal reflection fluorescence microscopy in cultured astrocytes is used to monitor single endocytic vesicles in response to the neurotransmitter glutamate. We find that endocytosed pro-BDNF is routed into a fast recycling pathway for subsequent soluble NSF attachment protein receptor–dependent secretion. Thus, astrocytes contain an endocytic compartment competent for pro-BDNF recycling, suggesting a specialized form of bidirectional communication between neurons and glia.

Introduction

Activity-dependent secretion of brain-derived neurotrophic factor (BDNF) into the extracellular space is a key step in the induction of long-term synaptic modification (Poo, 2001). It has been suggested that the effect of BDNF is critically dependent on whether it is secreted in its precursor form (pro-BDNF), which preferentially binds to the pan-neurotrophin receptor p75 ($p75^{NTR}$), or in its mature form, which activates the tropomyosin-related kinase B receptor (TrkB), because activation of these distinct receptors has opposite effects on synaptic strength (Lu, 2005). In addition, pro-BDNF can be processed in the extracellular space by tissue plasminogen activator/plasmin (Pang et al., 2004), further modulating synaptic modification by the neurotrophin. However, it was recently reported that processing of pro-BDNF occurs intracellularly in cultured hippocampal neurons and that the neurotrophin is secreted only in its mature form (Matsumoto et al., 2008).

To improve our understanding of the mechanisms controlling the extracellular availability of endogenous BDNF and the termination of its action, we examined the fate of both pro-BDNF and mature BDNF in cortical brain slices after θ-burst stimulation (TBS), a well-established paradigm inducing both long-term potentiation of synaptic transmission and secretion of BDNF (Aicardi et al., 2004). We provide compelling evidence that BDNF, which is newly synthesized in neurons after TBS, is secreted in its pro-form and is then rapidly internalized in perineuronal astrocytes, thereby restricting the availability of the neurotrophin

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Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; Map2, microtubule-associated protein 2; MDC, monodansylcadaverine; QD, quantum dot; TBS, θ-burst stimulation; TeNT, tetanus neurotoxin; TIRF, total internal reflection fluorescence; TrkB, tropomyosin-related kinase B receptor; TrkB-t, truncated TrkB; Vamp2, vesicle-associated membrane protein 2.

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at neuron–astrocyte contacts. After internalization, the neurotrophin can undergo a recycling process, endowing astrocytes with the ability to resecrete the neurotrophin upon stimulation.

Results and discussion

Field recordings were performed in layers II/III of rat perirhinal cortex slices (Fig. 1 A) subjected to either basal (0.033 Hz) or TBS (100 Hz) stimulation. BDNF secretion was measured in the collected perfusion medium by ELISA (Fig. 1 C). Although BDNF levels remained constant during basal stimulation, TBS induced a rapid, transient increase in BDNF in the perfusate, which correlated with induction of synaptic potentiation of the field potential as described previously (Aicardi et al., 2004). Fig. 1 C shows representative examples of pro-BDNF immunoreactivity in the perirhinal cortex upon basal and TBS stimulation using an antibody directed specifically (Fig. 1 B) against the pro-region of the neurotrophin. Basal levels of pro-BDNF were detected proximal to (Fig. 1 A, A1) and distal from (Fig. 1 A, A2) the stimulation electrode in controls. A marked increase in pro-BDNF immunoreactivity was observed after TBS, which gradually declined distally from the stimulation electrode. This effect was blocked by prior treatment with the protein synthesis inhibitor anisomycin (unpublished data), consistent with the pro-BDNF increase depending on activity-dependent local protein synthesis (Kandel, 2001). Similar results were obtained using an antibody directed against the mature portion of the neurotrophin that recognizes both mature and pro-BDNF (Fig. 1 D).

High resolution confocal analysis of individual neurons in the A1 region confirmed that pro-BDNF immunoreactivity was increased after TBS (Fig. 1 E and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200806137/DC1). Surprisingly, pro-BDNF was also localized in perineuronal astrocytes (Fig. 1 E). As astrocytes lack mRNA for BDNF (Ernfors et al., 1990; Conner et al., 1997), these data suggest that pro-BDNF was taken up by these cells upon secretion from nearby activated neurons. Notably, pro-BDNF immunoreactivity was typically punctate within astrocytic processes in contact with nearby neurons, whereas it appeared to be concentrated in larger clusters in the cell body (Fig. 1 E). This immunoreactivity pattern suggests intracellular trafficking of pro-BDNF after its transfer from nearby neurons to astrocytes at sites of neuron–glia contacts. We thus decided to follow the time course of pro-BDNF distribution within astrocytes after TBS (Fig. 1 F). Pro-BDNF uptake in individual astrocytes was determined by measuring colocalization of pro-BDNF immunoreactivity with that of the glial fibrillary acidic protein (GFAP), an astrocytic-specific cytoskeletal protein. In accordance with a transfer of pro-BDNF from neurons to astrocytes, colocalization of pro-BDNF to GFAP was first found within the periphery of astrocytic processes 5 min after TBS. At later time points (10–30 min), the overall quantity of pro-BDNF found in astrocytes had increased, and the intracellular distribution gradually shifted from the processes to the cell body. Finally, pro-BDNF levels returned to basal levels in both compartments after 3 h.

Although astrocytes were largely devoid of pro-BDNF under basal stimulation, 10–12% of GFAP-labeled individual astrocytes were positive for pro-BDNF 10 min after TBS (Fig. 1 G and Fig. S1). This effect was prevented by prior incubation with anisomycin, indicating that accumulation of pro-BDNF in astrocytes required activity-dependent synthesis in neurons, or with TrkB-Fc, a scavenger for secreted BDNF in both precursor and mature forms (Fayard et al., 2005). Strikingly, the astrocytic uptake was selective for pro-BDNF, as it was markedly reduced upon prior incubation with plasmin (Fig. 1 G and Fig. S1), an enzyme responsible for the extracellular proteolysis of pro-BDNF to mature protein.

The fact that we observe pro-BDNF secretion stands in contrast to a recent study that BDNF may only be released in its processed, mature form (Matsumoto et al., 2008). This discrepancy may be due to the fact that, in the latter study, BDNF processing was studied in unstimulated hippocampal tissue and hippocampal cultures after prolonged (1 d) stimulation, whereas we obtained evidence for transient pro-BDNF secretion within a few minutes after TBS. Much of this secreted pro-BDNF has been newly synthesized and, given the rapid transfer to astrocytes, is likely to derive from locally translated BDNF mRNA. Interestingly, another recent study has shown that BDNF mRNA occurs in two splice variants, one of which is selectively transported to the dendrites of hippocampal neurons, and BDNF derived from this transcript is required for synaptic and morphological modifications (An et al., 2008). Local synthesis may indeed favor secretion of pro-BDNF given that the machinery to process the neurotrophin is likely to be absent from dendrites.

We next investigated whether pro-BDNF uptake by astrocytes occurs by receptor-mediated internalization. We observed that pro-BDNF immunoreactivity within astrocytes colocalized with that of $p75^{NTR}$ 10 min after TBS. Clusters of pro-BDNF–$p75^{NTR}$ were found at sites in contact with nearby neurons and astrocytic processes, a pattern suggestive of $p75^{NTR}$-mediated internalization of pro-BDNF (Fig. 2 A). Accordingly, in slices of $p75^{NTR}$ knockout mice ($p75^{NTR-/-}$; Naumann et al., 2002) and different from slices of wild-type mice ($p75^{NTR+/+}$), pro-BDNF did not accumulate in astrocytes (Fig. 2 B and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200806137/DC1). However, preventing TrkB internalization with the kinase inhibitor K252a did not affect GFAP/pro-BDNF colocalization (Fig. 2 C), which is consistent with the notion that astrocytes do not express the full-length form of TrkB (Rose et al., 2003). Because data obtained in slices do not rule out the involvement in BDNF endocytosis of truncated TrkB (TrkB-t) forms (Rubio, 1997) lacking the catalytic domains (Klein et al., 1990), experiments were extended to primary cultures of cortical astrocytes. Surface biotinylation experiments showed that, in addition to $p75^{NTR}$, cultured astrocytes express TrkB-t but not full-length TrkB (Fig. 2 D). However, upon exposure of astrocytes to BDNF, a mixture of precursor and mature isoforms (mix; Fig. 1 B), only the membrane expression of $p75^{NTR}$, but not TrkB-t, was reduced. As a consequence, the same lysate in which plasma membrane levels of $p75^{NTR}$ were reduced by BDNF (mix) contained a high quantity of BDNF protein. Altogether, these data indicate that pro-BDNF uptake occurs primarily by $p75^{NTR}$, which is consistent with the notion that pro-BDNF binds preferentially to this receptor (Teng et al., 2005).

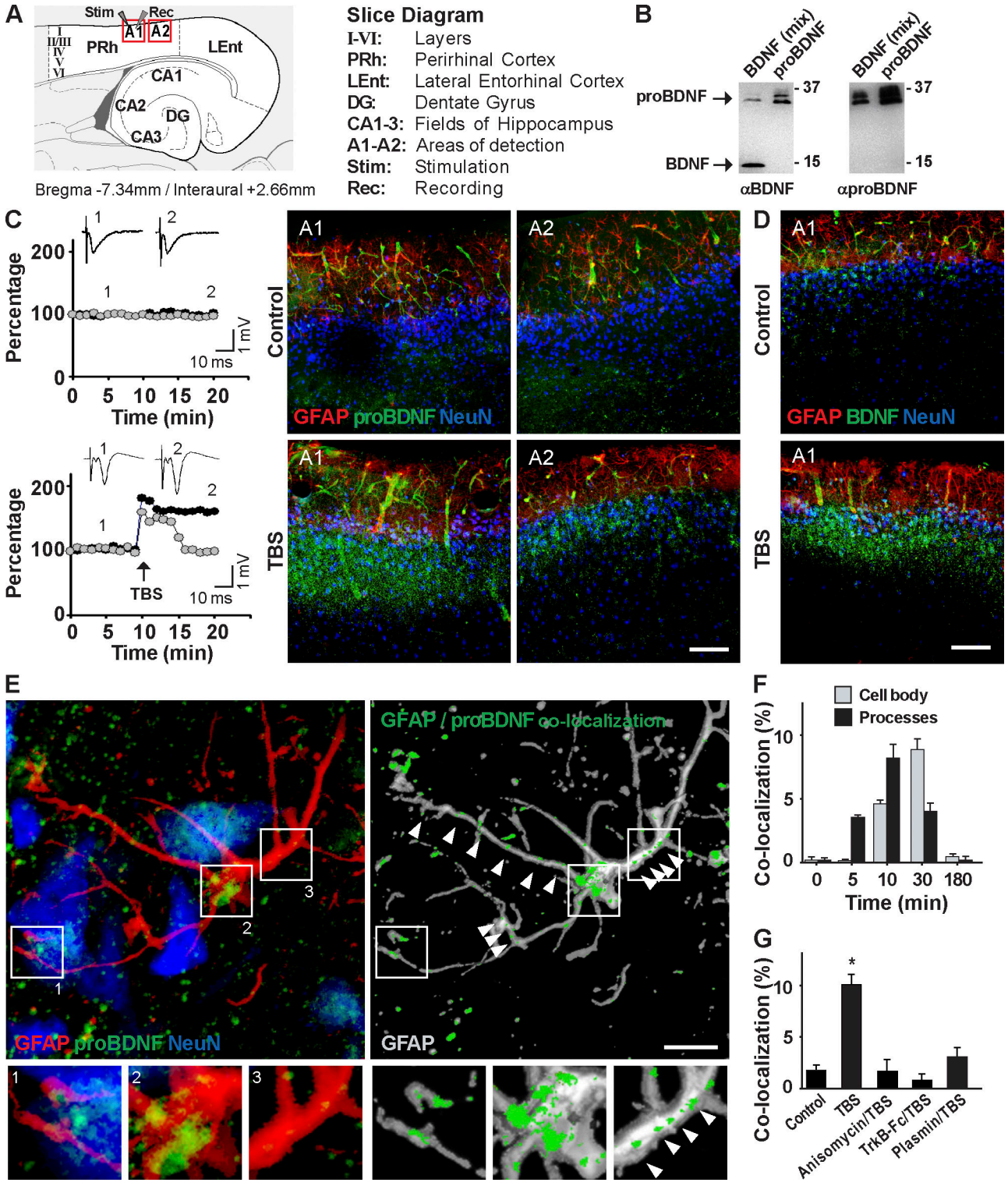

Figure 1. **Transfer of pro-BDNF from neurons to perineuronal astrocytes.** (A) Schematic representation of the slice preparation. (B) Western blot analysis of BDNF (mix) or cleavage-resistant pro-BDNF (Mowla et al., 2001) using α-BDNF– or α–pro-BDNF–specific antibodies. (C) Field potential amplitudes (black circles) and BDNF levels (gray circles) upon basal (control) or TBS stimulations. After recording, slices were immunostained using α–pro-BDNF. Immunoreactivity is shown in two adjacent areas corresponding to areas A1 and A2 of A. (D) Immunohistochemistry using α-BDNF. Bars, 100 μm. (E) High resolution confocal images of A1 in a slice 20 min after TBS. Pro-BDNF immunoreactivity is shown at the site of astrocytic contact with a neuron (box and inset 1), the astrocytic cell body (box and inset 2), and processes (box and inset 3). Colocalization of pro-BDNF with GFAP immunoreactivity is shown and superimposed onto the 3D reconstruction of the GFAP signal. Arrowheads indicate pro-BDNF immunoreactive puncta distributed along the astrocytic processes. Bar, 20 μm. (F) Time course of pro-BDNF/GFAP colocalization (four slices and nine cells). (G) Pro-BDNF/GFAP colocalization in astrocytes of control (three slices and 12 cells) or TBS slices in the absence (six slices and 24 cells) or presence of anisomycin (five slices and 11 cells), TrkB-Fc (four slices and nine cells), and plasmin (five slices and 24 cells) 10 min after stimulation. NeuN, neuronal nuclei. Data are means ± SEM (error bars). *, $P \leq 0.05$.

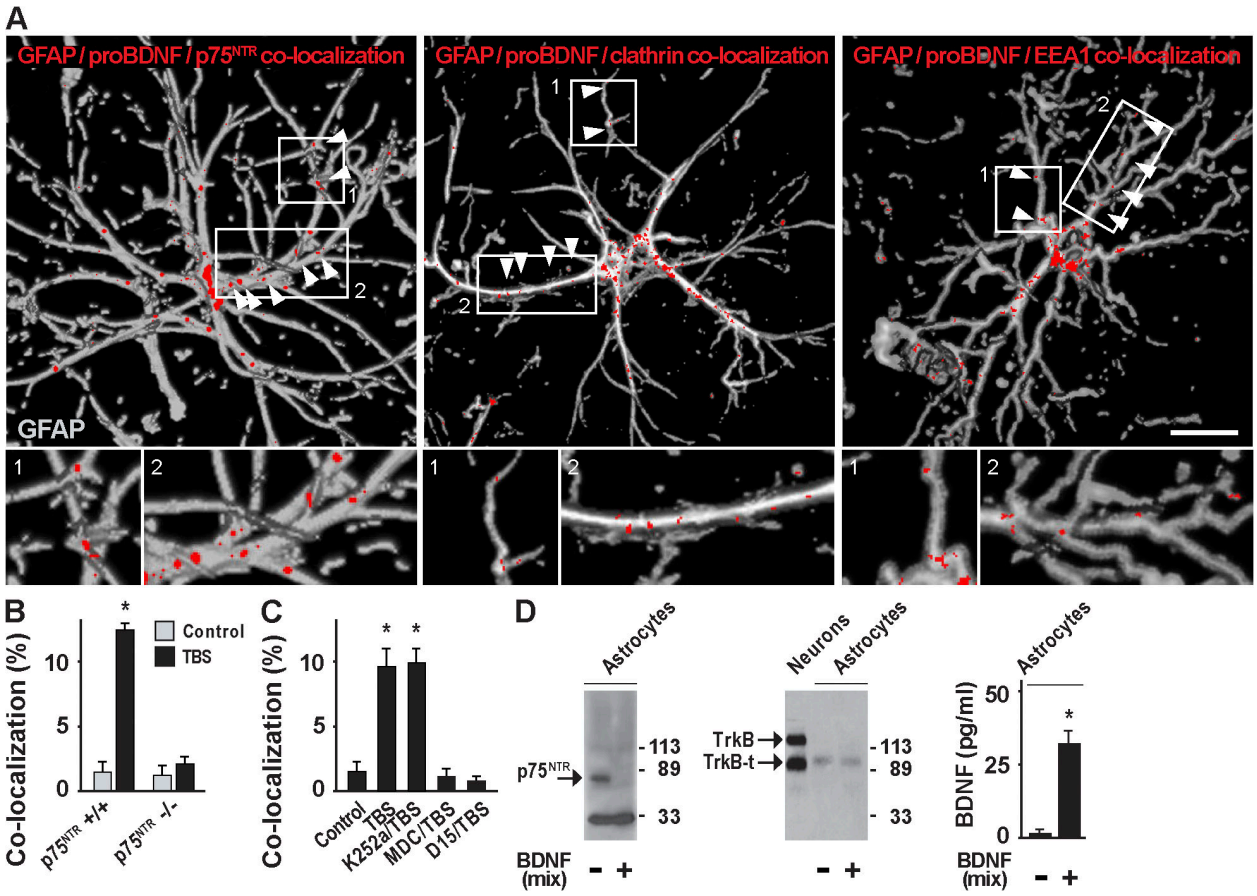

Figure 2. **p75^{NTR}–clathrin-mediated internalization of pro-BDNF in astrocytes.** (A) Colocalization between GFAP, pro-BDNF and p75^{NTR}, clathrin, or EEA1 immunoreactivity in astrocytes 10 min after slice exposure to TBS. Colocalization signal is shown at the site of astrocytic contact with a neuron (box and inset 1) and astrocytic processes (box and inset 2). Bar, 10 µm. (B) Pro-BDNF/GFAP colocalization in astrocytes of control (five slices and 22 cells) or TBS slices (five slices and 18 cells) in p75$^{NTR+/+}$ and p75$^{NTR-/-}$ mice. (C) Pro-BDNF/GFAP colocalization in astrocytes of control (six slices and nine cells) or TBS slices in the absence (four slices and 12 cells) or presence of K252a (four slices and nine cells), MDC (three slices and 11 cells), or D15 (three slices and 11 cells). (D) Western blot showing surface expression of p75^{NTR}, TrkB, or TrkB-t from control astrocytes or astrocytes exposed to BDNF (mix). TrkB and TrkB-t expression from cultured neurons is shown for comparison. The right panel shows ELISA measurement of BDNF concentration in astrocytes. Data are means ± SEM (error bars). *, $P \leq 0.05$.

To further investigate the molecular mechanism of pro-BDNF–p75^{NTR}-mediated internalization, we examined whether endocytic pro-BDNF colocalized with clathrin (Bronfman et al., 2003). We found that clathrin immunoreactivity colocalized with that of pro-BDNF after TBS (Fig. 2 A). This effect was blocked by prior treatment with the clathrin inhibitor monodansylcadaverine (MDC) or the dynamin blocker D15 (Fig. 2 C and Fig. S2; Wigge and McMahon, 1998). Likewise, we observed colocalization of pro-BDNF with the early endosomal marker EEA1 (Fig. 2 A). Overall, these data provide evidence that astrocytic uptake of endogenous pro-BDNF occurs via p75^{NTR}–clathrin-mediated internalization in endocytic compartments.

To obtain further insight into the possible regulation of pro-BDNF uptake in astrocytes, total internal reflection fluorescence (TIRF) microscopy (Thompson and Steele, 2007) was used to visualize the formation of single endocytic vesicles in real time (Fig. 3 A and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200806137/DC1). Cultured astrocytes were transfected with p75^{NTR} tagged with GFP (p75-GFP) for evanescent light excitation of p75-GFP residing within or in close proximity to the plasma membrane. The binding of pro-BDNF to p75-GFP was imaged in real time using pro-BDNF immunocomplexed with 10 nM of quantum dots (QDs; Fig. S3). Once a pro-BDNF–QD was found in the vicinity of the plasma membrane of a p75-GFP–expressing astrocyte, p75-GFP fluorescence became concentrated at the site of the QD within a few seconds, presumably reflecting the formation of endocytic vesicles and internalization of the pro-BDNF–QDs. Confocal microscopy (Fig. 3 B) showed that internalization of pro-BDNF–QDs was inhibited at a nonpermissive temperature (ice cold) for endocytosis and was restored by raising the temperature to 37°C for 10–20 min. QD uptake also depended on the level of p75^{NTR} expression: although astrocytes overexpressing p75-GFP showed high levels of QD internalization, significantly fewer QDs were taken up in astrocytes transfected with plasma membrane–linked GFP (Lck-GFP), which only relies on endogenous p75^{NTR} for internalization (Fig. 3, B and D). Likewise, QD uptake was virtually abolished in astrocytes prepared from p75$^{NTR-/-}$ mice (Fig. 3 C). Moreover, QD internalization required prior coupling to pro-BDNF, as it ceased when the α–pro-BDNF antibody was omitted from the immunocomplexes for control (Fig. 3 B). Lastly, internalized QDs colocalized with clathrin and EEA1

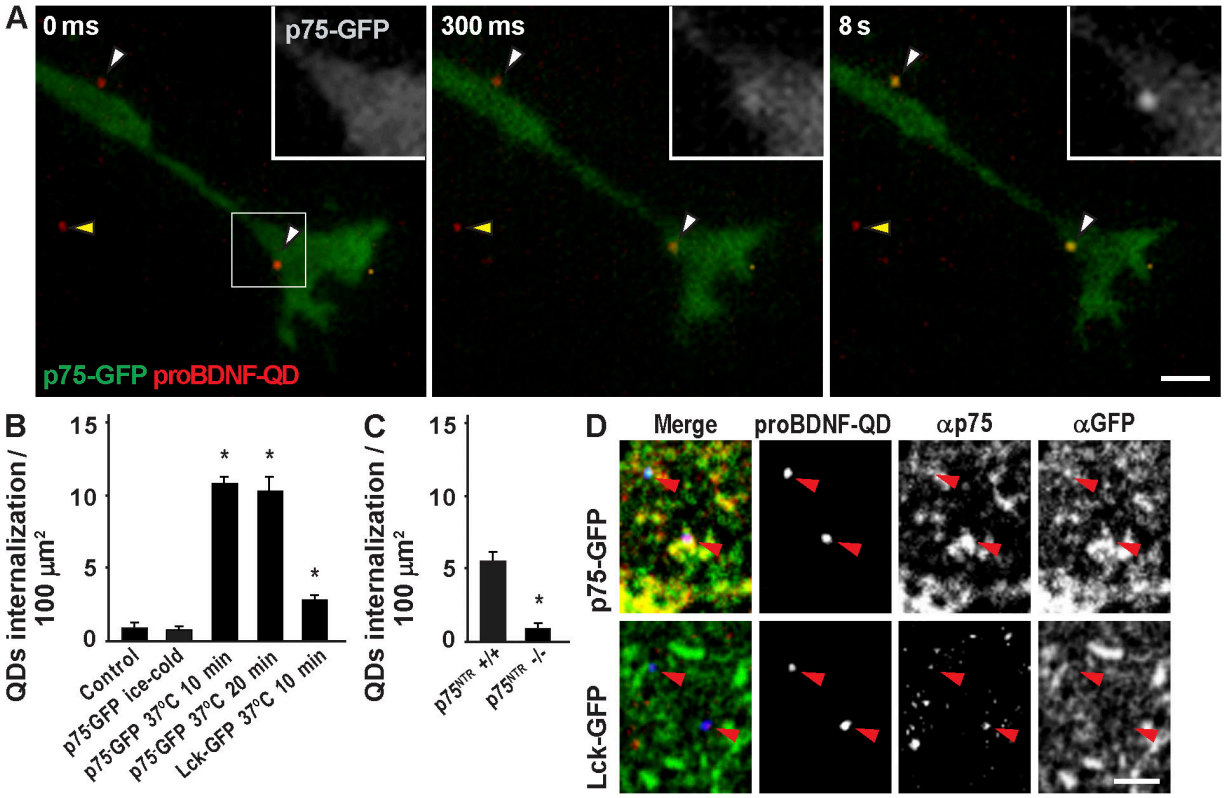

Figure 3. **Internalization of the pro-BDNF–p75^{NTR} complex in cultured astrocytes.** (A) Time sequence of pro-BDNF–QD–p75-GFP internalization in cultured astrocytes by TIRF imaging. White arrowheads indicate pro-BDNF–QDs (red) in close proximity to the membrane of an astrocyte transfected with p75-GFP (green). Yellow arrowheads point to reference QDs. Insets depict p75-GFP fluorescence that concentrates at the site of the QD. Bar, 5 µm. (B) Pro-BDNF–QD internalization in astrocytes transfected with p75-GFP (nine cells) or Lck-GFP (six cells). (C) Pro-BDNF–QD internalization in astrocytes from p75$^{NTR+/+}$ (22 cells) and p75$^{NTR-/-}$ (11 cells) mice. (D) Immunocytochemistry showing colocalization (arrowheads) between QDs (blue) and p75^{NTR} (red) in astrocytes transfected with p75-GFP or Lck-GFP (green). Bar, 2 µm. Data are means ± SEM (error bars). *, P ≤ 0.05.

(Fig. S3), confirming in primary cultures the mechanism of pro-BDNF endocytosis shown in stimulated slices.

What is the fate of internalized pro-BDNF? The sorting of endocytic vesicles has been suggested to lead to either vesicle recycling or vesicle entering the degradation pathway (Maxfield and McGraw, 2004; Soldati and Schliwa, 2006). This prompted us to investigate whether internalized pro-BDNF can eventually be recycled for regulated secretion. To this end, cultures were incubated for 10 min with a mixture of both precursor and mature BDNF tagged with YFP (BDNF-YFP mix; Fig. 4 A). Internalized BDNF-YFP showed a punctate pattern concentrated at the cell periphery of cultured astrocytes (Fig. 4 B). Ultrastructural characterization by preembedding experiments using immunogold-labeled BDNF-YFP (BDNF-YFP gold; Fig. S3) disclosed gold particles within vesicular structures (mean diameter, 125 ± 22 nm; n = 32; Fig. 4 C). Whether vesicles containing internalized BDNF-YFP are eventually destined for recycling was next evaluated by exploiting the pH sensitivity of BDNF-YFP. YFP fluorescence quenching inside acidic compartments followed by its unquenching upon BDNF-YFP secretion into the extracellular medium revealed fluorescent flashes by TIRF imaging (Santi et al., 2006). After loading astrocytes by brief exposure (1–5 min) to BDNF-YFP (mix), fluorescent vesicles appeared near the plasma membrane (Fig. 4 D and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200806137/DC1). Challenging astrocytes with glutamate triggered flashes lasting several hundred milliseconds. Only when fluorescence increased, spread, and subsequently declined was the flash considered an exocytic event. Quantitative analysis demonstrated that glutamate induced about a 10-fold increase in exocytic events (73 ± 12 mean flashes/astrocyte ± SD; n = 18) with respect to the control bath solution (6 ± 3 mean flashes/astrocyte ± SD; n = 6; Fig. 4 E). Most of the fusion events took place during the first 10 s of glutamate application. Because recycling is visualized by TIRF rapidly after exogenous BDNF-YFP administration, it is likely that endocytic vesicles containing BDNF-YFP can enter the exocytic process directly. Thus, endocytic vesicles may represent the main storage compartment for endocytosed BDNF-YFP before routing to the secretory pathway.

Lastly, neurotrophin recycling was determined by ELISA measurement of BDNF in supernatants collected from cultured astrocytes or astrocytes exposed to BDNF (mix) for 10 min and thoroughly washed (Fig. 4 F). Although challenge with glutamate for 5 min did not result in endogenous BDNF secretion from control cells, the same stimulation increased the neurotrophin secretion in BDNF preincubated cells. Neurotrophin release was strongly reduced by overnight intoxication with 40 nM tetanus neurotoxin (TeNT), a protease known to cleave the SNARE protein vesicle-associated membrane protein 2 (Vamp2)/synaptobrevin2 (Montana et al., 2006) and implicated in the

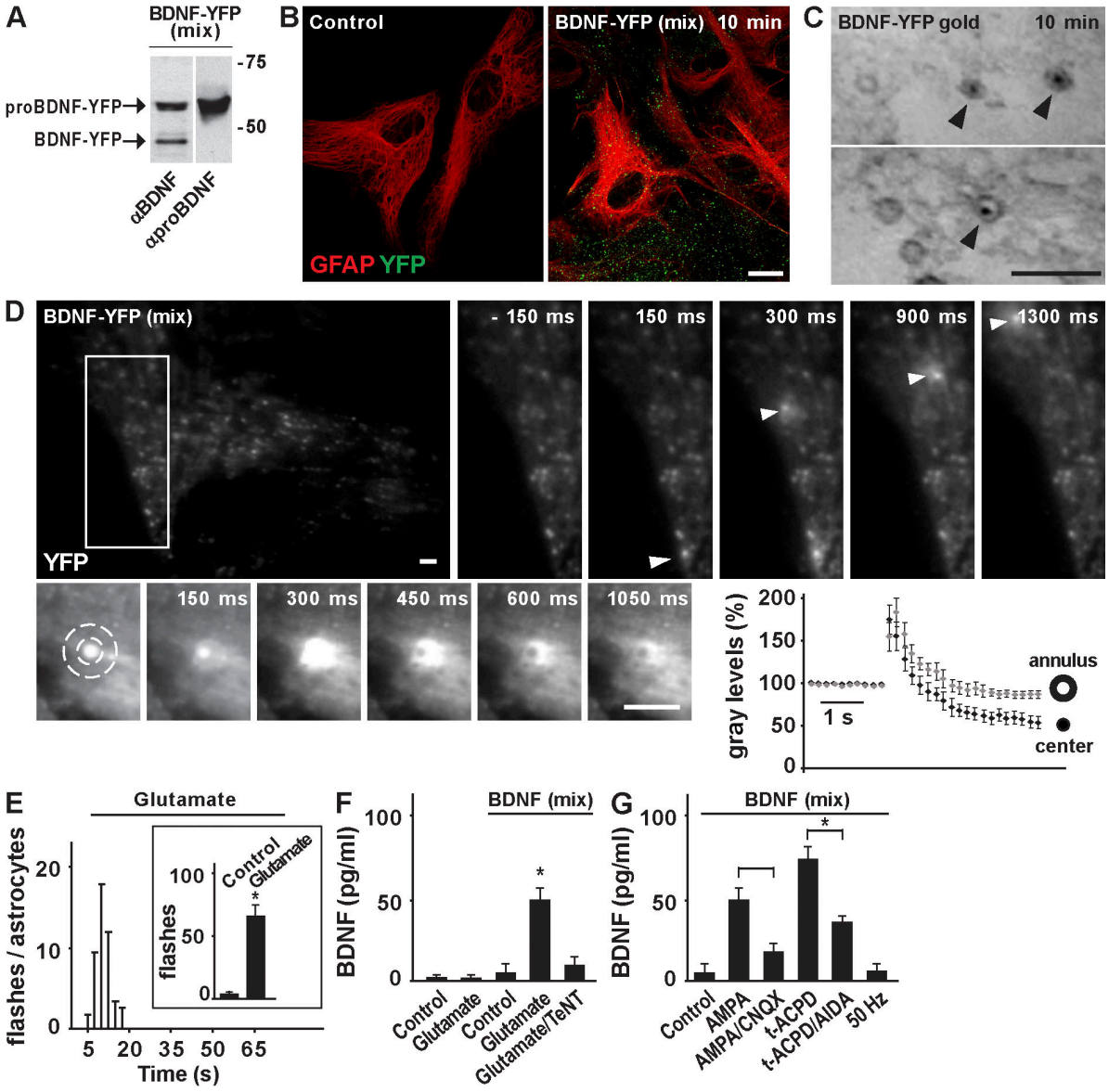

Figure 4. **Astrocytes recycle endocytic pro-BDNF for regulated secretion.** (A) Western blot analysis of BDNF-YFP (mix) using α-BDNF or α–pro-BDNF antibodies. (B) Immunocytochemistry in astrocytes untreated (n = 12) or incubated for 10 min with BDNF-YFP (mix; n = 18) followed by acid strip. Bar, 10 µm. (C) Ultrastructural characterization of astrocytes exposed to BDNF-YFP gold for 10 min. Arrowheads point to gold particles contained in vesicular organelles. Bar, 500 nm. (D) Representative TIRF images of astrocytes incubated with BDNF-YFP for 5 min. The top sequence depicts exocytic fusion (arrowheads) in a selected astrocytic area (white rectangle) before and after perfusion with glutamate. The bottom sequence shows fusion of a single vesicle. Fluorescence intensity is measured in a circular mask centered over the vesicle and in a concentric annulus on the circle. Bars, 2 µm. (E) Time distribution of fusion events (flashes) after glutamate application. The inset shows the total number of flashes per astrocyte before (n = 17) and after (n = 13) glutamate. (F) ELISA quantification of BDNF secretion from astrocytes before (n = 18) and after (n = 22) glutamate application for 5 min. Astrocytes previously exposed to BDNF (mix) for 10 min were stimulated with glutamate in the absence (n = 14) or presence (n = 4) of TeNT. (G) Secretion of BDNF induced by AMPA or t-ACPD in the absence (n = 23) or presence (n = 17) of the respective antagonists CNQX or AIDA and by 50 Hz (n = 6). Data are means ± SEM (error bars). *, $P \leq 0.05$.

regulated release of neurotransmitters from astrocytes (Bezzi et al., 2004). The effect of glutamate was mimicked by 50 µM AMPA or 100 µM t-ACPD, which activate AMPA or metabotropic group I/II glutamate receptors, respectively (Fig. 4 G). BDNF secretion induced by AMPA or t-ACPD was heavily reduced by the AMPA antagonists CNQX or by the metabotropic group I receptor antagonist AIDA. High frequency (50 Hz) electrical stimulation, which is known to trigger secretion of BDNF in cultured neurons (Balkowiec and Katz, 2000), was not effective (Fig. 4 G), indicating that BDNF secretion from astrocytes cannot be directly regulated by activity.

How is the endocytic pro-BDNF recycled for exocytosis? One potential mechanism making endocytic vesicles available for secretion involves the molecular machinery deputed to exocytic fusion. Astrocytes are known to express components of the core SNARE complex, including Vamp2 (Montana et al., 2006). Colocalization of pro-BDNF with Vamp2 was detected within astrocytes in slices 10 min after TBS (Fig. 5 A) or in cultured astrocytes transfected with p75-GFP and exposed to pro-BDNF–QDs (Fig. 5 B). The expression of Vamp2 on BDNF-containing vesicles was confirmed by Western blot analysis of endocytic vesicles purified by magnetic beads coated with α-$p75^{NTR}$ antibodies

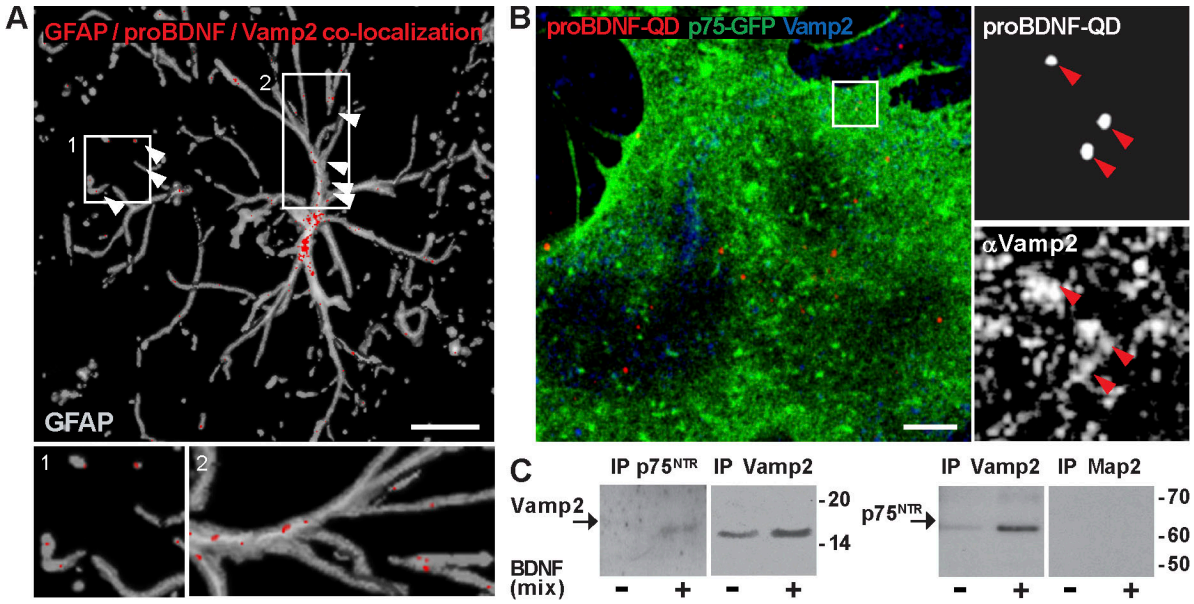

Figure 5. **Vesicles containing pro-BDNF–p75^{NTR} express the Vamp2 component of the SNARE core complex for vesicle fusion.** (A) Colocalization between GFAP, pro-BDNF, and Vamp2 immunoreactivity in astrocytes 10 min after TBS. Colocalization signal (arrowheads) is shown at the site of astrocytic contact with a neuron (box and inset 1) and astrocytic processes (box and inset 2). Bar, 20 µm. (B) Immunocytochemistry showing colocalization between pro-BDNF–QDs and Vamp2 in astrocytes transfected with p75-GFP. Right panels depict QD/Vamp2 colocalization (arrowheads) in a selected astrocytic area (boxed area). Bar, 2 µm. (C) Western blot showing p75^{NTR} and Vamp2 expression in endocytic vesicles immunopurified (IP) by magnetic beads coated with α-p75^{NTR}, α-Vamp2, or α-Map2 from astrocytes untreated or treated with BDNF (mix).

(Fig. 5 C). Conversely, endocytic vesicles purified using beads coated with α-Vamp2 antibodies were immunoreactive for p75^{NTR}. Interestingly, treatment with BDNF (mix) for 10 min enhanced the recovery of vesicles expressing both Vamp2 and p75^{NTR}. Beads coated with antibodies against the neuronal marker microtubule-associated protein 2 (Map2) were used as a control. These data indicate that endocytic vesicles expressing p75^{NTR} may represent the main storage compartment for endocytosed pro-BDNF before routing to the secretory pathway. This process might take place either by recycling of pro-BDNF–p75^{NTR} complexes to the surface or by pro-BDNF recycling upon its dissociation from p75^{NTR}. Moreover, given that TeNT prevented BDNF secretion (Fig. 4 F), all of these data indicate that after endocytosis in astrocytes, vesicles containing the neurotrophin may undergo regulated recycling via a SNARE-dependent mechanism.

Our overall findings indicate that astrocytes exert an important function in the neuronal clearance of pro-BDNF secreted upon neuronal activity and subsequent recycling of the endocytic neurotrophin, thus regulating both its spatial and temporal availability. In this respect, astrocyte-mediated clearing and recycling of BDNF share some similarities with the astrocyte clearance of neurotransmitters from the synaptic cleft. Recycling of BDNF by these cells may thus contribute to the regulation of synaptic plasticity by glia (Haydon, 2001; Fields and Stevens-Graham, 2002; Allen and Barres, 2005; Haydon and Carmignoto, 2006).

Materials and methods

Electrophysiology

Slice preparation and recordings were performed as previously reported (Aicardi et al., 2004). Slices were preincubated with 50 µM anisomycin, 1 µg/ml TrkB-Fc (Regeneron Pharmaceuticals, Inc.), 200 nM K252a, 50 µM MDC, and 20 µM D15 for 20 min or 100 nM plasmin for 2 h and were maintained throughout the recording by a recirculation system.

Immunohistochemistry and immunocytochemistry

Samples were subjected to conventional experimental procedures using chicken α–pro-BDNF (Millipore), chicken α-BDNF (Promega), rabbit or mouse α-GFAP (Sigma-Aldrich), mouse α–neuronal nuclei (Millipore), goat α-p75^{NTR} (R&D Systems), rabbit α-EEA1 (Abcam), rabbit α-clathrin (Abcam), mouse α-Vamp2 (Synaptic Systems), and mouse (Invitrogen) or chicken α-GFP (Aves Laboratory) primary antibodies. Immunoreactivity was evaluated using a confocal laser-scanning microscope (Radiance 2000; Bio-Rad Laboratories) equipped with krypton–argon and red diode lasers and 20×/0.75, 60×/1.40, and 100×/1.40 oil objectives (Nikon). Image processing and volume rendering were performed using the ImageSpace software (Molecular Dynamics) running on an Indigo Workstation (Silicon Graphics).

Biotinylation assay

Cell surface biotinylation of intact astrocytes was performed as previously described (Santi et al., 2006).

Western blotting

Samples were subjected to a conventional experimental procedure using chicken α-BDNF (R&D Systems), rabbit and chicken α–pro-BDNF (Millipore), mouse α-TrkB (BD Biosciences), rabbit α-p75^{NTR} (Abcam), mouse α-Vamp2 (Synaptic Systems), and mouse α-Map2 (a gift from A. Matus, Friedrich Miescher Institut, Basel, Switzerland) primary antibodies.

Cell cultures

Primary cultures of cortical astrocytes were prepared from postnatal day 1–2 Wistar rats and p75$^{NTR+/+}$ or p75$^{NTR-/-}$ mice (provided by Y.-A. Barde, Biozentrum, University of Basel, Basel, Switzerland) as previously described (McCarthy and de Vellis, 1980).

Characterization of BDNF immunocomplexes

Pro-BDNF–QD. QD-565 surfaces immobilized with α-rabbit antibodies (Invitrogen) were used to passively bind rabbit α–pro-BDNF (Millipore). According to the manufacturer, single QD-565 shows the potential to be bioconjugated to <10 antibody molecules; thus, the desired degree of immunocoupling was set by adjusting the mixing stoichiometry between QDs and α–pro-BDNF to a 1:10 concentration. Incubation was performed

for 10 min in a neutral buffer at 37°C. The final immunocomplex was obtained in a second step by incubating the QD/α–pro-BDNF mixture with 100 ng/ml BDNF (mix), which we show (Fig. 1 B) to contain BDNF in both precursor and mature forms. The correct formation of the pro-BDNF–QD immunocomplex was confirmed by immunocytochemistry (Fig. S3). The intensity profile of pro-BDNF–QD fluorescence was determined (Fig. S3) and compared with individual QDs as a reference. Although individual pro-BDNF–QDs showed high fluorescent fluctuation (blinking), pro-BDNF–QD clusters produced a larger variation in brightness but lower blinking. Confocal imaging revealed that 10 nM pro-BDNF–QDs were sufficient to visualize individual dots internalized into cultured astrocytes transfected with p75-GFP or Lck-GFP (provided by H. Lickert, Helmholtz Zentrum Muenchen, Neuherberg, Germany; Fig. 3 B). Higher concentrations (20–500 nM) increased QD clustering at the cell interspace and QDs decorated the surface of the cells, but internalization was strongly impaired.

BDNF-YFP gold. The immunocomplex was prepared by two-step coupling procedures in which BDNF-YFP (mix; provided by O. Griesbeck, Max Planck Institute of Neurobiology, Martinsried, Germany) was first coupled with α-GFP for 10 min and, with a secondary antibody, coupled with 5-nm gold particles.

Electron microscopy

In preembedding experiments, cultured astrocytes were treated with BDNF-YFP gold for 10 min, fixed in 2.5% glutaraldehyde (0.1 M phosphate buffer) for 30 min at 4°C, and postfixed in 1% OsO_4 for 60 min at room temperature. After dehydration, samples were embedded in epon 812 and counterstained with uranyl acetate–lead citrate. Sections were examined with an electron microscope (EM 109; Carl Zeiss, Inc.). The images were captured with a charge-coupled device camera (DMX 1200F; Nikon). Digital images were collected and analyzed using Image Pro+ software (Media Cybernetics, Inc.). Analysis was performed on at least 200 micrographs disclosing intracellular structures containing gold particles. The vesicle diameters were measured referring to calibrated latex beads.

Time-lapse TIRF imaging

TIRF imaging experiments were performed as described previously (Santi et al., 2006).

Characteristics of the perfusion setup and release experiments

Release experiments were performed as described previously (Canossa et al., 1997). Stimulations were obtained by adding 50 and 500 µM glutamate, 50 µM AMPA, and 100 µM t-ACPD over a 5-min period. Treatment with specific receptor antagonists (50 µM CNQX and 500 µM AIDA) started 30 min before the beginning of the perfusion and was maintained throughout the collection period. Intoxication with TeNT started 12 h before the beginning of perfusion and was not maintained throughout the collection period. High frequency stimulation was performed as reported previously (Santi et al., 2006). The amount of BDNF in each fraction was determined by a two-site ELISA (Canossa et al., 1997).

Vesicle immunopurification

Purifications were performed as previously described (Santi et al., 2006). Vesicles were immunoisolated from the postnuclear supernatant with α-mouse Dynabeads (M-280) coated with mouse α-$p75^{NTR}$ (Sigma-Aldrich), mouse α-Vamp2 (Synaptic Systems), or mouse α-Map2 (a gift from A. Matus) antibodies according to the manufacturer's instructions.

Online supplemental material

Figs. S1 and S2 show pro-BDNF internalization in individual astrocytes from control or TBS slices in the absence or presence of anisomycin, TrkB-Fc, plasmin and MDC, or in $p75^{NTR+/+}$ and $p75^{NTR-/-}$ mice. Fig. S3 shows (a) a schematic representation of pro-BDNF–QD and BDNF-YFP gold immunocomplexes and (b) colocalization between pro-BDNF–QDs and clathrin or EEA1 in cultured astrocytes expressing p75-GFP. Video 1 shows real-time visualization of pro-BDNF–QD endocytosis in cultured astrocytes expressing p75-GFP obtained by TIRF imaging. Video 2 shows the exocytic fusion of BDNF-YFP–containing vesicles analyzed by TIRF microscopy. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200806137/DC1.

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