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The localization, trafficking and retrograde transport of BDNF bound to p75NTR in sympathetic neurons

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BDNF, through p75NTR, promotes apoptosis and inhibits axonal growth of sympathetic neurons, antagonizing the pro-survival and axon growth-promoting actions of NGF through TrkA. While the trafficking of the TrkA:NGF complex is well characterized, little is known about p75NTR:BDNF trafficking in these neurons. Here we show that BDNF binds to and appears inside sympathetic neurons relatively slowly, although the temperature-sensitive internalization step itself is rapid. P75NTR internalization is partially sensitive to disruption of clathrin- or raft-mediated internalization, while that of TrkA is entirely clathrin-mediated. P75NTR, but not Trk, associates with neurotrophins in lipid rafts and coimmunoprecipitates with the truncated beta-caveolin-1 isoform. Finally, we directly visualize the retrograde transport of p75NTR ligands to cell bodies, which is insensitive to inhibitors of Trk retrograde transport, suggesting mechanistic differences. We postulate that beta-caveolin-1-containing lipid rafts and possibly intracellular endosomes might be compartments to which p75NTR:BDNF complexes are trafficked separately from Trk.
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Introduction

During neural development, neurotrophins act through the p75 neurotrophin receptor (p75NTR) and the Trk receptors to ensure that developing sympathetic neurons appropriately innervate their target organs (reviewed in Miller and Kaplan, 2001a). In these developing neurons, TrkA promotes neuronal survival and axonal growth, while p75NTR opposes TrkA, inhibiting axon growth and promoting apoptosis (Bamji et al., 1998; Kohn et al., 1999; Majdan et al., 2001). The mechanisms underlying their functional antagonism in sympathetic neurons are not yet fully understood, although genetic studies demonstrate that the pro-apoptotic actions of p75NTR can occur in the absence of TrkA and that these actions can be inhibited by TrkA (Majdan et al., 2001). P75NTR does, however, initiate some signals in the presence of TrkA activation, notably to influence axonal growth (Kohn et al., 1999). Together, these observations suggest that p75NTR may function, in part, in a compartment separate from TrkA. One approach to identify candidates for such a compartment is to investigate and compare the locations in which each receptor interacts with its neurotrophin ligands and the kinetics and mechanisms of their subsequent trafficking.

Trk receptor trafficking in neurons has been well characterized. Trk is internalized within 30 min of neurotrophin binding via a PI3-kinase-dependent mechanism, and is transported retrogradely along the axon, carrying a pro-survival signal from the distal axon to the cell body (Miller and Kaplan, 2001b; Neet and Campenot, 2001; Ginty and Segal, 2002). While Trk is found in lipid rafts of cell lines (Huang et al., 1999; Peiro et al., 2000), it is normally excluded from the lipid rafts of cortical neurons, only entering the rafts when activated by neurotrophins (Guirland et al., 2004; Suzuki et al., 2004). This translocation is prevented if p75NTR is also expressed by those neurons (Suzuki et al., 2004).

Less is known about the localization and trafficking of p75NTR. P75NTR mediates neurotrophin internalization to PC12 cells through clathrin-coated pits (Kahle and Hertel, 1992; Bronfman et al., 2003; Saxena et al., 2004), regulates retrograde transport of neurotrophins (Curtis et al., 1995, 1998; Helke et al., 1998; Kramer et al., 1999; Lalli and Schiavo, 2002) and localizes to the lipid rafts of central neurons when bound to neurotrophins (Higuchi et al., 2003). To what extent these observations extend to peripheral neurons has not yet been examined.

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We have characterized the localization, trafficking and retrograde transport of the p75NTR:ligand complex in primary sympathetic neurons. In developing sympathetic neurons, p75NTR expression becomes elevated when their axons reach their target organs, functioning to switch the neurons to an absolute dependence on NGF for their survival and to inhibit the growth and survival of the neurons (Bamji et al., 1998; Kohn et al., 1999; Majdan et al., 2001; Kuruvilla et al., 2004). Consequently, p75NTR first begins to act in a spatially complex, well-developed neuron, which has already formed its long axons. That these complex neurons are readily grown in culture has allowed the extensive characterization of the Trk-mediated events described above and has allowed us herein to establish similar investigations of the p75NTR. We used BDNF as a p75NTR-specific ligand, since sympathetic neurons do not express TrkB, the other BDNF receptor. We show that, in these neurons, p75NTR is internalized through clathrin-dependent and independent routes, in contrast to its singular, clathrin-mediated route into PC12 cells (Bronfman et al., 2003; Saxena et al., 2004). On the cell surface, p75NTR, unlike Trk, associates with neurotrophins in lipid rafts and coimmunoprecipitates endogenously with the raft marker beta-caveolin-1. In addition, BDNF, internalized with p75NTR, is retrogradely transported intact, providing a second potential p75NTR-signalling compartment. This transport occurs with a similar rate to, but is initiated by a different mechanism than that of Trk, indicating that this compartment may also be segregated from that used by Trk. Together these spatial, temporal and mechanistic differences between the trafficking of Trk and p75NTR are consistent with the ability of p75NTR to initiate signals on its own, independent of TrkA (Majdan et al., 2001), and we identify two cellular compartments, lipid rafts and retrograde transport vesicles, from which this may occur.

Results

A biologically active GFP-tagged BDNF protein binds specifically to cell surface p75NTR on sympathetic neurons

To visualize trafficking of the p75NTR:ligand complex, we stably expressed a GFP-tagged form of the neurotrophin BDNF (BDNF-GFP), a ligand for p75NTR, in 293A cells. Both the BDNF-GFP precursor (proBDNF-GFP) and mature processed BDNF-GFP were produced, as determined by Western blotting of the cell lysates with anti-BDNF (Fig. 1A) and anti-GFP (Fig. 1B). The mature BDNF-GFP was secreted, as shown by Western blotting of the conditioned media with anti-BDNF and anti-GFP. In order to prove that the same protein was being detected with both antibodies, the BDNF-GFP was immunoprecipitated from the conditioned medium using anti-GFP and subsequently detected on Western blots with anti-BDNF (Fig. 1C). The BDNF-GFP band was not detected in conditioned medium from untransfected 293A cells. The concentration of the secreted BDNF-GFP was estimated as approximately 1.4–7 nM by Western blot analysis and ELISA relative to known concentrations of recombinant BDNF (data not shown). To determine whether BDNF-GFP was biologically active, the ability of this protein to induce tyrosine phosphorylation of TrkB was assessed in 3T3 cells stably expressing TrkB. Lysates from cells treated for 10 min with conditioned media from either BDNF-GFP expressing cells or control 293A cells, or with 100 ng/ml recombinant BDNF were immunoprecipitated with anti-Trk and activated TrkB detected by probing with anti-phosphotyrosine. TrkB was activated to a similar extent by BDNF-GFP present in the conditioned medium or recombinant BDNF (Fig. 1D).

We have previously shown that neonatal sympathetic neurons express p75NTR but not the other BDNF receptor TrkB (Belliveau et al., 1997; Bamji et al., 1998). To determine whether, as this observation predicts, BDNF-GFP binds to sympathetic neurons in a p75NTR-specific manner, we first asked if BDNF-GFP binding to neurons could be abolished by incubating cells with a p75NTR antibody that blocks binding of neurotrophin to this receptor (REX, Weskamp and Reichardt, 1991; Kohn et al., 1999) or with excess recombinant neurotrophin. Cultured neurons were maintained overnight in medium containing BDNF-GFP with or without the addition of a 10-fold excess of BDNF, a 50-fold excess of nerve growth factor (NGF) or anti-p75NTR, and the total fluorescent signal on the surface and inside the cell bodies was quantified using confocal microscopy. The presence of excess NGF, BDNF or anti-p75NTR completely abolished the association of BDNF-GFP with neurons (Fig. 1E), indicating that p75NTR is required for BDNF-GFP binding. As an additional confirmation of the specificity of BDNF-GFP for p75NTR on these neurons, we cultured wild type or $p75NTR^{-/-}$ sympathetic neurons, incubated them overnight with BDNF-GFP and then analyzed them by confocal microscopy. Only the $p75NTR^{+/+}$ neurons displayed a fluorescent signal due to binding of the BDNF-GFP (Fig. 1F) indicating that minimal non-specific binding occurs even after extended incubations with the ligand. The lack of binding of BDNF-GFP to $p75NTR^{-/-}$ neurons was confirmed by the following biochemical assay. Wild type or $p75NTR^{-/-}$ neurons were incubated with BDNF-GFP for 5 h, washed extensively and then assayed for BDNF-GFP binding by Western blotting of cell lysates with anti-GFP. No binding of BDNF-GFP to $p75NTR^{-/-}$ neurons (Fig. 1G), or binding of free GFP to wild type or knockout neurons (data not shown) was evident. Finally, neurons were incubated for 5 h with BDNF-GFP and Cy3 labeled anti-p75NTR before imaging both fluorophores consecutively by confocal microscopy (Fig. 1H). The distributions of these two ligands were strikingly similar, as would be predicted as BDNF-GFP is a p75NTR specific ligand.

The p75NTR:ligand complex is internalized with a slower time course than the Trk:ligand complex

Previous data from cell lines indicated that p75NTR internalizes neurotrophins, but less efficiently than does Trk (Green et al., 1986; Loeb and Greene, 1993). In particular, this internalization occurs with a much slower time course than that through Trk (Kahle and Hertel, 1992; Mahadeo et al., 1994; Bronfman et al., 2003; Saxena et al., 2004). However, the internalization of neurotrophins by p75NTR to primary neurons has not been thoroughly characterized. We used several independent assays to characterize the internalization of BDNF to primary sympathetic neurons by p75NTR. As an initial assay, neurons were incubated with BDNF-GFP for various times, and the live cells imaged by confocal microscopy. After 1 h, BDNF-GFP was bound to the surface of sympathetic neurons, with little or no fluorescence detected in the cells (Fig. 2A). By 5 h, BDNF-GFP fluorescence was both surface-bound and distributed within cells in a punctate pattern (Fig. 2A), consistent with slow internalization. The internalization of p75NTR was also assessed in a continuous time series using

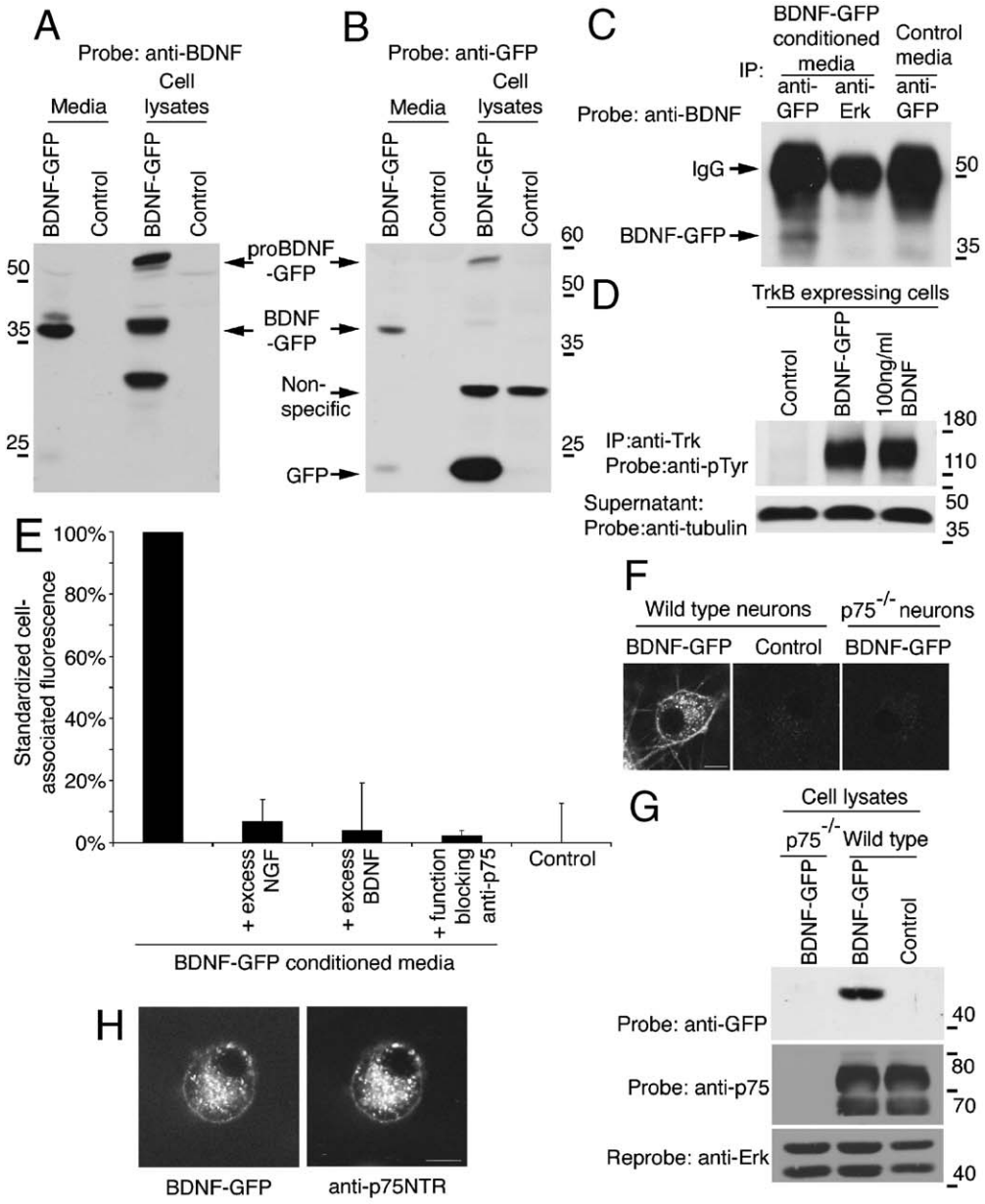
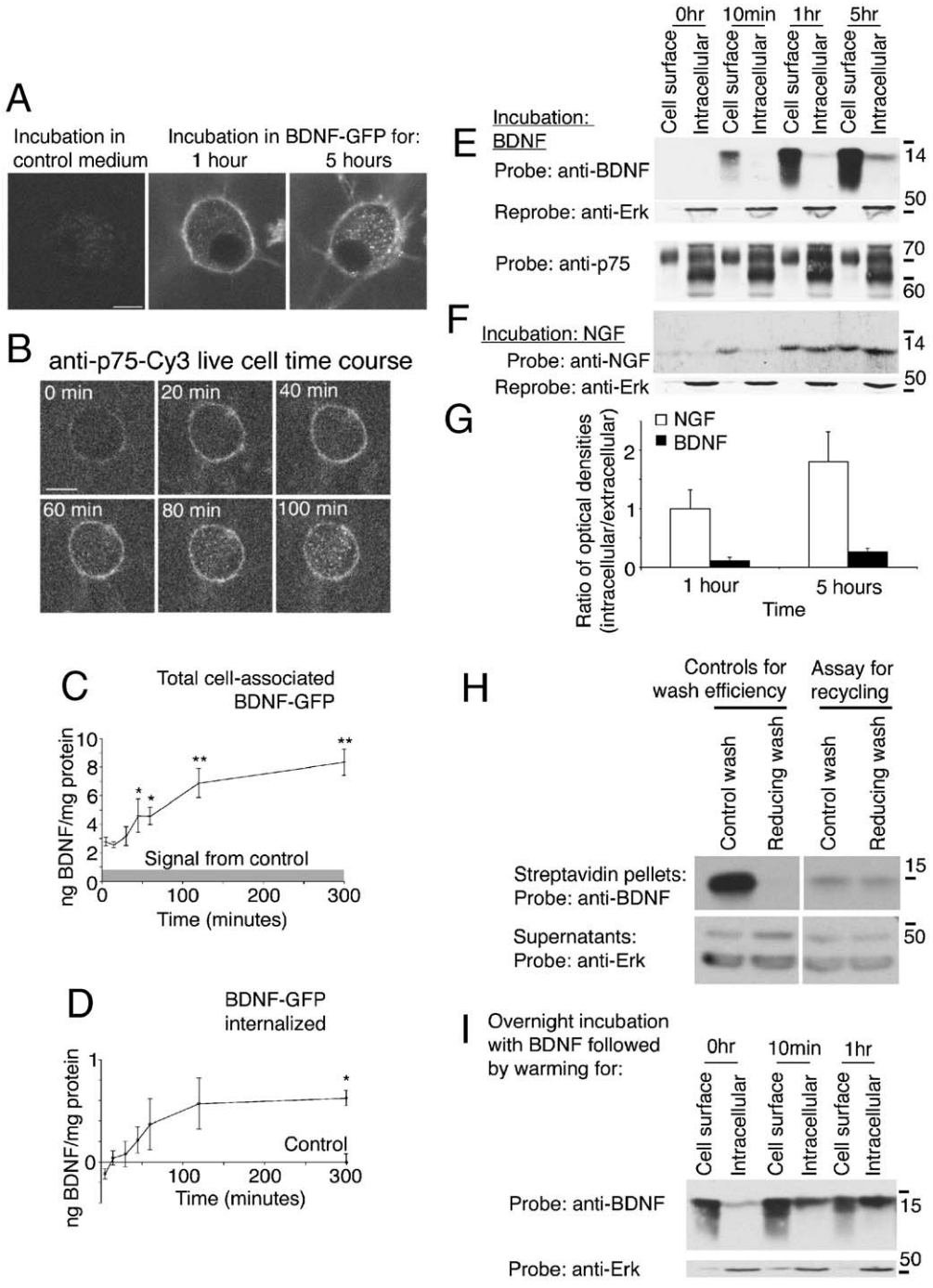


Fig. 1. Biologically active BDNF-GFP is appropriately processed and secreted by a 293 stable line and binds specifically to p75NTR on sympathetic neurons. (A, B) Medium conditioned by, or cell lysates collected from, control 293A cells or 293A cells stably expressing the BDNF-GFP construct was collected and subjected to Western blot analysis using antibodies raised against BDNF (A) or GFP (B). (C) Conditioned media from BDNF-GFP expressing or control 293A cells were subjected to immunoprecipitation (IP) using antibodies against GFP, or Erk as a negative control followed by Western blot analysis using an antibody against BDNF. (D) 3T3 cells stably expressing TrkB were incubated for 10 min with control medium, or with medium containing BDNF-GFP or 100 ng/ml recombinant BDNF. The cells were lysed and TrkB immunoprecipitated (IP) using a pan-Trk antibody. Both the precipitates and supernatants were separated by SDS-PAGE and transferred to nitrocellulose. Phosphotyrosine (p-Tyr) was detected in the precipitates and tubulin in the supernatants by Western blotting. (E) Sympathetic neurons were incubated with control medium (conditioned with non-transfected HEK-293 cells) or with BDNF-GFP conditioned medium alone or plus an excess of NGF or BDNF, or a function-blocking antibody against p75NTR. Quantitation of the relative fluorescent signal is shown. (F) Wild type or p75NTR$^{-/-}$ mouse sympathetic neurons were incubated overnight with either BDNF-GFP containing or control conditioned media. Representative fluorescent confocal images are shown. Scale bar = 10 μm. (G) Wild type or p75NTR$^{-/-}$ neurons were incubated with BDNF-GFP containing or control conditioned media for 5 h before lysis. The lysates were subjected to Western blot analysis using antibodies to GFP (to detect the tagged BDNF), to p75NTR (to confirm the genotype) and to the Erk proteins (as a loading control). (H) Sympathetic neurons were incubated for 5 h with BDNF-GFP and Cy3 labeled anti-p75NTR. Both ligands were then imaged consecutively in the same live neuron. Scale bar = 10 μm.

an activating Cy3-labeled antibody to p75NTR (MC192), which has been previously used as a marker for p75NTR trafficking in vivo (Johnson et al., 1987; Hartig et al., 1998), as a ligand. This antibody was added to sympathetic neurons with 100 ng/ml BDNF, and the cells imaged over time at 37°C. Similar results were obtained if BDNF was not included, consistent

with the ability of this antibody to act as an activating ligand for p75NTR. While the antibody rapidly bound to the cell surface, the fluorescent signal appeared in the cell body only after 60 to 90 min (Fig. 2B), in agreement with the BDNF-GFP internalization results (Fig. 2A).

To more quantitatively assess the time course of internalization of BDNF and p75NTR, an ELISA assay was performed to detect internalized BDNF-GFP as compared to total cell-associated BDNF-GFP. Our cultures contained only neurons, as glia were eliminated by the transient addition of cytosine arabinoside. For total BDNF-GFP, neurons were incubated with BDNF-GFP for 5 to 300 min, lysed and analyzed by ELISA for BDNF. To measure only internalized BDNF-GFP, cells were washed with acid to remove surface-bound ligand. A small amount of BDNF-GFP (2–3 ng/ml) bound to cells within 5 min (Fig. 2C). This rapid phase of binding was followed by a slow increase in the amount of total bound BDNF-GFP between 30 and 120 min. This slow increase in the amount of neurotrophin bound to p75NTR over time, normally after an initial rapid phase, has been previously detected in several cell lines (Fabricant et al., 1977; Kahle and Hertel, 1992; Saxena et al., 2004). Internalized BDNF-GFP was first detected only at 60 min, and the amount increased until 2 h, at which point it reached a plateau (Fig. 2D). As a final assay, we examined the binding and internalization of native, recombinant BDNF using a



biotinylation approach. Neurons were incubated with 100 ng/ml BDNF for 10 min to 5 h, cell surface proteins were biotinylated and the biotinylated proteins precipitated from cell lysates with streptavidin beads. Any precipitated, biotinylated proteins will, by definition, have been on the cell surface while non-biotinylated proteins must derive from an intracellular pool. This analysis (Figs. 2E, G) revealed a similar time course of BDNF binding and internalization to that of BDNF-GFP. The amount of cell surface-associated BDNF greatly increased between 10 min and 2 h, while BDNF accumulated inside the cells between 1 and 5 h. The slow phase of BDNF binding, described here in neurons and previously in cell lines (Fabricant et al., 1977; Kahle and Hertel, 1992; Saxena et al., 2004), does not reflect the recruitment of p75NTR to the cell surface, as similar amounts of cell surface-localized p75NTR were observed at all time points (Fig. 2E).

We next analyzed the binding and internalization kinetics of NGF bound to TrkA. NGF binds to both TrkA and p75NTR on sympathetic neurons, albeit at different functional concentrations; neurotrophins induce TrkA-mediated responses at concentrations from 1 to 50 ng/ml (Belliveau et al., 1997) and p75NTR-mediated responses at higher concentrations (Bamji et al., 1998; Kohn et al., 1999). We used the same biotinylation assay as described above to assess the binding and internalization of 50 ng/ml NGF. After 10 min, NGF was bound to the cell surface, and by 1 h the amounts of cell-surface bound and intracellular NGF were similar (Figs. 2F, G), in contrast to the small amount of intracellular BDNF that was observed at this time point (Figs. 2E, G). Thus, NGF, presumably bound to TrkA, is internalized much more rapidly than is BDNF bound to p75NTR.

One possible explanation for the minimal intracellular BDNF seen after 1 h is that BDNF bound to p75NTR is internalized rapidly but then recycles rapidly back to the cell surface, as can occur for Trk (Eveleth and Bradshaw, 1988; Buxser et al., 1990; Zapf-Colby and Olefsky, 1998). To address this, we asked whether BDNF biotinylated on the cell surface and then internalized would recycle back to cell surface. BDNF bound to sympathetic neurons for 1 h at 4°C was labeled using a cell-impermeable biotinylation reagent with a reducible linker. The cells were warmed for 1 h to allow some of the biotinylated BDNF to be internalized, and washed with a buffer containing reduced glutathione to remove all biotin still associated with the cell surface. The neurons were then incubated for 1 h at 37°C before a further wash using either the glutathione buffer (reducing wash) or a control buffer. Cells were then lysed, biotinylated proteins precipitated on streptavidin beads and BDNF detected by Western blot analysis (Fig. 2H). This analysis did not detect any further decrease in the amount of biotinylated BDNF induced by the second reducing wash, indicating that BDNF had not recycled back to the cell surface between the two washes.

Our results and those of others show that the increase in BDNF binding overlaps in time with its internalization (Figs. 2C–E), suggesting that it may be the binding, rather than the internalization of BDNF that is the rate-limiting step, a possibility that has not been previously addressed. To examine this possibility, we allowed neurons to bind BDNF overnight at 4°C, at which temperature internalization is inhibited, before warming the cells to allow internalization to proceed. Cell surface-bound BDNF was then biotinylated, precipitated and both the precipitates and the supernatant were analyzed for BDNF by Western blotting (Fig. 2I). In this experiment, a substantial proportion of the BDNF entered the cells within 10 min and approximately equal proportions of BDNF were inside the cells and on the surface after 1 h (Fig. 2I). Thus, the slow rate of internalization of BDNF is determined by the rate of ligand binding and/or by an as yet uncharacterized, rate limiting step prior to internalization, rather than by the internalization process itself.

BDNF bound to p75NTR or to TrkB is distributed differently

We next asked whether BDNF bound to p75NTR versus TrkB occupies different compartments on the cell surface, by visualizing BDNF-GFP bound to each of these receptors with confocal microscopy. In neurons incubated overnight with BDNF-GFP to allow it to bind p75NTR, BDNF-GFP fluorescence was detected in a relatively diffuse distribution near or on the cell surface (Figs. 3A, B). To compare this distribution to that of the Trk:neurotrophin complex, neurons were grown in a low concentration of NGF (10 ng/ml) that we previously showed greatly reduces the expression of p75NTR (Ma et al., 1992; Toma et al., 1997), infected with a recombinant adenovirus expressing rat TrkB or β-galactosidase (Atwal et al., 2000), and exposed to BDNF-GFP for 1 h. Under these conditions, very little BDNF-GFP binding was observed to control, β-galactosidase-expressing neurons (Fig. 3C). In contrast, robust BDNF-GFP fluorescence was observed bound

Fig. 2. BDNF is internalized to sympathetic neurons with a slow time course as a consequence of its slow association with p75NTR. (A) Sympathetic neurons were incubated with control medium for 5 h or with BDNF-GFP containing conditioned medium for the times indicated. Live cells were imaged by confocal microscopy. Scale bar = 10 μm. (B) Anti-p75-Cy3 plus 10 ng/ml NGF and 100 ng/ml BDNF in a buffered medium was added to sympathetic neurons maintained on a confocal microscope at 37°C. Immediately following addition of the ligands, a continuous time series was started, collecting images at the times indicated. Scale bar = 10 μm. (C, D) Neurons were incubated for various times in conditioned medium containing BDNF-GFP, and lysed either with (internalized, panel D) or without (bound to cells, panel C) prior acid wash. The amount of BDNF in the lysates was determined by ELISA relative to known concentrations of recombinant BDNF, and then expressed relative to total protein. In the analysis of total binding, the BDNF concentration in the negative control (cells incubated with control conditioned medium) was below the limit of detection, indicated by the grey region. In the analysis of internalized BDNF, the concentration of BDNF in the negative control was subtracted from all measurements. Error bars are SEM. $*P < 0.05$, $**P < 0.01$, one-way ANOVA followed by Dunnett's Post hoc comparisons versus the negative control. (E) Neurons were incubated with 100 ng/ml BDNF for the times indicated before the cell surface proteins were biotinylated and the cells were lysed. Biotinylated proteins were precipitated on streptavidin beads and proteins in the bead pellet (cell surface) and supernatant (intracellular) separated by SDS-PAGE and subjected to Western blot analysis for BDNF or Erk (as a loading control). (F) Internalization of NGF was visualized using a similar procedure to that used in panel E, except that neurons were exposed to 50 ng/ml NGF for the times indicated. (G) The optical densities of the bands immunoreactive for neurotrophin from blots obtained as for E and F above were determined. For NGF (open bars) and BDNF (solid bars), the ratio of the optical densities corresponding to the intracellular versus the extracellular protein was determined. (H) Recycling of BDNF was assessed as described in the experimental procedures. Western blotting for BDNF revealed no effect of the reducing wash in the recycling assay, indicating that recycling did not occur. (I) To visualize the kinetics of internalization of BDNF by p75NTR following maximal BDNF binding, 100 ng/ml BDNF was allowed to bind to sympathetic neurons overnight at 4°C, at which temperature internalization is inhibited. Following binding, cells were warmed to 37°C for the times indicated. Internalization was assayed as in panel E.

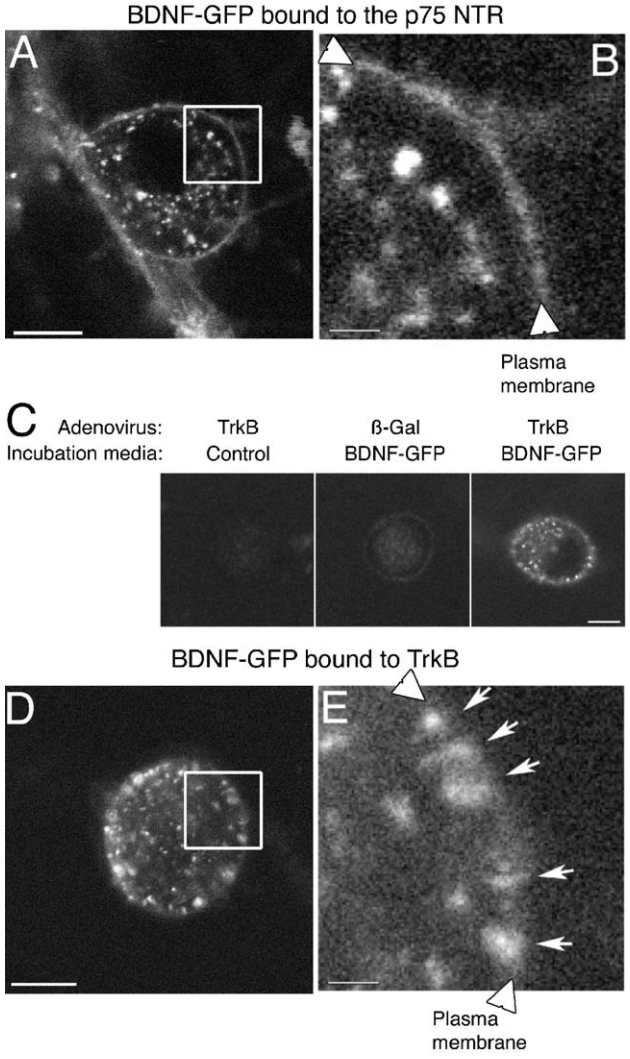


Fig. 3. The distribution of BDNF-GFP binding to p75NTR versus TrkB in sympathetic neurons. (A) Sympathetic neurons were incubated overnight with conditioned medium containing BDNF-GFP and confocal images were collected. (B) An enlargement of the area outlined in panel A illustrates the diffuse distribution on the cell surface. Arrowheads highlight the plasma membrane. (C) To visualize the trafficking of BDNF-GFP bound to TrkB, sympathetic neurons were infected with an adenovirus encoding TrkB and incubated for 2 days in a reduced concentration of NGF without added serum. As a control, sister cultures were infected with an adenovirus expressing β-galactosidase (βGal). TrkB-infected neurons were incubated with control medium or medium containing BDNF-GFP, or control, β-galactosidase-expressing neurons were incubated with BDNF-GFP. After 1 h, images were collected by confocal microscopy. (D) Under the same conditions as used in panel C, TrkB-expressing sympathetic neurons were exposed to BDNF-GFP for 1 h, and images were collected by confocal microscopy. (E) Puncta on the cell surface are illustrated by arrows in an enlargement of the area indicated in panel D. Arrowheads highlight the position of the plasma membrane. Scale bars are 10 μm in panels A and D, 2 μm in panels B and E.

to neurons expressing TrkB (Figs. 3C–D). The TrkB:BDNF-GFP complex was present in a markedly punctate distribution near or at the cell surface (Fig. 3D), most clearly seen in a magnified view (Fig. 3E). Moreover, in the TrkB-expressing neurons, a large percentage of the total BDNF-GFP was observed in punctate structures inside the cells after 1 h (Figs. 3C–E). This is consistent with the more rapid internalization of neurotrophins through Trk than p75NTR described above (Fig. 2).

BDNF internalization does not occur exclusively through a clathrin- or a lipid raft-dependent mechanism

In PC12 cells, clathrin-coated pits have been implicated in p75NTR signaling (Bronfman et al., 2003). In order to examine whether p75NTR internalization to neurons might also occur through a clathrin-dependent mechanism, we used sucrose, which functions as an inhibitor of clathrin-mediated internalization, and nystatin, which depletes cholesterol from membranes, thereby disrupting lipid rafts. These inhibitors were selected because they did not exhibit toxicity during the prolonged time courses required for examination of p75NTR internalization (data not shown). Other inhibitors of internalization were either toxic or non-specific under our experimental conditions (data not shown). To show that these inhibitors were effective in sympathetic neurons, neurons were incubated for 5 h with fluorescent conjugates of transferrin (TFN), which is internalized through clathrin-coated pits (Pearse, 1982; Harding et al., 1983) or cholera toxin subunit B (CTX-B), which is internalized in a raft-dependent manner (Orlandi and Fishman, 1998). Sucrose and nystatin inhibited the internalization of TFN and CTX-B, respectively, confirming their efficacy (Figs. 4A–D). We next examined the effect of these inhibitors on BDNF-GFP internalization through p75NTR. When studied by confocal microscopy, neither inhibitor prevented BDNF-GFP internalization to the extent seen with TFN and cholera toxin subunit B, although both appeared to reduce the amount of BDNF-GFP inside the cells (Figs. 4E–G). To better quantify this effect and to compare p75NTR:BDNF and TrkA:NGF internalization, neurons were treated for 1 h with 10 ng/ml NGF or 100 ng/ml BDNF either alone or in the presence of the inhibitors. Cell-surface bound BDNF or NGF was biotinylated, precipitated from cell lysates with streptavidin beads to separate it from the non-biotinylated intracellular neurotrophin and detected with anti-BDNF or anti-NGF in Western blots. NGF internalization was inhibited by sucrose but not nystatin (Fig. 4H), indicating a clathrin-mediated route of internalization, consistent with previous reports (Grimes et al., 1996; Howe et al., 2001). In contrast, BDNF internalization was decreased, but not eliminated, by both sucrose and nystatin (Fig. 4H), suggesting that both pathways might mediate internalization. We were, however, unable to rule out the possibility that a third, alternative pathway is involved, as the combination of both inhibitors proved to be toxic to the cells (data not shown). These results reveal that, unlike in PC12 cells, p75NTR does not mediate internalization exclusively through clathrin-coated pits in sympathetic neurons. Rather, it also uses a cholesterol-dependent route.

The p75NTR:ligand and Trk:ligand complexes differ in their association with cell surface lipid rafts

The observation that the lipid raft-disrupting drug nystatin inhibited p75NTR:BDNF but not TrkA:NGF internalization suggested that Trk and p75NTR differ in their localization to lipid rafts. We therefore assessed the presence of total p75NTR and TrkA in lipid rafts, isolated from sympathetic neurons by virtue of their insolubility in 1% Triton X-100 at 4°C. The effectiveness of the fractionation was confirmed by the presence of caveolin, a lipid raft marker, in the raft fraction (Fig. 5A) and

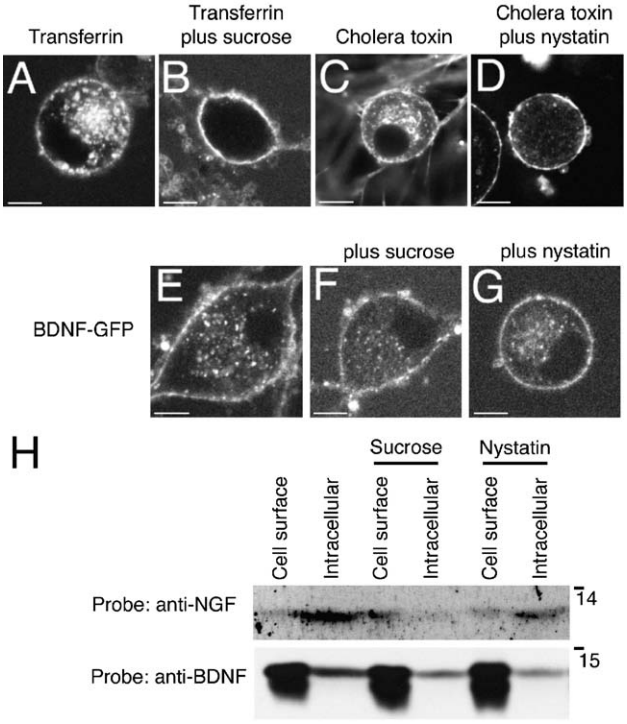


Fig. 4. BDNF internalization is partially prevented by either inhibition of clathrin-mediated endocytosis or disruption of lipid rafts. (A, B) Sympathetic neurons were incubated with Cy3 labeled TFN for 5 h after a 1-h incubation in serum free medium either in the absence (A) or presence (B) of 500 mM sucrose. The distribution of the ligand was determined by confocal microscopy. (C, D) Sympathetic neurons were incubated for 5 h with alexa-555 conjugated cholera toxin subunit B in the absence (C) or presence (D) of 50 μg/ml nystatin and imaged by confocal microscopy. (E–G) BDNF-GFP was incubated for 5 h with sympathetic neurons either alone (E) or with the addition of 500 mM sucrose (F) or 50 μg/ml nystatin (G) and its distribution determined by confocal microscopy. (H) Sympathetic neurons were incubated with 50 ng/ml NGF, after washing out residual NGF, or 100 ng/ml BDNF in the presence or absence of the indicated inhibitors. Cell surface proteins were labeled with biotin and detected as described for Fig. 2. Scale bars = 10 μm.

the exclusion of BiP (an endoplasmic reticulum marker not expected to enter lipid rafts) from that fraction (Fig. 5B). Both p75NTR and TrkA were found in the lipid raft fraction, although the majority of both receptors were found in the non-raft fraction (Figs. 5C, D). To confirm that these receptors were in lipid rafts, the 1% Triton-insoluble fraction containing the rafts was subjected to sucrose density centrifugation, and the purified raft fraction, which floats in this gradient, identified by the presence of Fyn, a lipid raft marker. Both p75NTR and TrkA were present to some extent in purified lipid rafts (Fig. 5E).

While both receptors were present in lipid rafts, it is important to ascertain whether neurotrophin binding occurs in this compartment. It is possible that the receptors move into or out of the rafts in response to ligand or even that the rafts detected in the above assay are intracellular, for example in the Golgi apparatus. To determine whether neurotrophins bound to p75NTR are localized within lipid rafts, sympathetic neurons were incubated with 100 ng/ml BDNF for 10 min to 1 h, and then fractionated to separate the lipid raft and non-raft portions of the plasma membrane as described above. After 10 min or 1 h, comparable levels of BDNF were found in the raft and non-raft fractions (Fig. 5F). Thus, approximately half of the p75NTR:BDNF complex seen at these time points is associated with lipid rafts, even though only a small percentage of total cellular p75NTR is present in this compartment (Fig. 5F). Reprobing of this blot for the non-raft protein BiP showed that non-raft protein does not contaminate the raft fractions. In neurons treated with NGF, NGF was mainly associated with the non-raft fraction both after 10 min and 1 h (Fig. 5G), suggesting that neurotrophins bound to Trk primarily localize in the non-raft fractions. To confirm this finding, the distribution of BDNF bound to TrkB in neurons expressing this receptor via recombinant adenovirus and expressing only low levels of p75NTR was assessed. BDNF was detected only in the non-raft fraction 10 min after addition to the TrkB-expressing cells, and primarily in this fraction at 1 h (Fig. 5H). A small amount of BDNF was present in the raft fraction at 1 h, although a similar amount was found in the control, β-galactosidase expressing adenovirus-infected neurons, likely indicating a low level of binding to p75NTR. Thus, much of the BDNF bound to p75NTR is found in lipid rafts while neurotrophins bound to Trk receptors are largely excluded from the rafts.

To confirm the biochemical data indicating that the p75NTR:ligand complex is associated with lipid rafts, we visualized the rafts by confocal microscopy. The neurons were incubated with BDNF-GFP and Alexa-555-conjugated CTX-B for 5 h, and confocal microscopy was performed on the living cells. As has been previously shown (Harder et al., 1998), CTX-B brings the lipid rafts, which are extremely small, into large patches on the plasma membrane. BDNF-GFP colocalized with CTX-B in these patches (Figs. 5I, J), confirming that BDNF-GFP bound to p75NTR localizes to a lipid raft population.

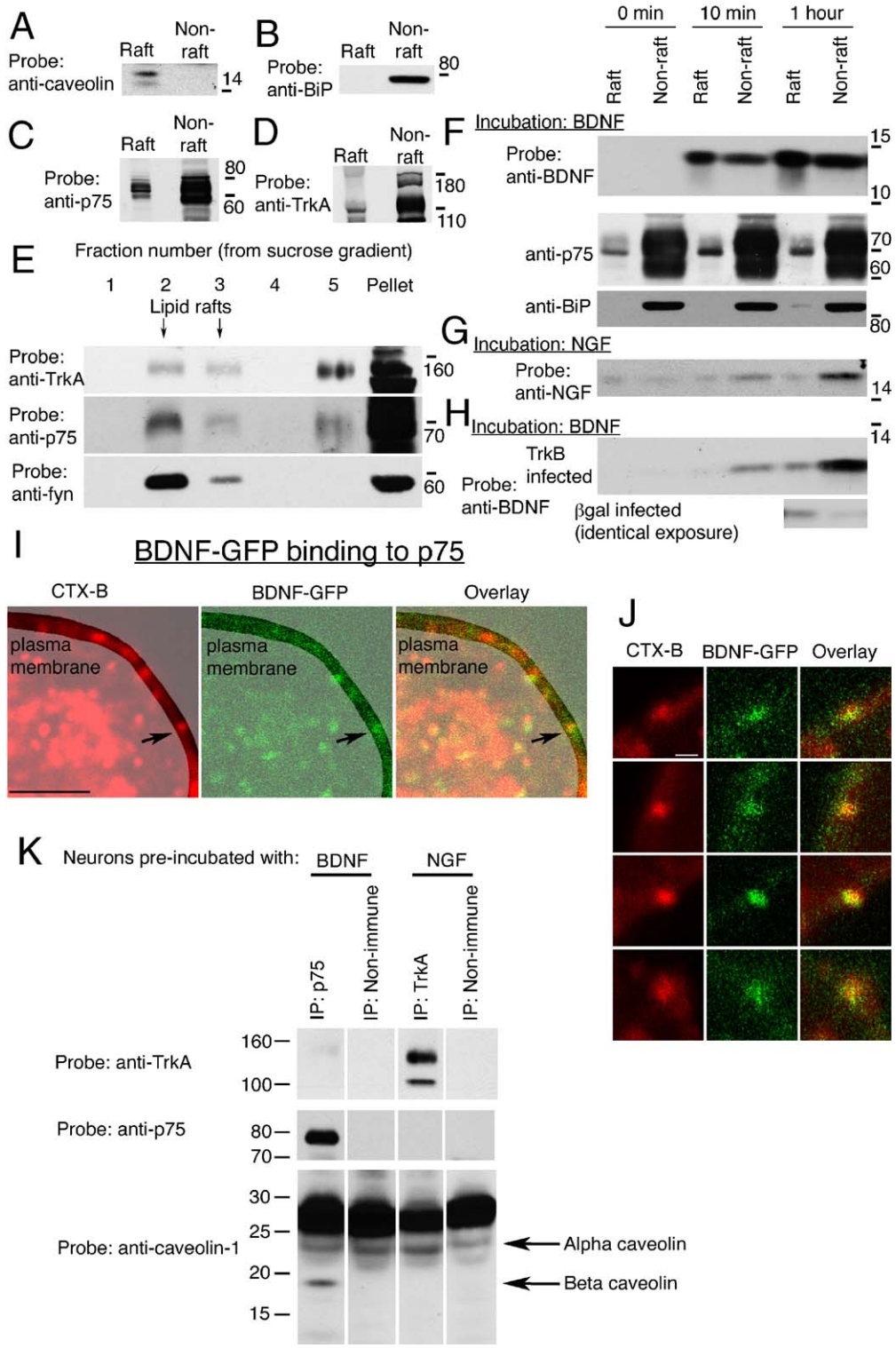
As a final confirmation that p75NTR was localized to lipid rafts, we asked whether it associated with the lipid raft marker caveolin-1. An association was identified between both TrkA and p75NTR with caveolin when these receptors were over-expressed in cell lines or in vitro (Bilderback et al., 1997, 1999). In view of this, we asked whether endogenous p75NTR or TrkA associated with caveolin-1. Sympathetic neurons were treated with NGF or BDNF overnight at 4°C, warmed to 37°C for 5 min to allow internalization to commence, lysed and immunoprecipitated with anti-p75NTR or anti-TrkA. Western blotting with anti-caveolin-1 revealed that p75NTR, but not TrkA, coimmunoprecipitated with a 17 kDa band corresponding to the predicted molecular weight of the truncated, beta isoform of caveolin-1 (Scherer et al., 1995) (Fig. 5K). Bands at 27 and 22 kDa (possibly alpha-caveolin) were also precipitated with non-immune serum, and were therefore precipitated non-specifically.

BDNF is degraded in lysosomes after its internalization with p75NTR

Since our data show that BDNF is internalized by p75NTR and does not recycle (Fig. 2), we asked whether it was ultimately degraded in lysosomes. Neurons were incubated overnight at 4°C with 100 ng/ml BDNF, and the surface-bound protein labeled with a cell impermeable biotinylation reagent with a reducible chain. The cells were warmed to 37°C for 1 h to allow BDNF internalization, and washed with a buffer containing reduced glutathione to remove all of the biotin associated with surface-bound BDNF. The cells were returned to 37°C for up to 24 h in

order to determine how rapidly this intracellular BDNF was lost from the cells. The cells were then lysed, the biotinylated proteins precipitated with streptavidin and the precipitates analyzed for biotin-labeled BDNF by Western blotting. This analysis revealed that the amount of internalized, intact BDNF decreased between 1 and 5 h (Figs. 6A, B). As this decrease cannot be attributed to recycling (Fig. 2H), this result indicates that BDNF, after being internalized via the p75NTR, is degraded within several hours.

We hypothesized that this degradation might take place in the lysosomal pathway. To address this, we incubated sympathetic neurons with BDNF-GFP plus 10 ng/ml NGF either alone or with the addition of one of two inhibitors of lysosomal function, ammonium chloride or chloroquine (Wibo and Poole, 1974;



Hopgood et al., 1977), and assessed the amount of BDNF-GFP in the cells by confocal microscopy. After 24 h, BDNF-GFP levels were much greater in the presence of these inhibitors than in their absence (Figs. 6C–D), indicating that it normally enters the degradative pathway. Little difference between the amounts of BDNF-GFP inside the cells with or without these inhibitors was found after 5 h, consistent with the combined time lags of several hours before internalization and then before degradation of the internalized protein (Figs. 2 and 6A–B).

It is possible that soon after internalization, BDNF dissociates from p75NTR and is transported for degradation independently of the receptor. To ask whether this was the case, we used confocal microscopy to determine whether BDNF-GFP colocalized with a Cy3-labeled p75NTR antibody (MC192) in the presence of chloroquine or ammonium chloride. In the presence of either inhibitor, strong colocalization between BDNF-GFP and p75NTR inside the cells was apparent (Figs. 6E, F). Quantitation revealed that this colocalization was statistically significant, as compared to a negative control in which the red channel was rotated 90° relative to the green (Fig. 6G). This colocalization indicates that the ligand and receptor remain in a common compartment at least until shortly before the ligand is degraded.

The retrograde transport of BDNF and p75NTR occurs in the absence of PI3-kinase and TrkA activation

A significant amount of research has focused upon the mechanisms underlying the retrograde transport of the Trk: neurotrophin complex in sympathetic and sensory neurons (reviewed in Miller and Kaplan, 2001b). However, little is known about the mechanisms that regulate retrograde transport of the p75NTR: neurotrophin complex. To address this question, we utilized sympathetic neurons in compartmented cultures, where the distal axons are separated from the cell bodies and proximal axons by a barrier (Fig. 7A). To determine whether p75NTR or its ligand, BDNF-GFP was retrogradely transported from distal axons to cell bodies, BDNF-GFP and Cy3-labeled p75NTR antibody were added to the side compartments but not the center compartment overnight (Fig. 7B), and the cell bodies analyzed by confocal microscopy. Anti-p75-Cy3 was detected in many cell bodies (Fig. 7C), while BDNF-GFP was not detected (Fig. 7D). One possible explanation for this difference is that BDNF-GFP is retrogradely transported, but is rapidly degraded once it reaches the cell body. To examine this, the experiment was repeated with the lysosomal inhibitors ammonium chloride or chloroquine added to all compartments to prevent BDNF-GFP degradation (Fig. 7B). Under these conditions, both anti-p75NTR-Cy3 (Figs. 7E, G) and BDNF-GFP (Figs. 7F, H) were readily observed in the cell bodies. Thus, both p75NTR and BDNF-GFP are retrogradely transported, presumably as a complex based upon the colocalization described above (Figs. 6E–G). Following retrograde transport, BDNF-GFP is then degraded in lysosomes.

To ask whether BDNF-GFP was retrogradely transported intact and then degraded in the cell bodies, and to estimate the rate of retrograde transport, we performed live imaging of the axons. To do so, we imaged a region under the barrier crossed by the axons, as indicated in Fig. 7I, using an inverted confocal microscope equipped with a heated stage. Only a narrow region was examined to allow rapid scans, enabling us to follow movement along the axons over time. Compartmented cultures were incubated with BDNF-GFP and anti-p75-Cy3 in the side compartments overnight in the absence of inhibitors, and a series of images were collected of the region under the barrier for each of these two fluorophores (Figs. 7J, K, movies provided in Supplementary data). Bright, p75NTR- or BDNF-GFP-positive structures were seen moving through the axons towards the cell bodies, corresponding to retrograde transport. Occasionally, structures were seen moving in the anterograde direction. Importantly, since BDNF-GFP was observed being transported in the absence of lysosomal inhibitors, these findings suggest that BDNF degradation occurs only at or near the cell body. Quantification of the transport of the BDNF-GFP-positive structures revealed that these structures were retrogradely transported at a rate of 1.52 ± 0.26 μm/s. This is similar to the estimated rate of transport of the TrkA receptor in vivo (Richardson and Riopelle, 1984) although slower than that reported in vitro (Ure and Campenot, 1997; Heerssen et al., 2004). The 15.5% of the structures that were moving in the anterograde direction traveled at a rate of 1.65 ± 0.26 μm/s.

It has been reported that PI3-kinase and Trk activity are required for the ligand-dependent retrograde transport of Trk receptors (Watson et al., 1999; Kuruvilla et al., 2000). We therefore asked whether this was also true for the retrograde transport of BDNF-GFP and p75NTR. Compartmented cultures were established and then chloroquine and either 50 μM LY294002 or 200 nM K252a, selective pharmacological inhibitors of PI3-kinase and TrkA respectively, were added to all compartments, and BDNF-GFP and Cy3-labeled p75NTR antibody added to the distal compartments. After 24 h, inhibition of PI3-kinase or TrkA activity had no detectable effect on the retrograde transport of BDNF-GFP or p75NTR (Fig. 7L). To confirm the efficacy of the inhibitors,

Fig. 5. P75NTR but not Trks bind to their ligands in lipid rafts. (A–D) Sympathetic neurons were collected, incubated in 1% Triton-X100 on ice and centrifuged. The insoluble pellet containing the lipid rafts (raft) and supernatants (non-raft) were separated by SDS-PAGE and the proteins indicated detected by Western blotting. (E) Sympathetic neurons were collected by scraping and homogenized on ice in 1% Triton-X100. The homogenate was loaded on a sucrose gradient. Following ultracentrifugation, fractions were collected from the gradient, with fraction 1 being at the top, along with the pellet. Proteins from the fractions and pellet were separated by SDS-PAGE and analyzed by Western blotting to detect the proteins indicated. (F) Sympathetic neurons were incubated with 100 ng/ml BDNF for the times indicated (with the "0 min" time point corresponding to cells not exposed to ligand) and the raft and non-raft proteins separated as for panels A–D. (G) The same procedure was followed as for panel F except that incubations were carried out using 50 ng/ml NGF after washing NGF from the cells. (H) Sympathetic neurons were infected with adenoviruses encoding TrkB or β-galactosidase and then grown in 10 ng/ml NGF for 2 days. The neurons were then incubated for the times indicated in 10 ng/ml BDNF and raft fractions collected as described above. (I) Confocal microscopy of uninfected neurons incubated overnight with BDNF-GFP (green) and cholera toxin B (CTX-B; red), a lipid raft marker that patches rafts, in live cells. The arrow highlights a cluster in which both fluorophores colocalize. A semi-transparent mask has been used to highlight the plasma membrane region. Scale bar = 5 μm. (J) Magnifications of four further patches formed by CTX-B (red) which also contain BDNF-GFP (green). The colocalization is shown in overlays of the two channels. Scale bar = 0.5 μm. (K) Sympathetic neurons were washed free of ligand, cooled to 4°C in buffered medium and allowed to bind 10 ng/ml NGF or 100 ng/ml BDNF overnight. The cells were warmed for 5 min, lysed, and immunoprecipitation was carried out with the indicated sera. Western analysis was carried out using the indicated antibodies. The positions of the alpha and beta forms of caveolin-1 are indicated. All lanes were obtained from the same gel.

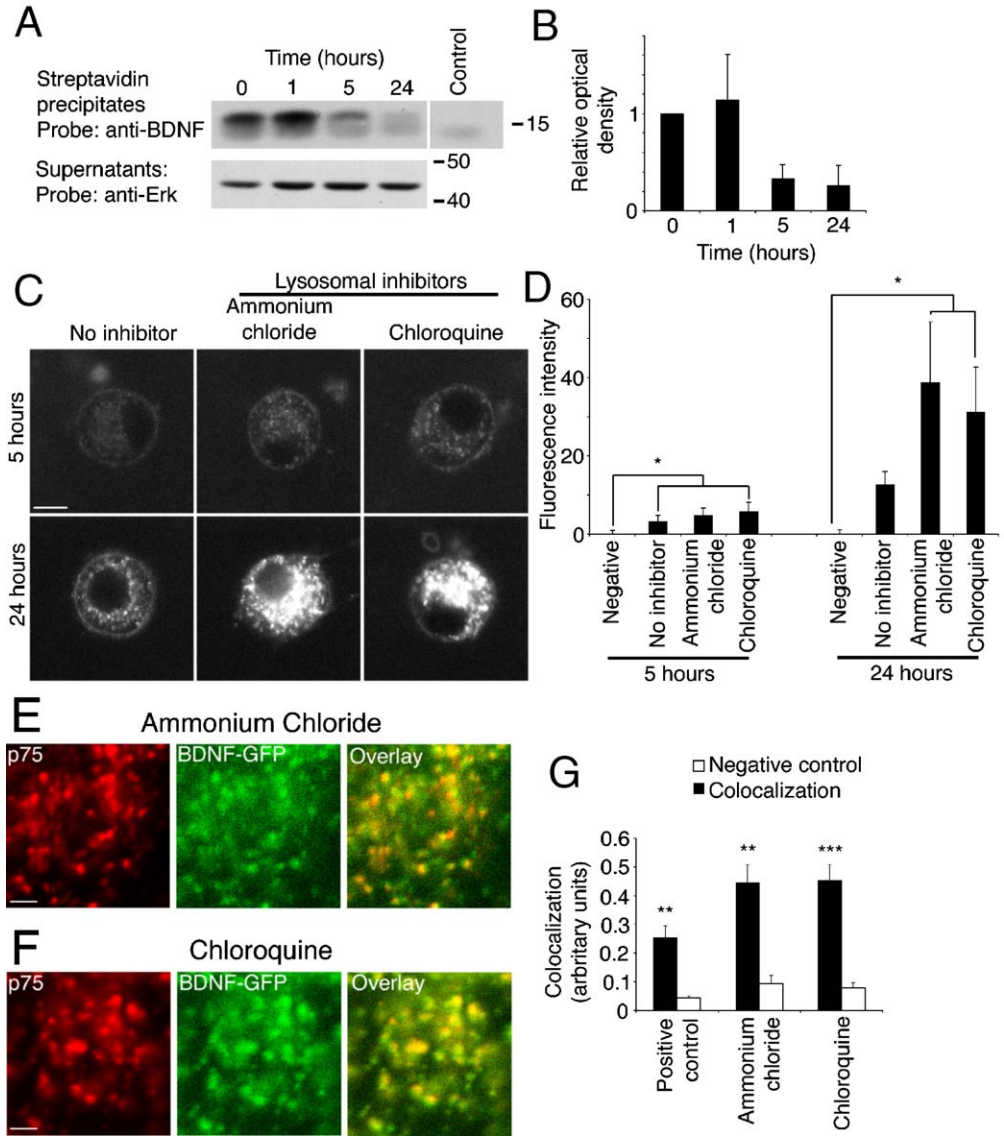


Fig. 6. Following internalization, BDNF-GFP is degraded in lysosomes. (A) The lifetime of internalized BDNF was assayed as described in Experimental methods. No BDNF was added to the cells used in the control sample. Internalized BDNF was lost between 1 and 5 h. Equal loading is evidenced by probing the supernatants for Erk. (B) Quantitation of the optical density of the bands corresponding to BDNF in panel A, illustrating the loss of signal between 1 and 5 h. (C, D) Sympathetic neurons were incubated with BDNF-GFP either alone or plus the lysosomal inhibitors ammonium chloride or chloroquine, as indicated. Images were collected after 5 or 24 h (C; Scale bar = 10 μm). The intensity of the fluorescence in a stack of images through the intracellular domain of the cell body was quantified under each treatment and time point (D). The intensities were compared by ANOVA with Student–Newman–Keuls post hoc testing (* $P < 0.05$). (E, F) Neurons were incubated with BDNF-GFP and anti-p75-Cy3 for 24 h in the presence of ammonium chloride (E) or chloroquine (F). Stacks of confocal images were collected spanning the intracellular region of the neuronal cell body. Colocalization between the markers was evidenced by the yellow signal in the overlay of the channels. Scale bars = 2 μm. (G) Quantitation of the colocalization between anti-p75-Cy3 and BDNF-GFP by thresholding. Images were collected as for panels E, F above. The relative proportion of the voxels containing a strong signal in the red channel that also had a signal in the green channel was determined. As a positive control, BDNF-GFP was detected in both channels. In each case, the calculation was repeated after rotating one channel 90° relative to the other as a negative control (open bars) to compare to the measured colocalization (solid bars). In every case, significant colocalization was seen over the negative control (paired *t* test with Bonferroni correction; ** $P < 0.01$, *** $P < 0.001$).

sympathetic neurons were washed free of NGF, and then exposed for 30 min to 10 ng/ml NGF in the presence or absence of LY294002 or K252a. Western blot analysis with antibodies specific for the phosphorylated, activated forms of Akt, ERK or TrkA indicated that LY294002 only inhibited Akt activity, while K252a inhibited activation of TrkA, Akt and ERK, as predicted (Fig. 7M). Thus, the internalization and retrograde transport of the p75NTR:ligand complex in sympathetic neurons occurs through a mechanism distinct from the internalization and transport of the Trk:neurotrophin complex.

Discussion

Here we compare the localization, trafficking and retrograde transport of the two neurotrophin receptor:ligand complexes,

p75NTR and Trk, in primary sympathetic neurons using live cell imaging and biochemical approaches. Our data support the conclusion that p75NTR is found in a cellular compartment segregated from TrkA, and that it can be trafficked by different mechanisms than TrkA. Following ligand exposure, the p75NTR:BDNF complex is localized largely in lipid rafts and associates with the beta isoform of caveolin-1. In contrast, Trk:ligand complexes are excluded from lipid rafts for the first hour following ligand exposure, and no association of TrkA with beta-caveolin-1 was detected. Unlike Trk, p75NTR is slowly internalized. This is not due to a slow internalization step per se, since BDNF is rapidly internalized if it is first bound to cells overnight under conditions where internalization is blocked, and internalization is then allowed to proceed. This result suggests that it is an earlier step, likely the slow rate of association of BDNF with neurons, which has been previously described in cell lines (Fabricant et al., 1977; Kahle and Hertel, 1992; Saxena et al., 2004), that limits p75NTR-mediated internalization. Further characterization of this step will provide additional insight into p75NTR-mediated internalization. Differences in the rates of association of different neurotrophins with p75NTR have been described (Rodriguez-Tebar and Barde, 1988; Rodriguez-Tebar et al., 1990). While Trk internalization is entirely mediated by clathrin-coated pits, the internalization of p75NTR is partially inhibited by disruption of clathrin-mediated endocytosis or of lipid rafts in neurons. This is in contrast to p75NTR internalization to PC12 cells, which is completely dependent upon clathrin-mediated endocytosis (Bronfman et al., 2003; Saxena et al., 2004). Previous experiments have found that TrkB moves into lipid rafts when bound to BDNF in central neurons (Suzuki et al., 2004), which express low levels of p75NTR. When the expression of p75NTR was artificially elevated, TrkB no longer moved into lipid rafts (Suzuki et al., 2004). Thus, the high level of p75NTR expressed in sympathetic neurons may function to exclude TrkA:NGF from rafts. We demonstrate that the P75NTR:BDNF complex is retrogradely transported in cultured neurons in a PI3-kinase and Trk activity-independent manner, in contrast to the mechanism of Trk retrograde transport (Watson et al., 1999; Kuruvilla et al., 2000), and the internalized BDNF-GFP is rapidly degraded in lysosomes at or near the cell bodies.

The Trk:neurotrophin complex is internalized to PC12 cells or sympathetic neurons, through clathrin-coated pits, to "signaling" endosomes containing the activated Trk receptor and its associated downstream signaling complex (Grimes et al., 1996; Howe et al., 2001; Delcroix et al., 2003; Ye et al., 2003). These signaling endosomes are thought to retrogradely carry a survival signal from the axon terminal to the cell body (Neet and Campenot, 2001; Ginty and Segal, 2002). TrkA retrograde transport requires Trk and PI3-kinase activity (Watson et al., 1999; Kuruvilla et al., 2000). The lack of such a requirement for p75NTR:BDNF transport argues for a distinct route of transport for p75NTR.

It will be interesting to determine whether p75NTR signals after internalization or during retrograde transport. Previous studies have shown that p75NTR is required for retrograde transport of neurotrophins in vivo (Johnson et al., 1987; Yan et al., 1988; Curtis et al., 1995, 1998; Helke et al., 1998; Kramer et al., 1999; Crockett et al., 2000; Janiga et al., 2000). While none of these studies have demonstrated cotransport of a p75NTR:neurotrophin complex, an intriguing recent study demonstrated that motor neurons contain a retrogradely transported vesicle population that cotransports NGF, p75NTR and a non-toxic tetanus toxin fragment (Lalli and Schiavo, 2002). Since tetanus toxin binds to its neuronal receptors in lipid rafts (Herreros et al., 2001), these vesicles may represent specialized raft-derived retrograde transport vesicles, and may well be analogous to the vesicles that we observe here.

We have previously shown that p75NTR is able to promote apoptosis in a TrkA-independent manner but that TrkA can inhibit this function of p75NTR (Majdan et al., 2001). Conversely, p75NTR has a negative influence on axon growth in the presence of the TrkA ligand NGF (Kohn et al., 1999). Together, these observations suggest that p75NTR may function, in part, in a compartment separate from TrkA. By describing the localization and trafficking of p75NTR and TrkA, we have identified two candidate compartments where this might occur, namely lipid rafts and possibly an endosomal compartment accessed by p75NTR in a PI3-kinase independent manner. It is important to note that p75NTR:BDNF and TrkA:NGF complexes might also coexist, and so be able to interact, in common compartments in addition to the segregated pool of p75NTR. Such a possibility is evidenced by the partial inhibition of p75NTR-mediated internalization by inhibitors of clathrin-mediated endocytosis, which is responsible for TrkA internalization.

While this work reinforces many important themes in neurotrophin signaling, we describe several important differences from results obtained in other systems. For example, in central neurons TrkB is trafficked into lipid rafts in response to BDNF, while we show that in sympathetic neurons it is not. Since such translocation in central neurons is inhibited by ectopic expression of p75NTR (Suzuki et al., 2004), which is expressed at high levels in sympathetic neurons, it may be the level of expression of this receptor that accounts for the difference between the neuronal types. In PC12 cells, clathrin-coated pits are entirely required for p75NTR internalization (Bronfman et al., 2003; Saxena et al., 2004) while we find that at least one alternative, cholesterol-dependent route exists in sympathetic neurons. This may indicate the existence of lipid-raft compartments or internalization mechanisms in sympathetic neurons that are lacking in PC12 cells. These differences emphasize the importance of examining p75NTR trafficking in the same model system, sympathetic neurons, in which we and others have previously characterized its signaling (Bamji et al., 1998; Kohn et al., 1999; Majdan et al., 2001; Palmada et al., 2002; Yeiser et al., 2004; Linggi et al., 2005).

Experimental methods

Cell culture

Sympathetic neuron cultures were prepared from P1 to P3 rats supplied by Charles River Canada as described previously (Vaillant et al., 1999). Media used were supplemented with 2 mM L-glutamine (Cambrex, Walkersville, MD) and 100 μg/ml penicillin/streptomycin (Cambrex). On day 1 neurons were plated in Ultraculture medium (Cambrex) including 3% rat serum (Wisent, St. Bruno, QC), 0.7 mM cytosine arabinoside (CA; Sigma, Oakville, ON) and 50 ng/ml nerve growth factor (NGF; CedarLane, Hornby, ON) to a density of 1 l per 6 well plate or 8 chamber cover slip (Nalge Nunc, Rochester, NY). On day 2, additional medium was added and all medium replaced on day 4. On day 6, the medium was changed to Ultraculture (or conditioned medium in some experiments) including 10 ng/ml NGF. Experiments were carried out on day 7.

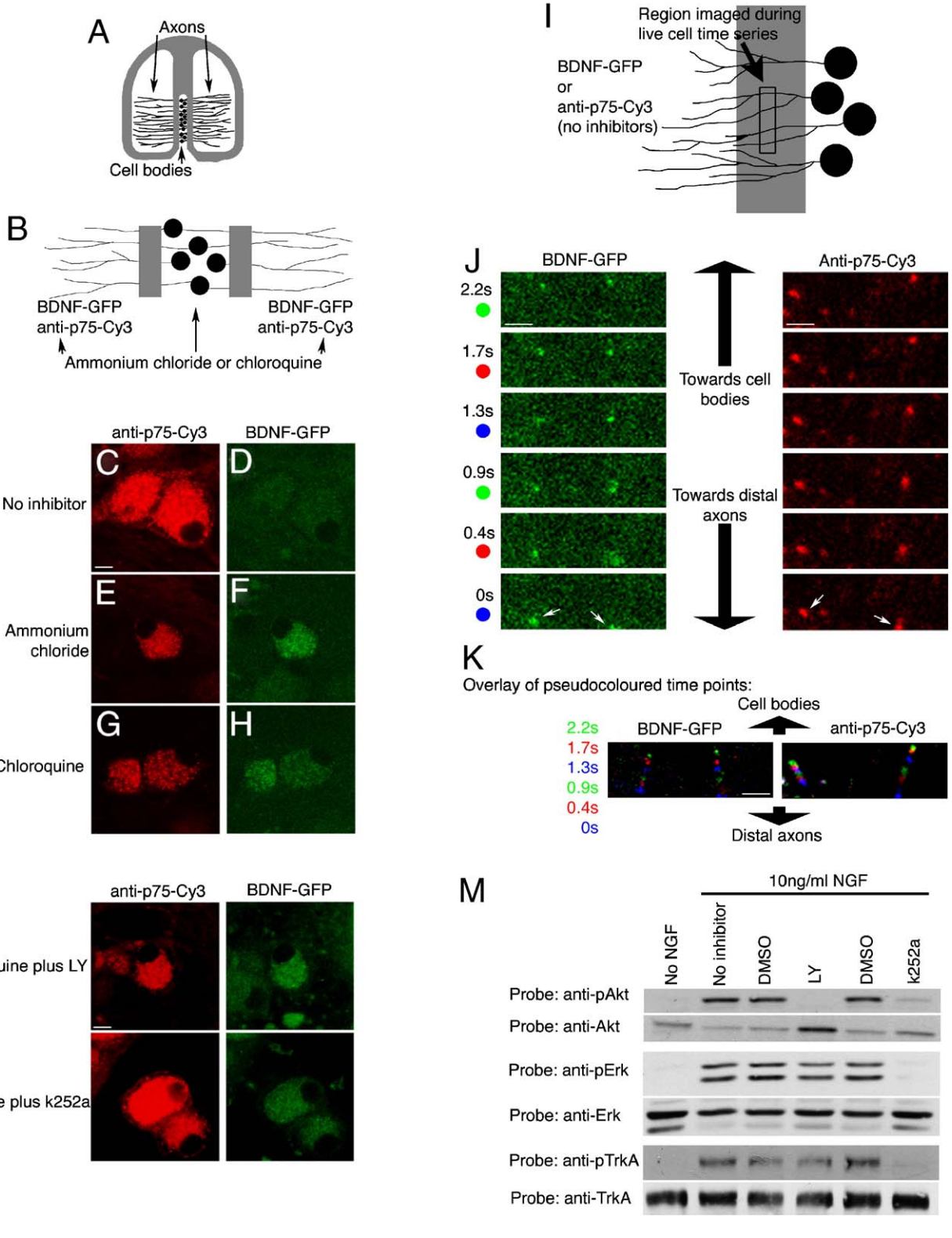
Mouse sympathetic neurons were cultured in an identical manner except that the plating medium contained 3% fetal bovine serum (FBS; Cambrex) instead of rat serum and CA was not added to the cells until the day after

plating, after which it was used at 0.5 mM. The p75 exon III knockout mice have been described previously (Lee et al., 1992; Bamji et al., 1998; Majdan et al., 2001).

Cell lines were maintained in DMEM (Cambrex) plus 10% FBS. Gentamicin (0.7%, Invitrogen) was also added to cells stably expressing BDNF-GFP or TrkB.

Compartment cultures

Compartment cultures were prepared as previously reported (Campenot and Martin, 2001) on a single-well chambered glass cover slip (Nalge Nunc) that was coated with collagen. Scratches were made individually using a diamond pen, taking care not to fracture the cover glass. The



neurons were plated in the center compartment and grown with 50 ng/ml NGF in all compartments, plus 3% rat serum and 0.7 mM CA in the center compartment only for the first 5 days.

Generation of the BDNF-GFP construct

The vector encoding BDNF-GFP has been previously described (Mowla et al., 1999). 293A cells stably expressing this construct were the generous gift of Dr S. Morris (Aegera Therapeutics, Montreal, QC). Conditioned media were prepared from cells plated in 75 cm^2 vented tissue culture flasks and grown to 80% confluency. The flasks were wrapped in aluminum foil and the cells incubated with 5 ml Ultraculture or DMEM per flask for 2 days. The conditioned medium was collected, cleared by centrifugation and stored at −80°C. After thawing, the conditioned medium was added undiluted to the cells.

Adenoviral infections

Adenoviruses encoding TrkB (wild type) and β-galactosidase have been previously described (Atwal et al., 2000; Toma et al., 2000). Neurons were infected on day 4 in medium DMEM plus 10% FBS and 50 ng/ml NGF with 3.0×10^5 plaque forming units of virus to one well of an 8 chamber cover slip or 3.2×10^6 plaque forming units to each well of a 6 well plate. On day 5, the neurons were switched to Ultraculture containing 10 ng/ml NGF and no serum. Experiments were carried out on day 7.

Live cell imaging

Neurons were plated on eight chamber glass cover slips (Nalge Nunc). Conditioned Ultraculture plus 10 ng/ml NGF was added to the cells, along with 4 μg/ml cholera toxin subunit B conjugated to Alexa Fluor 555 (Molecular Probes, Burlington, ON) or 3 μg/ml anti-p75NTR (MC192) conjugated to Cy3 (Advanced Targeting Systems, San Diego) as indicated in some experiments. In some experiments, 20 mM ammonium chloride, 100 μM chloroquine, 50 μM LY294002, 200 nM K252a, 500 mM sucrose or 50 μg/ml nystatin was added along with the ligands. In experiments assessing transferrin (TFN) localization, the cells were first washed with and then incubated for 1 h in serum-free DMEM plus 10 ng/ml NGF. DMEM plus 10 ng/ml NGF was added to the cells with 5 μg/ml human TFN conjugated to Cy3 (Jackson, West Grove, PA). Following incubation in the dark for the durations indicated in the figure legends, the cells were observed using a Zeiss Pascal confocal microscope through a 63× oil immersion objective (Plan aprochromat, NA = 1.4). Cells were at room temperature for the duration of the observations, which did not exceed 3 h. Following the observations, cells could be maintained in the incubator with no toxic effects. Images were collected using the manufacturer's software. To collect data from prolonged time courses following internalization, cells were first incubated in CO_2-independent medium (Invitrogen) for 1 h and then transferred to a heated stage on the confocal microscope, and 3 μg/ml anti-p75NTR conjugated to Cy3 plus 10 ng/ml NGF and 100 ng/ml BDNF in CO_2-independent medium was added to the cells. Stacks of confocal images were collected at regular time intervals using the manufacturer's software. Images were only enhanced by manipulation of the levels using Photoshop (Adobe, San Jose, CA) such that the entire intensity range was used.

Cells in compartmentalized cultures were imaged in a similar manner. Fluorescent ligands were added only to the side compartments and the cultures incubated overnight. Time courses were collected with the cultures maintained on the heated stage. A narrow field under the center of the barrier was examined. Consecutive confocal images were collected at a rate of over two frames per second. The rate of transport was quantified using a plug-in to ImageJ (NIH).

Image analysis

Image analysis was carried out using macros written in Northern Eclipse (Empix, Mississauga, ON). To determine the mean fluorescence signal reported in Fig. 1, all slices from a confocal z-stack collected at 4 μm intervals in which the cell body could be clearly distinguished were analyzed. The cell body was outlined manually and the fluorescence of all voxels determined. The mean voxel intensity across the stack was determined for each cell and the values standardized. To determine the fluorescence signal intensity in the presence of lysosomal inhibitors, a z-stack of 10 images, at 1.5 μm intervals, spanning the internal cell body was collected. The mean fluorescence of all voxels was determined for each stack.

To quantify colocalization inside the cell, a z-stack of 10 images, at intervals of 1.5 μm was assessed. Thresholds at which most background noise was eliminated were first determined. These thresholds were maintained for all images collected in a single experiment but varied between experiments as ligand concentrations and the properties of the light path varied slightly. The percentages of voxels above threshold in each channel separately, or in both, were determined and ratios calculated. Random colocalization was assessed by rotating the red channel 90° from its starting orientation using Photoshop before analysis.

Western blot analysis

Cell lysates were prepared, protein concentrations determined and Western blotting performed as previously described (Vaillant et al., 1999). The antibodies used were: BDNF (Santa Cruz, Santa Cruz, CA, 0.2 μg/ml), GFP (Santa Cruz, 0.2 μg/ml), tubulin (Sigma, 1:5000), p75 (Promega, Mississauga, ON, 1 μg/ml), Erk (Santa Cruz, 0.02 μg/ml), NGF (Santa Cruz, 0.2 μg/ml), TrkA (RTA, 1:5000; Clary et al., 1994), Fyn (BD Biosciences, Mississauga, ON, 1 μg/ml), BiP (BD Biosciences, 0.5 μg/ml), caveolin-1 (BD biosciences, 0.5 μg/ml) and phosphotyrosine 4G10 (Upstate, Waltham, MA, 1:50).

The optical densities of the immunoreactive bands were quantified using ImageJ (NIH). The band optical density was subtracted from a

Fig. 7. BDNF-GFP is retrogradely transported in sympathetic neurons in a PI3-kinase and Trk-independent fashion. (A) Schematic diagram to illustrate the compartment culture system. Neurons are plated in the central compartment and grow axons under the barrier to the sides. Ligands can be added to the sides and do not diffuse into the central compartment. (B) Schematic diagram to illustrate the reagents added to each compartment imaged in panels C–H below. (C–H) Sympathetic neurons were grown in compartment cultures on borosilicate cover slips and incubated with BDNF-GFP and anti-p75 Cy3 applied only to their axons for 24 h with either no inhibitor (C, D), ammonium chloride (E, F) or chloroquine (G, H) in all compartments. Anti-p75-Cy3 (C, E, G) and BDNF-GFP (D, F, H) were detected by confocal microscopy. Scale bar = 10 μm. (I) Schematic diagram showing the region of the axons imaged in live cell observations of retrograde transport. (J) Time series illustrating the transport of single vesicles containing anti-p75-Cy3 (red) or BDNF-GFP (green) along the axons. Each image was collected at the time indicated. Ligands were applied to the distal axons. Vesicles (indicated by arrows at the first time point) can be seen progressively closer to cell bodies as the time series progresses, consistent with retrograde transport. (K) Overlay of the images from panel J to illustrate the transport of the vesicles. The images have been pseudocolored, with each image colored as indicated by the spot below the time indicator in panel J. This key is also indicated by the colors of the times marked beside the images. The movement of the vesicles is indicated by their appearance in the sequence blue–red–green–blue–red–green from the bottom to the top of the image. Scale bars in panels J and K = 2 μm. (L) Sympathetic neurons were cultured and incubated as for panels C–H above, except that both chloroquine and either LY or k252a were added to all chambers. The cell bodies were imaged as for panels C–H. Scale bar = 10 μm. (M) Sympathetic neurons were washed free of NGF, preincubated with or without LY294002 or K252a, or DMSO at an equivalent concentration to that in which each drug was mixed, and then incubated either without NGF or in the presence of 10 ng/ml NGF, with the same pharmacological agents for 30 min. Cells were lysed and the indicated proteins detected by Western analysis using phosphorylation-specific antibodies. The blots were stripped and reprobed for total Erk, Akt or TrkA, as indicated.

background region of the same size. Equal areas were quantified for the comparison of bands from the same gels.

Immunoprecipitation

Immunoprecipitations of equal protein concentrations of cell lysates or equal volumes of conditioned medium and Western blotting were performed as previously described (Belliveau et al., 1997) using 0.4 μg anti-GFP (Santa Cruz), 3 μl anti-pan Trk (203, Stephens et al., 1994) or 2 μl of anti-p75NTR (REX, Weskamp and Reichardt, 1991; Kohn et al., 1999).

Cell surface biotinylation

Cells were incubated with medium containing the ligand of interest for the times indicated. Where BDNF or BDNF-GFP was used, the medium was supplemented with 10 ng/ml NGF. In the case of NGF stimulation, the cells were first washed with Ultraculture twice in quick succession followed by two washes at 1-h intervals; NGF was added 1 h or more after the last wash. Incubations were normally carried out at 37°C. In some experiments, the last wash was done using CO_2-independent medium after which cells were cooled to 4°C over 1 h and then incubated with the ligands in CO_2-independent medium overnight at 4°C before warming to 37°C. Following the incubations, cells were washed 3 times with phosphate buffered saline (PBS) and then incubated for 1 h in 0.5 mg/ml EZ-link sulfo-NHS-biotin (Pierce) in PBS. The cells were washed twice in TBS to remove any unreacted biotin, and lysed in TBS lysis buffer. Equal amounts of protein were incubated for 2–3 h with 40 μl of a 50% slurry of streptavidin-conjugated beads (Amersham). The supernatants were saved and the beads washed four times with TBS lysis buffer. The supernatants and bead pellets were boiled in sample buffer and subjected to Western blot analysis as described above. In the experiments with BDNF, a small amount of BDNF was observed to bind non-specifically to the streptavidin beads, but this amount was low relative to the experimental values.

Recycling assay

Sympathetic neurons were incubated in Ultraculture with 10 ng/ml NGF and 100 ng/ml BDNF for 1 h, washed three times with PBS and incubated with a cell-impermeable biotinylation reagent with a reducible linker (EZ-link sulfo-NHS-SS-biotin, Pierce, 1 mg/ml in PBS) to label cell surface proteins. As a control, some cells were washed using a reducing or control wash and lysed immediately as described below. The remaining cells were washed once with Ultraculture plus 10 ng/ml NGF and incubated in the same medium for 1 h at 37°C. The cells were then washed with a reducing buffer (50 mM Tris, pH 8.6, 100 mM NaCl, 1 mg/ml glucose, 1 mg/ml BSA, 50 mM reduced glutathione) twice for 30 min each at 4°C to remove all biotin still associated with the cell surface and then washed 3 times with Ultraculture plus 10 ng/ml NGF included in the final wash. The neurons were then incubated for 1 h at 37°C before a further two 30-min washes using either the reducing buffer or a control buffer (in which the reduced glutathione was omitted). The cells were washed 3 times with TBS and lysed using TBS lysis buffer. Biotinylated proteins were precipitated on streptavidin beads and analyzed by Western blotting as described above.

Degradation assay

Sympathetic neurons were incubated in CO_2-independent medium plus 10 ng/ml NGF for 1 h at 37°C and then cooled to 4°C for 1 h. Ice-cold CO_2-independent medium plus 10 ng/ml NGF and 100 ng/ml BDNF was added to the cells which were incubated at 4°C overnight. The following day, cells were labeled with NHS-SS-biotin as for the recycling assay before incubation for 1 h in Ultraculture plus 10 ng/ml NGF at 37°C. They were then washed with reducing buffer before incubation in Ultraculture plus 10 ng/ml NGF at 37°C for various times. The cells were washed 3 times with TBS and lysed using TBS lysis buffer. Biotinylated proteins were precipitated and analyzed by Western blotting as described above.

Acid wash

Cells were incubated with conditioned media containing BDNF-GFP plus 10 ng/ml NGF for various times, washed twice with PBS and incubated twice for 30 min in 0.5 M NaCl with 0.2 M acetic acid. Following four washes with TBS, the cells were lysed in TBS lysis buffer. To measure total binding, cells were washed 3 times with TBS then lysed in TBS lysis buffer. The BDNF-GFP concentration was measured using an ELISA kit (Promega) according to the manufacturer's instructions. Total protein concentrations were determined and used to standardize samples for Western analysis.

Triton insolubility

Following incubation with ligands as for the cell surface biotinylation experiments, cells were washed 3 times with TBS and scraped into 250 μl TBS. The wells were washed with a further 250 μl TBS and the two suspensions were combined. The cell fragments were pelleted at 16,000 × *g* for 10 min at 4°C and the supernatant removed. The pellet was resuspended in 200 μl 25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100 by passing 10 times through a 200 μl pipette tip. The suspension was incubated on ice for 30 min before centrifugation at 16,000×*g* for 10 min at 4°C. The supernatant and pellet were collected and subjected to Western blot analysis.

Sucrose gradient purification of lipid rafts

Sympathetic neurons from 4 wells of a 6 well plate were collected and homogenized in buffer containing 1% Triton X-100 as described above. Protease inhibitors (Roche) were added to all solutions used. Following a 30-min incubation on ice, 300 μl TBS was added. The suspension passed 5 times through a Dounce homogenizer and 500 μl 80% sucrose was added. The suspension was mixed well and transferred to a 5 ml ultracentrifuge tube. Subsequently, 2 ml 36% sucrose followed by 10% sucrose was sequentially layered over the sample. The samples were centrifuged at 200,000 × *g* overnight at 4°C in a swinging bucket rotor (Beckman, Fullerton, CA), after which a white band containing the lipid rafts was clearly seen floating in the gradient. Fractions of 1.05 ml were collected and diluted in TBS plus sucrose such that all samples contained 8.4% sucrose. The samples were centrifuged at 200,000 × *g* for 90 min, and the pellets from this centrifugation and material from a pellet at the bottom of the original gradient were subjected to SDS-PAGE and Western blot analysis.

Statistical analysis

Statistical testing was carried out using SigmaStat (Systat software, Point Richmond, CA). All error bars represent the standard error of the mean. All experiments were performed independently at least three times. Approximate standard sizes, in kilodaltons, are indicated next to Western blots.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2006.06.001.

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