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RESEARCH ARTICLE

The p75 neurotrophin receptor evades the endolysosomal route in neuronal cells, favouring multivesicular bodies specialised for exosomal release

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ABSTRACT

The p75 neurotrophin receptor (p75, also known as NGFR) is a multifaceted signalling receptor that regulates neuronal physiology, including neurite outgrowth, and survival and death decisions. A key cellular aspect regulating neurotrophin signalling is the intracellular trafficking of their receptors; however, the post-endocytic trafficking of p75 is poorly defined. We used sympathetic neurons and rat PC12 cells to study the mechanism of internalisation and post-endocytic trafficking of p75. We found that p75 internalisation depended on the clathrin adaptor protein AP2 and on dynamin. More surprisingly, p75 evaded the lysosomal route at the level of the early endosome, instead accumulating in two different types of endosomes, Rab11-positive endosomes and multivesicular bodies (MVBs) positive for CD63, a marker of the exosomal pathway. Consistently, depolarisation by KCl induced the liberation of previously endocytosed full-length p75 into the extracellular medium in exosomes. Thus, p75 defines a subpopulation of MVBs that does not mature to lysosomes and is available for exosomal release by neuronal cells.

KEY WORDS: MVBs, Traffic, Endocytosis, Neurotrophins, p75

INTRODUCTION

The neurotrophin receptor p75 (also known as NGFR) is a multifaceted signalling receptor that regulates different aspects of neuronal physiology, including neurite outgrowth, and survival and death decisions. p75 belongs to the tumour necrosis factor receptor family and binds to all of the neurotrophins (nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4, or NGF, BDNF, NT3 and NT4, respectively) and proneurotrophins (non-processed forms of neurotrophins) (Bronfman, 2007; Ibáñez and Simi, 2012; Roux and Barker, 2002). During the development of the sympathetic nervous system, p75 potentiates cell survival, together with the NGF-specific tyrosine kinase receptor TrkA or induces neuronal cell death and axonal pruning in the presence of BDNF, allowing the formation of the proper architecture and connectivity of the sympathetic nervous system (Glebova and Ginty, 2005).

It is now well accepted that the endocytosis of a receptor determines its signalling outcome, because the choice of post-endocytic trafficking route can regulate the duration of receptor signalling through the degradation of a receptor in the lysosome (e.g. EGFR), prolong signalling (e.g. TrkA) and/or allow signalling within the cell (e.g. the TGFβ receptor and Sara endosomes) (Di Guglielmo et al., 2003; Liu et al., 2007; Saxena et al., 2005a; Sorkin and Von Zastrow, 2002; Sorkin and von Zastrow, 2009). The endosomal pathway defines distinct endocytic organelles where different functions are regulated and/or defined. For example, when receptors are first internalised, they are localised to small vesicles at the periphery of the cell; as these endosomes move towards the nucleus, they mature and their associated protein contents, including components of the signalling machinery, change (Huotari and Helenius, 2011; Kenchappa et al., 2006; Platta and Stenmark, 2011). As a result, several signalling kinases, such as PKD1, ERK1 or ERK2 and mTORC1, are associated with specific intracellular endocytic membranes (Flinn et al., 2010; Mashukova et al., 2012; Wan et al., 2008).

A key cellular aspect regulating neurotrophin signalling is the intracellular trafficking of the neurotrophin receptors. p75 is not an exception; for example, in PC12 cells, p75 interacts with signalling adaptors in endosomes, which are also a platform for the γ-secretase-mediated cleavage of p75 C-terminal fragments, suggesting that p75 signals within the cell (Bronfman et al., 2007; Bronfman et al., 2003; Urra et al., 2007). Although the internalisation and post-endocytic trafficking of p75 are relevant to understanding the mechanisms of p75 signalling, relatively few studies examining these cellular processes have been conducted.

The aim of this work was to characterise the internalisation and post-endocytic trafficking of p75 in relevant neuronal models, including sympathetic neurons and PC12 cells (Greene and Tischler, 1976). We found that p75 evades the lysosomal route and accumulates in two different organelles, Rab11-positive endosomes and multivesicular bodies (MVBs) that are positive for CD63, a marker of secreted microvesicles that are known as exosomes. After ligand-mediated endocytosis, full-length p75 is liberated in exosomes upon depolarisation of the cell. Our findings reveal unforeseen trafficking capabilities of the p75 neurotrophin receptor and raise new questions regarding the role of exosomal p75 and MVBs in neurotrophin signalling.

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RESULTS

p75 is internalised through clathrin-coated pits by recruitment of the AP2 adaptor in PC12 cells and sympathetic neurons

We characterised the kinetics of the BDNF ligand-dependent internalisation of p75 in sympathetic neurons, as described previously in PC12 cells. Our results indicated that sympathetic neurons internalised p75 with kinetics that were similar to those of PC12 cells (Bronfman et al., 2003), but with a 30-minute delay (Fig. 1A,B).

To determine whether p75 internalisation requires dynamin (a GTPase that is essential for the pinching-off of nascent clathrin-coated vesicles and lipid-raft-derived vesicles), we used the dynamin inhibitor dynasore (Kirchhausen et al., 2008; Macia et al., 2006). We found that dynasore almost completely blocked the internalisation of p75 and transferrin (Trf, used as control) by PC12 cells and sympathetic neurons (Fig. 1C–H). The internalisation of transferrin depends on dynamin and clathrin (González-Gaitán and Stenmark, 2003; Macia et al., 2006). To confirm whether p75 used clathrin-coated vesicles for internalisation in sympathetic neurons, we transfected sympathetic

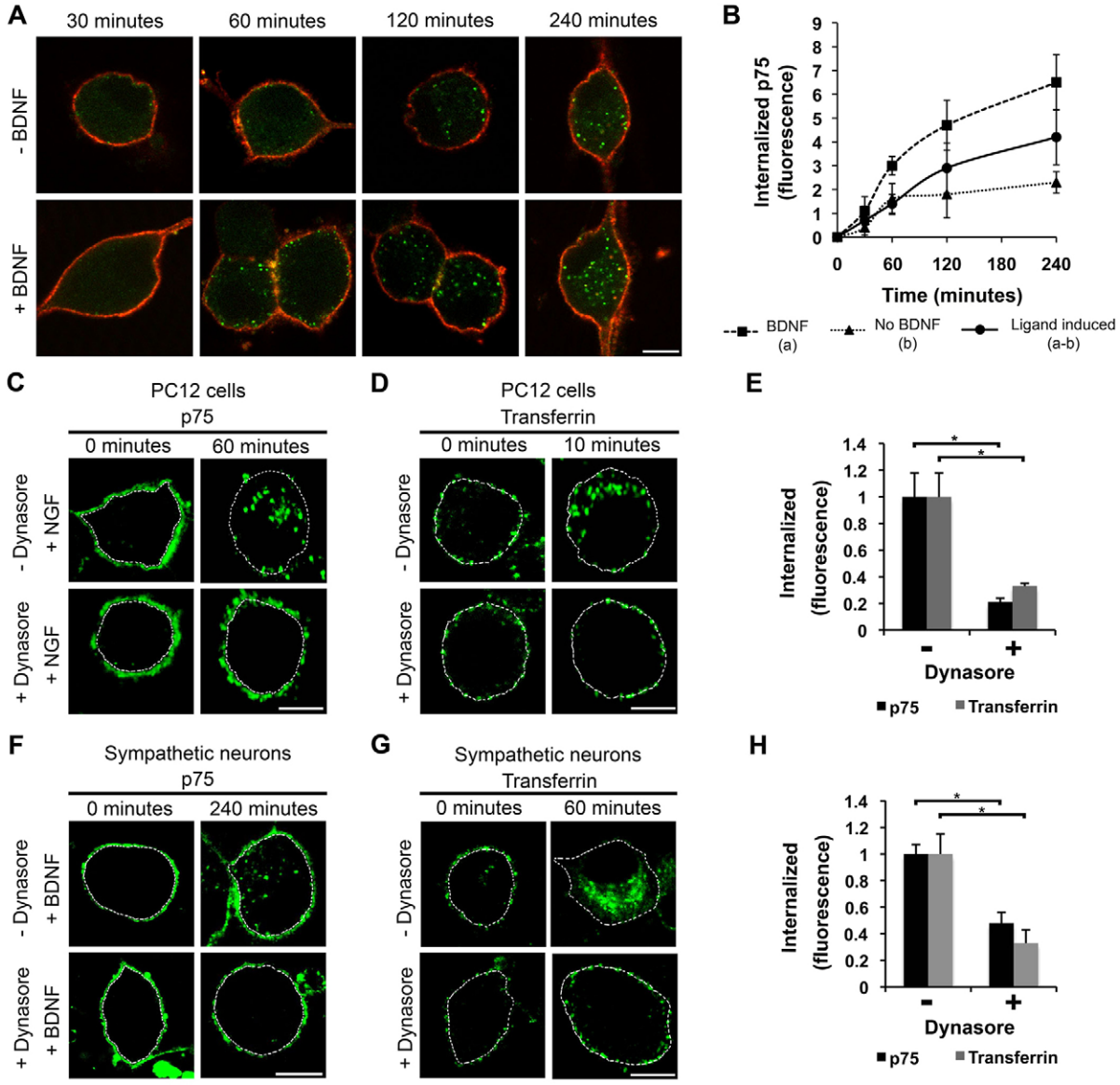

Fig. 1. p75 internalisation kinetics and inhibition of p75 internalisation by dynasore. (A) Confocal microscopy of p75 immunoendocytosis in the presence or absence of BDNF (150 ng/ml) in sympathetic neurons. p75 on the cell surface is shown in red and internalised p75 is shown in green. Scale bar: 10 μm. (B) Internalisation kinetics of p75 in sympathetic neurons. Basal internalisation (no BDNF, b), total internalisation (BDNF, a) and BDNF-dependent internalisation (calculated as a−b) are shown. The internalised fluorescence corresponds to the intracellular fluorescence normalised to the cell-surface-associated fluorescence. Data show the mean±s.e.m.; $n=3$ (80 cells). (C–H) p75 immunoendocytosis and transferrin internalisation by PC12 cells and sympathetic neurons with or without the addition of dynasore (80 μM). The confocal images show the initial (0 minutes) and final times of internalisation after addition of the ligand (neurotrophin or transferrin). For the immunoendocytosis of p75, PC12 cells (C) were treated with NGF (100 ng/ml, 60 minutes) and sympathetic neurons (F) were treated with BDNF (150 ng/ml, 240 minutes). The endocytosis of Trf–Alexa-Fluor-568 was induced for 10 minutes in PC12 cells (D) and for 60 minutes in sympathetic neurons (G). Scale bars: 10 μm. (E,H) Internalisation was quantified as indicated in B, but the numbers were normalised to the control (without dynasore treatment). White lines indicate the cell surface. Data show the mean±s.e.m.; $n=3$ (90 cells); $^*P<0.05$.

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neurons with an siRNA directed against the μ subunit of the AP2 clathrin adaptor protein (μ2). This treatment reduced the expression of μ2 by ~50% (Fig. 2C). Consistently, the internalisation of Trf and p75 was reduced by ~50% in μ2-siRNA-treated sympathetic neurons (Fig. 2A,B). In addition, co-immunoprecipitation assays revealed that upon the addition of NGF, p75 interacted with the β subunit of AP2 (β-AP2) in PC12 cells (Fig. 2D).

μ2 normally interacts with the specific sequence YXXθ (where θ is any hydrophobic amino acid) in the cytoplasmic tail of receptors (Pearse et al., 2000), and p75 possesses a LYSSL sequence (supplementary material Fig. S1). To gain further insight into the mechanism of p75 internalisation mediated by interaction with AP2, we mutated the putative μ2-binding sequence in the p75 intracellular domain by changing the wild-type (WT) p75 sequence LYSSL to AASSA, thus generating a p75 tyrosine mutant (p75-YXXθ). We found that the ligand-induced internalisation of p75-YXXθ was reduced in PC12 cells and sympathetic neurons compared with that of the exogenous WT receptor (Fig. 2E–H).

p75 enters the early endosome but is rapidly sorted to other organelles

Previously, we used subcellular fractionation to show that endocytosed p75 interacts with signalling adaptors in

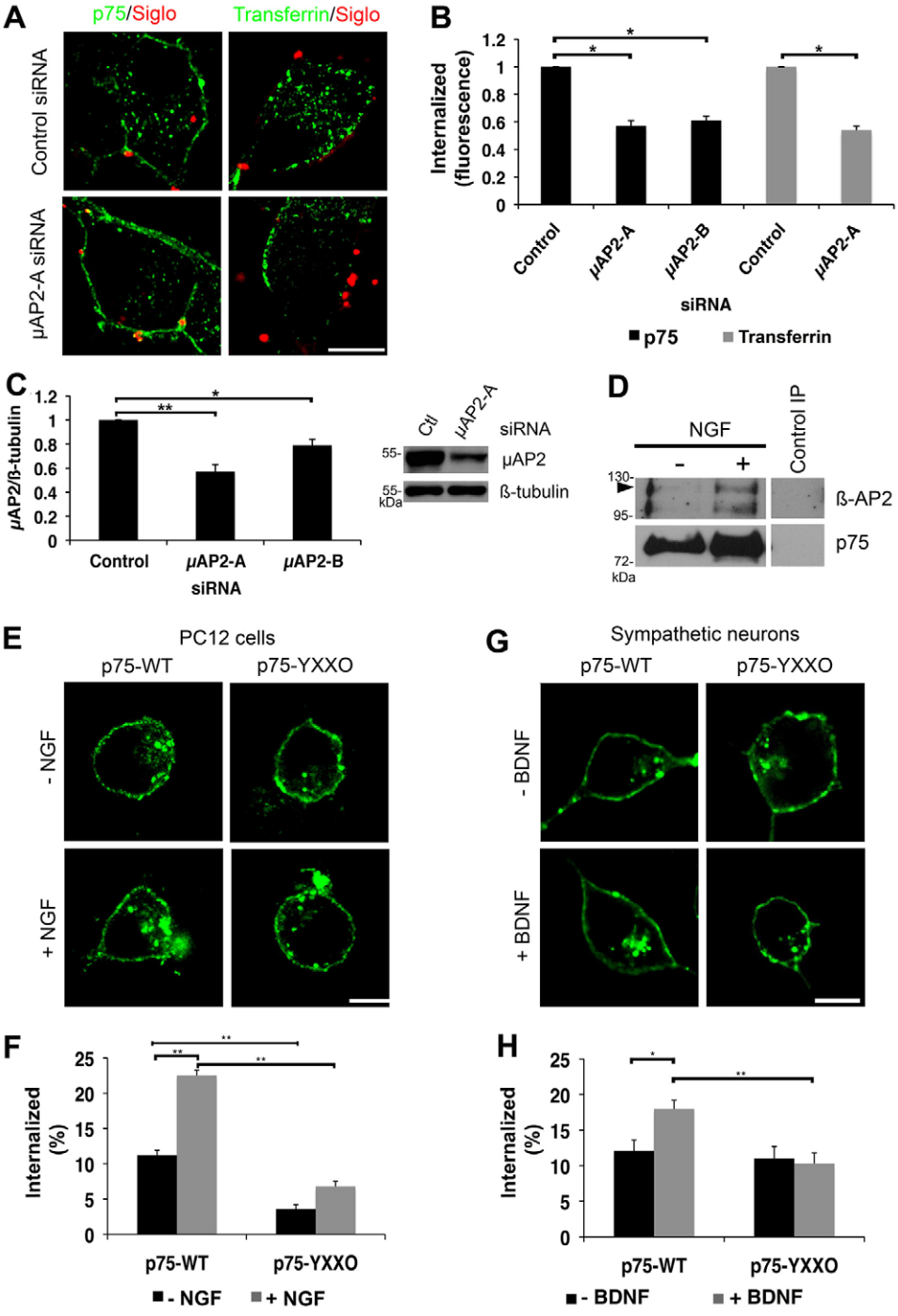

Fig. 2. AP2-dependent internalisation of p75. (A) Confocal microscopy of ligand-dependent p75 immunoendocytosis (240 minutes, 150 ng/ml BDNF) and Trf internalisation (60 minutes) in sympathetic neurons transfected with an siRNA sequence A directed against μ2 (μAP2A). p75 and Trf are shown in green, and siGLO Green (siGLO)-labelled transfected cells are shown in red. Scale bar: 10 μm. (B) Quantification of p75 and Trf internalisation (as in A) using siRNA sequence B against μAP2 (μAP2B). The internalisation was calculated as indicated in Fig. 1B and normalised to the control siRNA. Data show the mean±s.e.m.; $n=3$ (120 cells); $^{*}P<0.0001$. (C) Western blot (right) and quantification of μ2 protein levels (left) determined by western blotting of the lysates of sympathetic neurons transfected with an siRNA against μ2. Data show the mean±s.e.m.; $n=3$; $^{*}P<0.05$, $^{**}P<0.0004$. (D) Co-immunoprecipitation of p75 and β2. PC12 cells were treated with NGF (100 ng/ml) for 5 minutes, and p75 was immunoprecipitated from cell lysates by using anti-p75ECD. The arrowhead indicates the band for β2. The western blot is representative of two independent experiments. (E,G) Confocal microscopy of the immunoendocytosis of WT p75–HA (p75-WT) and tyrosine mutant p75–HA (p75-YXXθ) in PC12 cells (E) and sympathetic neurons (G). p75 immunoendocytosis (green) was followed by incubation with an anti-HA monoclonal antibody for 60 minutes in the presence of NGF (100 ng/ml) in PC12 cells (E) or BDNF (150 ng/ml) in sympathetic neurons (G). Scale bars: 10 μm. (F,H) Quantification of p75-WT and p75-YXXθ internalisation by PC12 cells (F) and sympathetic neurons (H), as shown in E and G. For PC12 cells (F), data show the mean±s.e.m.; $n=3$ (240 cells); $^{**}P<0.001$. For sympathetic neurons (H), $n=2$ (30 cells); $^{*}P<0.01$, $^{**}P<0.001$.

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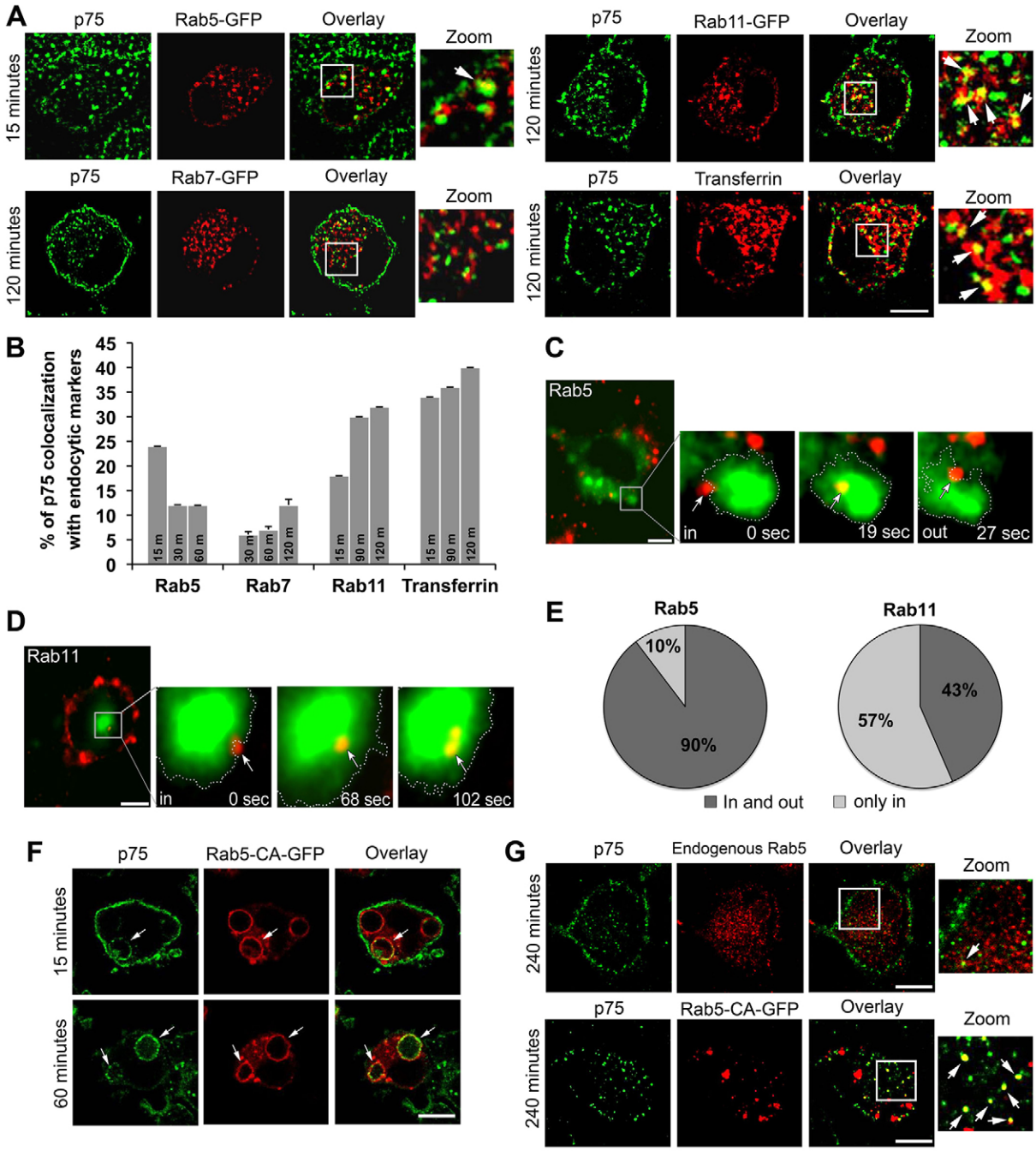

Fig. 3. See next page for legend.

endosomes (Bronfman et al., 2003). However, the nature of these endosomes has not been characterised. To address this point, we performed immunoendocytosis of endogenous p75 with different endocytic markers in PC12 cells and sympathetic neurons; as previously reported, we found that p75 was poorly colocalised with early endosomes (Rab5- and Trf-positive endosomes at short incubation times) (Fig. 3A; supplementary material Fig. S2C) and with late endosomes (Lamp2-positive) (Fig. 3A; supplementary material Fig. S2D) (Bronfman et al., 2003). When p75 was co-internalised with Trf at longer incubation times (to label the recycling endosome), the colocalisation of the two labels was evident (Fig. 3A; supplementary material Fig. S2E). These results, together with the results presented in Figs 1 and 2, indicate that p75 uses a similar mechanism of internalisation and post-endocytic trafficking in PC12 cells and sympathetic neurons. We then decided to quantitatively characterise the post-endocytic trafficking of p75 in PC12 cells. PC12 cells were transfected with different EGFP-tagged Rab GTPases, which regulate the post-endocytic trafficking of receptors such as Rab5 (an early endosome marker), Rab11 (a recycling endosome marker) and Rab7 (a late endosome marker). The colocalisation of p75 with these markers at different timepoints after ligand addition was evaluated. The maximum amount of p75 that was colocalised with Rab5 was ~24% at early timepoints (15 minutes), falling to 12% at later timepoints (30 and 60 minutes) (Fig. 3B; supplementary material Table S1). Here, it is important to clarify

Fig. 3. Colocalisation of endocytosed p75 with endocytic organelles. (A) Deconvoluted confocal images of internalised p75 (green) in PC12 cells transfected with Rab5–GFP (red), Rab7–GFP (red) or Rab11–GFP (red). Immunoendocytosis for different times at 37˚C (indicated to the left of each panel) was performed for endogenous p75 using an anti-p75ECD antibody in the presence of NGF (100 ng/ml), and the internalisation of Trf–Alexa-Fluor-568 is also shown (red). 'Zoom' represents magnification of the white square in the overlay images. Arrows, colocalization between p75 and endosomal markers. Scale bar: 10 μm. (B) Quantification of the colocalisation of internalised p75 with different endosomal markers shown in A. The number inside the columns indicates the time (m, minutes) for which p75 was allowed to internalise after adding NGF. The values shown in the graph are from supplementary material Table S1 and are corrected for the maximum colocalisation obtained by using the technique (colocalisation of anti-p75ECD–Alexa-Fluor-594 with anti-mouse-IgG–Alexa-Fluor-488). Data show the mean±s.e.m.; $n=3$ (20–30 cells). (C) Time-lapse microscopy of internalised p75 (red) entering (in) and leaving (out) the Rab5 endosome (green, outlined). p75 from the cell surface was labelled with an anti-p75ECD–Qdots antibody in PC12 cells transfected with Rab5–GFP, and time-lapse imaging was performed in the presence of NGF (100 ng/ml). Arrows, a p75-labelled vesicle. Scale bar: 2.5 μm. (D) Time-lapse microscopy shows that internalised p75 (red) enters (in) but does not leave the Rab11 compartment (green, outlined). PC12 cells were treated as indicated in C, but the cells were transfected with the Rab11–GFP plasmid. Arrows, a p75-labelled vesicle. See supplementary material Movies 1, 2. Scale bar: 4 μm. (E) Quantification of the events observed in C and D where a p75-positive vesicle enters and leaves the organelle (in and out) or just enters (only in) within the time frame of the time-lapse experiment (5 minutes). Quantification was conducted over 29 events from 4 cells for Rab5 and 23 events from 15 cells for Rab11. (F) Deconvoluted confocal images of PC12 cells transfected with a constitutively active Rab5 mutant (Rab5-CA–GFP, red) after p75 immunoendocytosis (green) in the presence of NGF (100 ng/ml). Arrows, p75 accumulating in giant early endosomes. The images are representative of two independent experiments. $n=3$. Scale bar: 10 μm. (G) Confocal images of p75 immunoendocytosis (green) by sympathetic neurons in the presence of BDNF (150 ng/ml), followed by immunofluorescence to detect endogenous Rab5 (upper panel, red) or Rab5-CA–GFP (lower panel, red). 'Zoom' represents magnification of the white square in the overlay images. Arrows, colocalization of the two labels. The images are representative of three independent experiments. Scale bar: 10 μm.

that 15 minutes after ligand addition (a time when other receptors have already been internalised) is considered an early time point to study p75 internalisation because most p75 is still at the cell surface (Bronfman et al., 2003; Cuitino et al., 2005; Li et al., 2001; Urra et al., 2007).

We hypothesised that after internalisation, p75 rapidly moves from the early endosome to a different endocytic organelle. To test this hypothesis, we transfected PC12 cells with constructs driving the expression of Rab5–GFP or Rab11–GFP and recorded the dynamics of the internalised p75 by live-cell imaging in a 5-minute window after ligand addition. We found that most of the p75-positive vesicles that enter Rab5-positive organelles left them within the time frame of the recording. However, more than half of the p75 vesicles that entered the Rab11 compartment did not leave the organelle within the same timeframe (Fig. 3C–E; supplementary material Movies 1, 2). We also transfected PC12 cells with a constitutively active Rab5 mutant (Rab5CA). This mutant has been shown to cause the formation of giant endosomes and the accumulation of endocytosed receptors, because the receptors enter but cannot leave the early compartment (Stenmark et al., 1994; Volpicelli et al., 2001). After 15 minutes of p75 immunoendocytosis by PC12 cells expressing Rab5CA, the internalised p75 was localised mainly to Rab5-positive giant endosomes. The same result was found at later timepoints, with p75 accumulating in these Rab5-positive giant structures (Fig. 3F). Similar results were found in sympathetic neurons (Fig. 3G). These results suggest that p75 effectively enters the Rab5-positive early endosome and has a short time of residence in this organelle.

p75 accumulates in Rab11 recycling endosomes and CD63-positive MVBs

Consistent with previous results indicating that most of the endocytosed p75 does not follow the late endocytic route after neurotrophin treatment (Bronfman et al., 2003; Saxena et al., 2005b; Urra et al., 2007), we found that endocytic p75 colocalised with Rab11, attaining a maximum of 32% colocalisation at 120 minutes after the addition of ligand (Fig. 3A,B; supplementary material Table S1). Consistent with the fact that p75 partially colocalised with the Rab11-positive endosomes, live-cell imaging of endocytosed p75 suggested that some of the p75-labelled vesicles fused with the plasma membrane (Fig. 4A,B; supplementary material Movie 3). To quantitatively assess the amount of endocytic p75 that was recycled back to the plasma membrane, we used a quantitative fluorescence technique. We observed that, in a period of 90 minutes, 25% of the p75 had recycled back to the plasma membrane. We compared this level of recycling with that of the receptor ApoER2. After 60 minutes, 75% of the ApoER2 had recycled back to the plasma membrane (Fig. 4C), indicating that the low capacity of p75 to recycle was not due to the assay used. Thus, these results indicated that only a proportion (25%) of the endocytic p75 is recycled back to the plasma membrane, consistent with the results indicating that 32% of p75 is colocalised with Rab11, the Rab GTPase that regulates the recycling of receptors to the plasma membrane (Hutagalung and Novick, 2011; Zerial and McBride, 2001).

During the live-cell imaging experiments, we also observed a group of small vesicles moving together, suggesting that a portion of the endocytic p75 was localised to structures that resembled multivesicular bodies (MVBs; Fig. 5A,B). To confirm this hypothesis, we performed quantitative electron microscopy of p75 in PC12 cells. After 30 minutes of internalisation, we observed that most of the receptor was still at the plasma membrane (74%), ~20% was located in simple vesicles near the plasma membrane and a small proportion was located in MVBs (~6%) (Fig. 5C,D). However, after 120 minutes of internalisation, the proportions of p75 in the different subcellular locations of PC12 cells had changed; after 120 minutes, ~43% was at the plasma membrane, ~14% was located in simple vesicles and 43% had moved to MVBs (Fig. 5C,D). MVBs containing endocytosed p75 were also evident in the sympathetic neurons treated with BDNF (Fig. 5E). By using live-cell imaging, round structures labelled with endocytosed p75 were observed leaving p75-positive MVBs, suggesting that these organelles represent dynamic structures (Fig. 5B).

Our quantitative studies of the colocalisation of endocytosed p75 with different endocytic organelles indicated that p75 did not significantly colocalise with late endocytic markers (Fig. 3A,B; supplementary material Table S1). These results suggest that the MVBs in which p75 accumulates are different from the classic Rab7-positive MVBs that mature into lysosomes. Other types of MVBs include those related to exosomal release (Denzer et al., 2000; Hanson and Cashikar, 2012; Ludwig and Giebel; Mathivanan et al., 2010; Raposo et al., 1996). A well-known marker of MVBs in the exosomal pathway is the tetraspanin CD63 (Ludwig and Giebel, 2012). Therefore, we hypothesised that a portion of p75 was accumulating in CD63-positive

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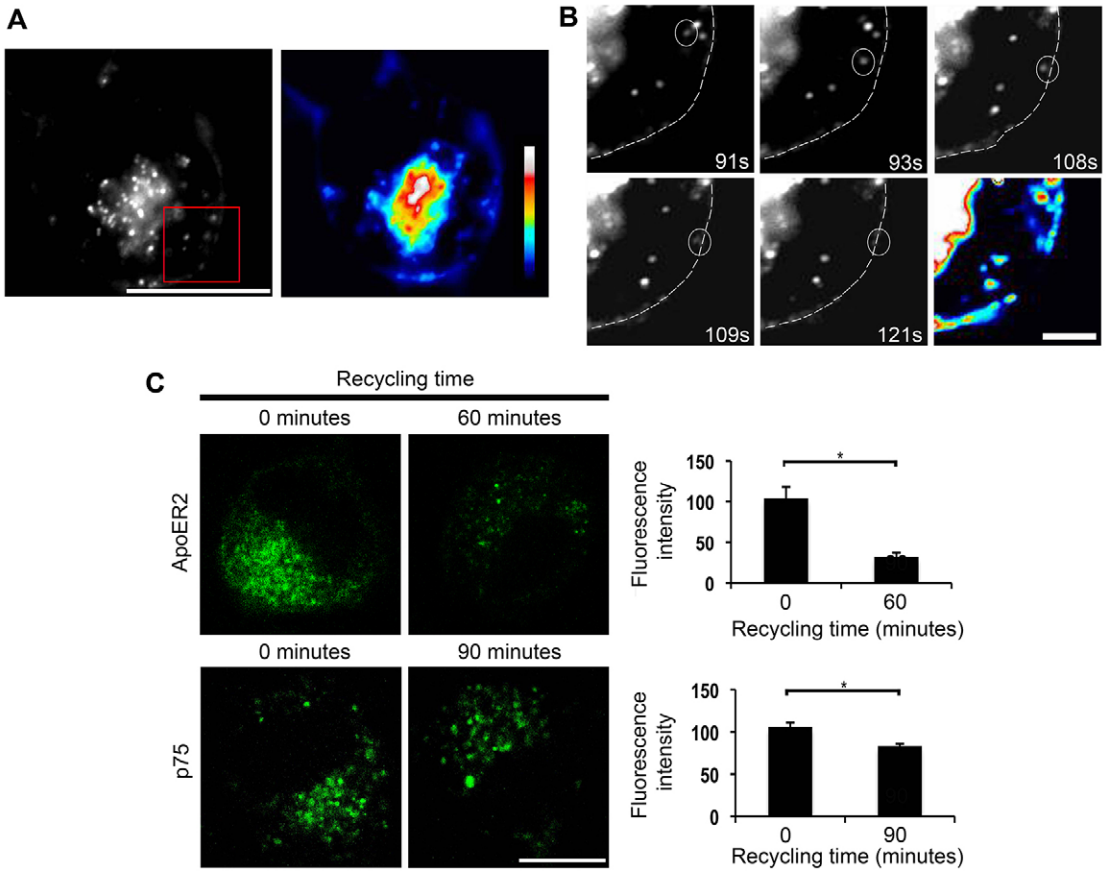

Fig. 4. Low rate of p75 recycling to the plasma membrane. (A) Real-time microscopy of internalised p75 in PC12 cells. PC12 cells were incubated with anti-p75ECD–Qdots in the presence of NGF (100 ng/ml), and live-cell imaging was performed (supplementary material Movie 3). Left, the first frame from supplementary material Movie 3 is shown. p75 is shown in white. Right, 500 frames (the total recording time was 4.15 minutes) from supplementary material Movie 3 were condensed into one image and transformed into a pseudocolour image; white represents the immobile particles and blue represents the most mobile particles. Scale bar: 10 μm. (B) High-magnification images of the area in the red square in A at different time points from supplementary material Movie 3. The white circle shows a particle moving towards the plasma membrane (white line). Scale bar: 2 μm. (C) Left, confocal microscopy showing ApoER2–HA and p75 in PC12 cells before and after a recycling assay. PC12 cells were transfected with ApoER2–HA, and an immunoendocytosis assay was performed using Alexa-Fluor-488-conjugated antibody against the HA tag to visualise ApoER2 and the anti-p75ECD–Alexa-Fluor-488 to visualise p75. Scale bar: 10 μm. Right, the quantification of the fluorescence remaining after the recycling assay. $n=3$ (150 cells). Data show the mean±s.e.m. *$P<0.008$.

organelles; we quantified the colocalisation of endogenous endocytosed p75 with EGFP-tagged CD63 at different timepoints after internalisation in PC12 cells. Our results indicated that p75 partially colocalised (34%) with CD63 in PC12 cells and sympathetic neurons (Fig. 6A–C; supplementary material Table S1). Moreover, we could visualise a direct interaction of endocytosed p75 with CD63-positive organelles by live-cell imaging. This is similar to the findings obtained for Rab11-positive organelles (Fig. 3C–E). We found that more than half of the p75-positive vesicles that enter CD63-positive organelles did not leave the organelle within the timeframe of the recording (5 minutes) (Fig. 6D,E).

Finally, to analyse whether the $p75^{+}Rab11^{+}$ endosomes were different from the $p75^{+}CD63^{+}$ endosomes, we performed triple-labelling experiments using endogenous p75 labelled with Alexa-Fluor-647-conjugated antibody against the p75 extracellular domain (anti-p75ECD–Alexa-Fluor-647), Rab11–mCherry and CD63–EGFP. By using two different types of masks, we observed that these endosomes mainly corresponded to two different populations of organelles (Fig. 6F,G; supplementary material Fig. S3).

The endocytosed p75 is released from the cell in exosomes upon KCl depolarisation

The results indicating that p75 accumulates in CD63-positive endosomes suggested that p75 is released into the culture medium by exosomes. To assess this possibility, we first characterised the diameter of the vesicles obtained after stimulating PC12 cells and sympathetic neurons with KCl to induce the release of exosomes. The mechanism that regulates the release of exosomes is not well understood, but depolarisation with KCl is a well-defined stimulus increasing the release of exosomes from neuronal cells (Fauré et al., 2006). After KCl treatment, the medium was collected and the exosome samples were observed by electron microscopy. The mean diameter of the vesicles obtained from PC12 cells was 68.1±1.1 nm, and it was 104±3.8 nm in the case of sympathetic neurons (±s.e.m.; supplementary material Fig. S4A,B). Both of these values are within the range established for exosomes (Kleijmeer et al., 1996; Simons and Raposo, 2009). To study the presence of p75 in exosomes, we incubated PC12 cells with BDNF and triggered exosomal release by adding KCl. We collected exosomal pellets from the media of

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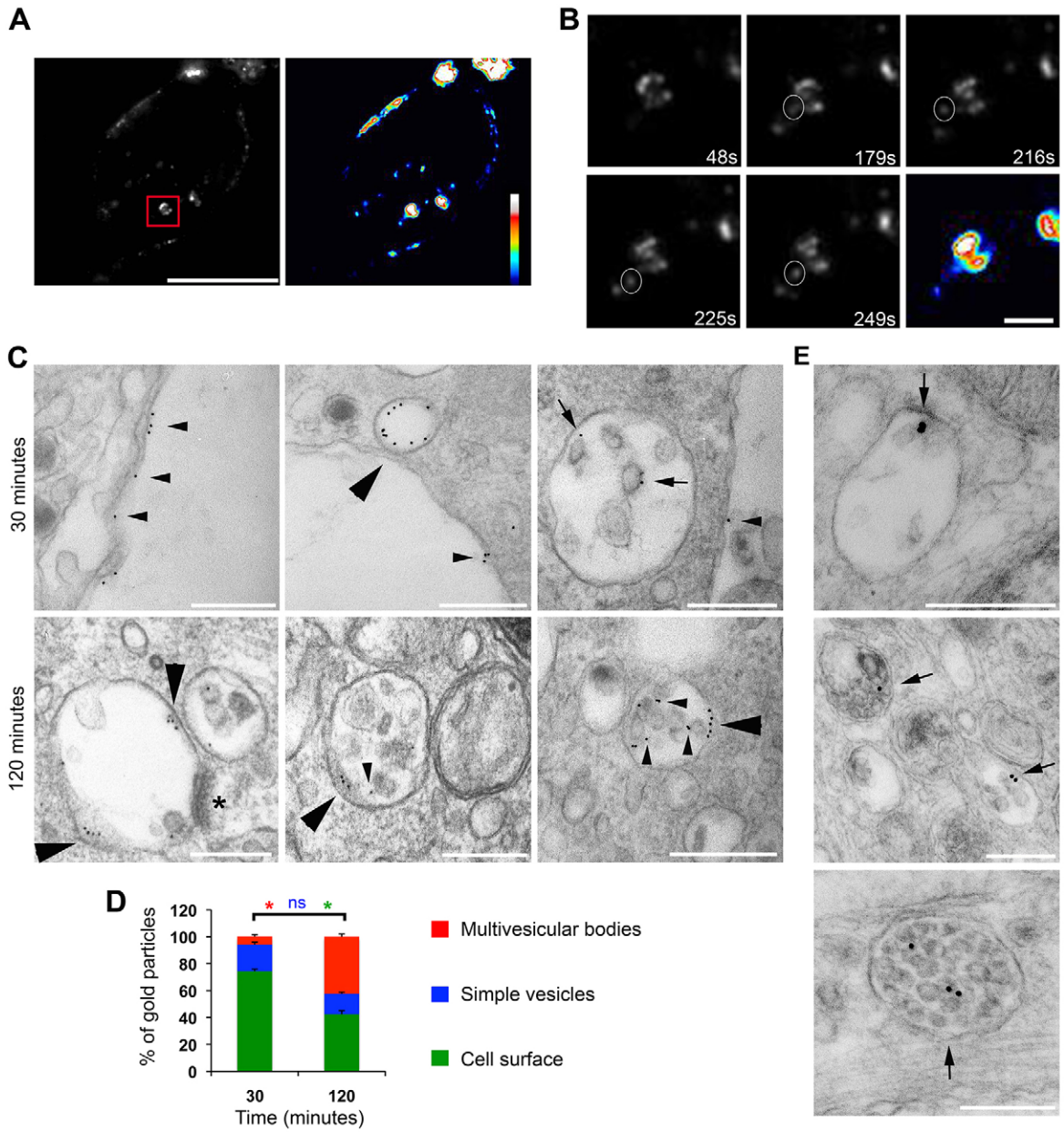

Fig. 5. p75 accumulates in MVBs. (A) Real-time microscopy of internalised p75 in PC12 cells. PC12 cells were incubated with anti-p75ECD–Qdots in the presence of NGF (100 ng/ml), and live-cell imaging was performed. Left, the first frame of a time-lapse movie is shown. Right, 500 frames (total recording time is 4.15 minutes) from a time-lapse movie were condensed into one image and transformed into a pseudocolour image; white represents the immobile particles, and blue represents the most mobile particles. Scale bar: 10 μm. (B) High-magnification images of the area in the red square in A at different time points during the time-lapse movie. The white circle shows a vesicle leave a group of organelles that moved together, reminiscent of MVBs. Scale bar: 1 μm. (C) Electron microscopy of p75 immunoendocytosis by PC12 cells, as visualised by using anti-p75ECD–gold in the presence of NGF (100 ng/ml). Upper panels, after 30 minutes of internalisation, p75 is detected on the cell surface (small arrowheads), in simple vesicles (large arrowhead) and in MVBs (arrows). Lower panels, after 120 minutes of internalisation, p75 is mainly detected in MVBs; it is localised to the outer membrane of MVBs (large arrowheads) and on the membranes of internal vesicles (small arrowheads). The asterisk (*) indicates a clathrin lattice associated with an MVB. Scale bar: 300 nm. (D) Quantification of p75 distribution in different subcellular compartments from electron micrographs as shown in C. Data show the mean±s.e.m.; $n=3$ (30 cells); $^{*}P<0.001$; ns, not significant. (E) Electron microscopy of p75 immunoendocytosis by sympathetic neurons as visualised by staining with the anti-p75ECD–gold antibody in the presence of BDNF (150 ng/ml) for 240 minutes. Arrows, MVBs. The images are representative of two independent experiments. Scale bar: 300 nm.

PC12 cells and sympathetic neurons, and verified the enrichment of CD63 compared with other membrane markers, such as markers for the endoplasmic reticulum (β-COP), early endosomes (Rab5) and the plasma membrane (Na^{+}/K^{+} ATPase) (Fig. 7A,B). We demonstrated that full-length p75 was enriched in the exosomal fraction obtained after the internalisation of p75 and the stimulation of the release of exosomes in both cell types. To confirm this result, we induced the internalisation of p75 by PC12 cells and sympathetic neurons with BDNF in the presence of a monoclonal antibody recognising the extracellular domain of p75 and then induced the release of exosomes with KCl. In these samples, we observed p75 labelled with gold in exosomes,

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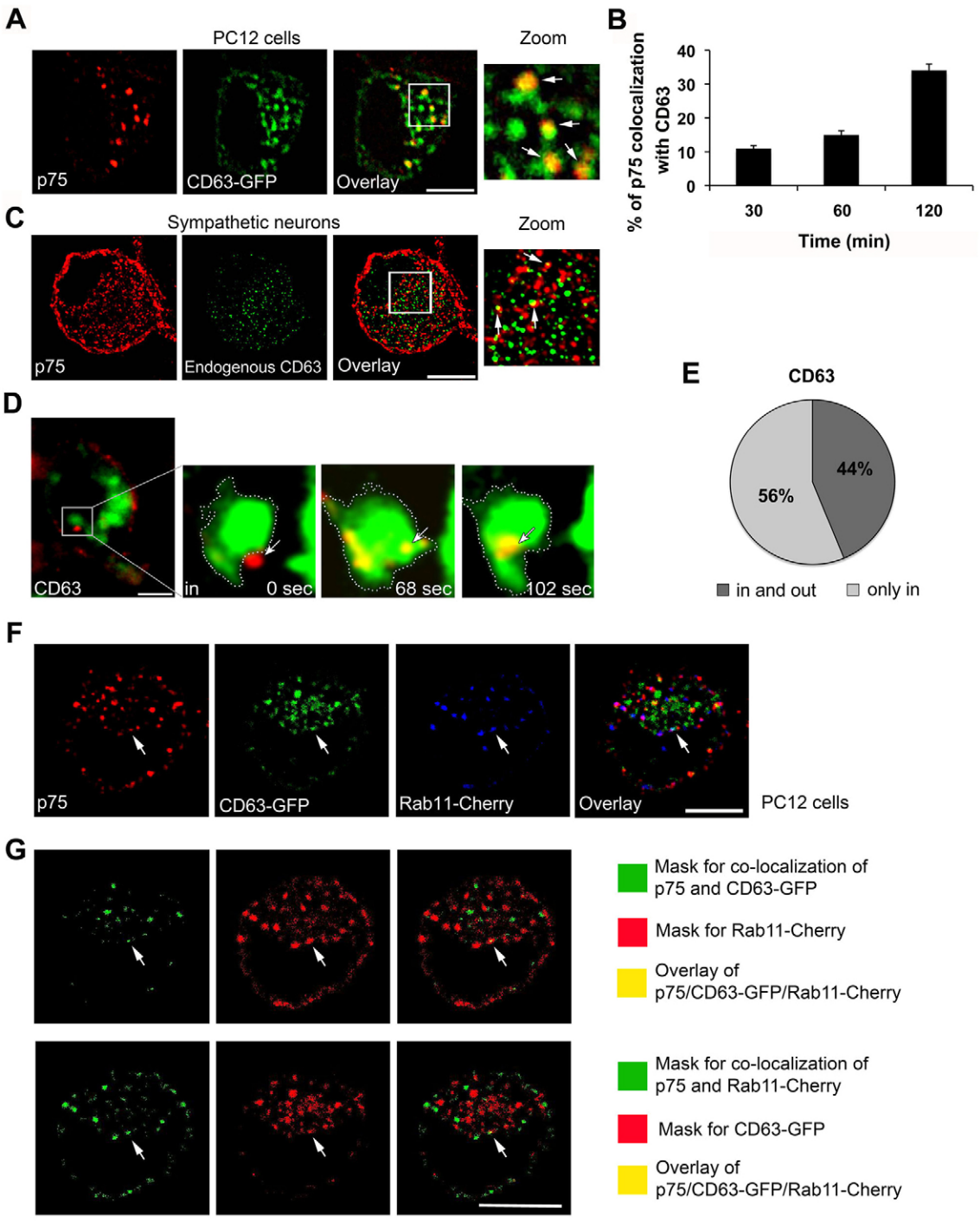

Fig. 6. See next page for legend.

demonstrating the presence of endocytic p75 in the exosomes of PC12 cells and sympathetic neurons (Fig. 7C).

To examine whether there is a correlation between the amount of internalised p75 and the amount of p75 found in exosomes after KCl treatment, we induced p75 internalisation in sympathetic neurons stimulated with BDNF and we compared them with non-stimulated cells. We found increased levels of p75-positive exosomes in the neurons treated with BDNF compared with control neurons (Fig. 7D,E). Similar results were obtained from a different assay in which a fluorescently labelled monoclonal antibody that recognises the extracellular domain of p75 was used to track endogenous p75 (supplementary material Fig. S4C).

Taken together, our results indicate that, after clathrin-mediated internalisation, p75 reaches Rab5-positive early endosomes, from which it is rapidly sorted (directly or indirectly) into two different types of organelle, Rab11-positive organelles and CD63-positive MVB organelles. Thus, p75 follows a non-canonical route of ligand-induced endocytic trafficking – a subpopulation is sorted into MVBs that do not mature into lysosomes; instead, p75 is available for exosomal release by neuronal cells.

Fig. 6. Internalised p75 colocalises with organelles that are positive for CD63 and distinct from Rab11-positive endosomes. (A) Deconvoluted confocal images of internalised p75 (red) in PC12 cells transfected with CD63–GFP (an MBV marker, green). Immunoendocytosis of p75 is visualised by using anti-p75ECD in the presence of NGF (100 ng/ml) for 120 minutes. 'Zoom' represents magnification of the white square in the overlay image. Arrows, colocalization between the two labels. Scale bar: 10 μm. (B) Quantification of internalised p75 colocalisation with CD63 as shown in A. The values shown in the graph were obtained from supplementary material Table S1 and are corrected for the maximum colocalization obtained by using the technique (colocalisation of anti-p75ECD–Alexa-Fluor-594 with anti-mouse-IgG–Alexa-Fluor-488). Data show the mean±s.e.m.; $n=3$ (20–30 cells). (C) Confocal microscopy of p75 immunoendocytosis (red) as visualised by using anti-p75ECD in the presence of BDNF (150 ng/ml) for 240 minutes in sympathetic neurons, followed by immunofluorescence to detect endogenous CD63 (green). 'Zoom' represents magnification of the white square in the overlay image. Arrows, colocalization between the two labels. Scale bar: 10 μm. (D) Time-lapse microscopy of internalised p75 (red) entering (in) the CD63 endosome (green, outlined). p75 from the cell surface was labelled with an anti-p75ECD–Qdots antibody in PC12 cells transfected with CD63–GFP, and time-lapse imaging was performed in the presence of NGF (100 ng/ml). Arrows, a p75-labelled vesicle entering the CD63-positive compartment. (E) Quantification of the events observed in D in which a p75-positive vesicle enters and leaves the organelle (in and out) or just enters (only in) within the time frame of the time-lapse experiment. Quantification was conducted over 16 events from 8 cells. (F) Deconvoluted images of internalised p75 in PC12 cells transfected with Rab11–mCherry and CD63–GFP. Immunoendocytosis assay for p75 was performed with anti-p75ECD–Alexa-Fluor-647 in the presence of NGF (100 ng/ml) for 120 minutes. Arrows, the colocalisation of p75, CD63–GFP and Rab11–mCherry. Scale bar: 10 μm. (G) The same cell shown in F with different masks applied. Upper panel: in green, the mask for the colocalisation of p75 and CD63–GFP; in red, the mask for Rab11–mCherry; and in yellow, the overlay of both masks. Lower panel: in green, the mask for the colocalisation of p75 and Rab11–mCherry; in red, the mask for CD63–GFP; and in yellow, the overlay of both masks. Arrows, the colocalisation between the three labels. In all cases, the images are representative of three independent experiments. Scale bar: 10 μm.

DISCUSSION

One of the major impediments to understanding how p75 intracellular trafficking and p75 signalling mechanisms are interrelated to allow neuronal responses is the lack of knowledge about p75 intracellular trafficking in neuronal models (Casademunt et al., 1999; Gentry et al., 2004; Kenchappa et al., 2006; Park et al., 2010). In this study, we found that p75 follows a route that has not been described previously for signalling receptors. In sympathetic neurons and PC12 cells, p75 was internalised in a clathrin and dynamin-dependent manner, evading the early-late endosomal route and accumulating in two discrete endosomal populations, the Rab11-positive recycling endosomes and MVBs that are positive for CD63, a marker of MVBs that fuse with the plasma membrane for exosomal release. We showed that the full-length p75 receptor was secreted in exosomes after KCl-mediated cell depolarisation. In addition, the amount of p75-positive exosomes increased when the cells had been previously treated with a ligand that allowed p75 internalisation. All of these results indicate that the inclusion of p75 defines a specific MVB that is distinct from the MVBs that fuse with lysosomes. These results further suggest a previously unknown mechanism of downregulating the p75 receptor in neurons – by releasing p75 in exosomes or transferring p75 signalling complexes from one cell to another.

We have shown previously that neurotrophin treatment increases the interaction of endocytosed p75 with signalling adaptors in endosomes that are distinct from late endosomes (Bronfman et al., 2003; Urra et al., 2007). In the present study, we defined two different populations of endosomes in which p75 might continue to signal inside the cell – Rab11-positive and CD63-positive endosomes.

Research using different cell types has suggested that p75 is internalised by a mechanism that involves both clathrin-mediated and lipid-raft-mediated internalisation (Bronfman et al., 2003; Deinhardt et al., 2007; Hibbert et al., 2006; Saxena et al., 2004). We confirmed these results by showing that p75 internalisation is completely dependent on dynamin in PC12 cells and sympathetic neurons. In addition, we showed that the clathrin-mediated internalisation of p75 through the AP2 adaptor is the predominant mechanism of ligand-mediated p75 internalisation in sympathetic neurons and PC12 cells. Other adaptors, such as AP180, also play a role in clathrin-dependent p75 internalisation in motoneurons and, similar to our results for sympathetic neurons, only the ligand-dependent internalisation appears to be clathrin-mediated (Deinhardt et al., 2007).

It is widely accepted that, after receptor internalisation, all of the newly formed endocytic vesicles fuse in a Rab5 GTPase-dependent manner to form early endosomes (Poteryaev et al., 2010; Woodman, 2000; Zeigerer et al., 2012). Considering this, it was a surprise to find low levels of colocalisation of internalised p75 with Rab5 at the different timepoints studied. We hypothesised that p75 is rapidly sorted from early endosomes to other compartments to avoid the late endocytic route. Supporting this idea, when a live-cell imaging experiment was conducted to visualise the interaction of endocytosed p75 with Rab5-positive endosomes, the transitory nature of this interaction was evident. Moreover, a constitutively active Rab5 mutant trapped p75 in early endosomes, suggesting that p75 passes through Rab5-positive endosomes and that a reduction in Rab5 activity is a requirement for p75 to leave the early endosomes. It was recently shown that p75 interacts with Rab5 in adipocytes, suggesting that there is a functional relationship between p75 and Rab5 that allows proper p75 trafficking (Baeza-Raja et al., 2012). The idea that p75 evades the endolysosomal system is supported by the fact that p75 colocalised poorly with Rab7-positive endosomes at all of the timepoints studied and that the majority of p75 accumulates in Rab11- and CD63-positive endosomes. Supporting these results, we have shown that endocytosed p75 is not localised in late endosomes or lysosomes by using subcellular fractionation techniques (Urra et al., 2007). However, it is important to mention that p75 might have different sorting steps after internalisation in axons compared with the cell body. It has been reported that the retrograde transport of p75 in axons depends on a Rab7-positive organelle in motoneurons (Deinhardt et al., 2006).

We studied the post-endocytic trafficking of p75 and found that p75 accumulates in two discrete populations of organelles – endosomes that are positive for Rab11 and transferrin, and MVBs that are positive for CD63. These organelles are different compartments because the $Rab11^{+}p75^{+}$ organelles did not colocalise significantly with the $CD63^{+}p75^{+}$ organelles. Consistently, we found that a proportion of the endocytic p75 was localised in intraluminal vesicles (ILVs) within the MVBs, as well as in the limiting MVB membrane.

The formation of ILVs from endosomes to originate MVBs is thought to occur through the ESCRT (endosomal sorting complex required for transport) proteins, which transiently associate with the endosomal membrane by recognising ubiquitylated endosomal cargo until the MVBs mature or fuse with lysosomes

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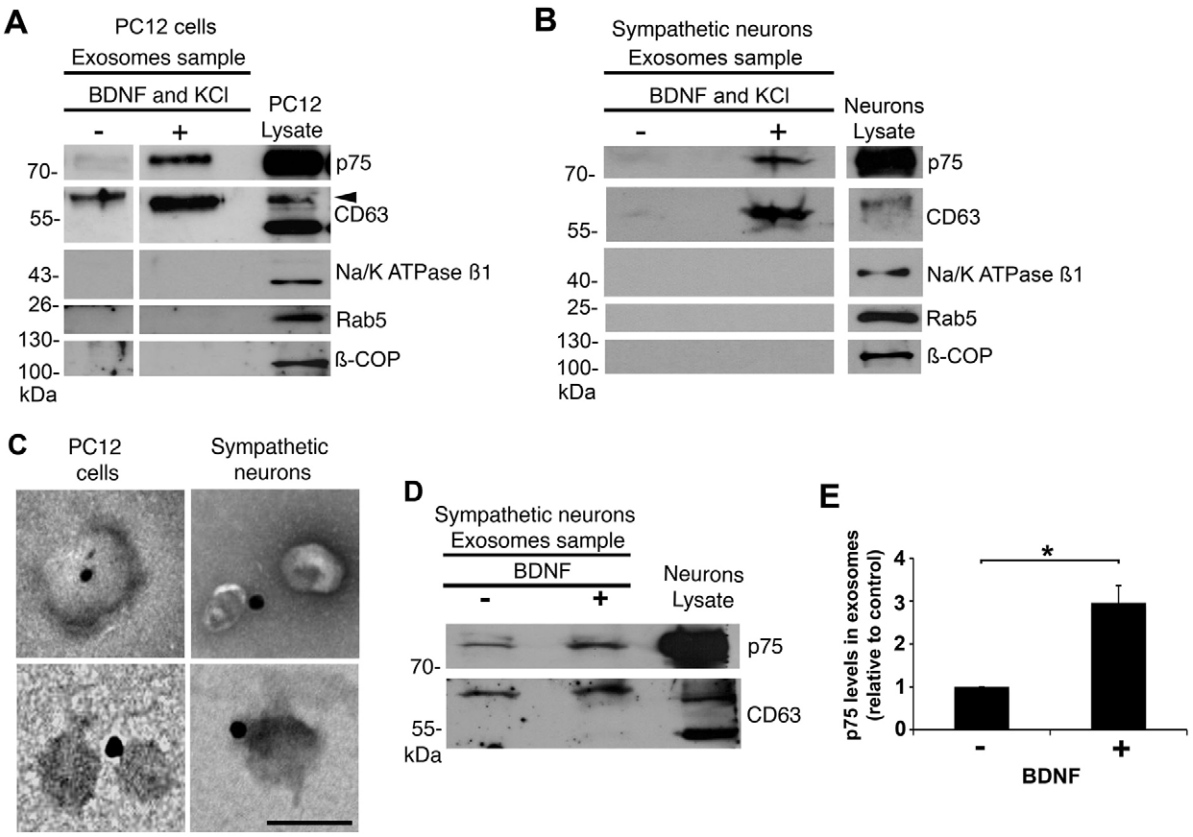

Fig. 7. Endocytosed p75 is localised in exosomes. (A,B) Western blots to show p75 enrichment in exosome samples. PC12 cells (A) and sympathetic neurons (B) were either untreated (−) or incubated with BDNF (150 ng/ml) for 2 or 4 hours, respectively (+). The cells then received either no treatment (−) or had KCl added to the medium to induce the release of exosomes for 30 minutes (PC12 cells) or 6 hours (sympathetic neurons) (+). After the treatments, the medium was ultracentrifuged to obtain an exosome-enriched sample and was analysed by western blotting. The result is representative of three experiments including all the markers in PC12 cells and four experiments including all markers (except Na/K ATPase, which was included in one experiment) in sympathetic neurons. The arrowhead labels the band corresponding to CD63. (C) Electron microscopy of intact exosomes in which endocytosed p75 labelled with gold. PC12 cells and sympathetic neurons were incubated with an anti-p75ECD antibody in the presence of BDNF (150 ng/ml) for 2 and 4 hours, respectively. The cells were then treated with KCl for 4 hours (PC12 cells) or 6 hours (sympathetic neurons) to induce exosomal release. The anti-p75ECD antibody recognising the intact exosomes was labelled with gold-conjugated protein A, and the samples were prepared for electron microscopy. The images are representative of three independent experiments. Scale bar: 100 nm. (D) Western blotting for p75 in the exosome samples. Sympathetic neurons were incubated with (+) or without (−) BDNF (150 ng/ml) for 4 hours to allow the internalisation of p75 and subsequently were treated with KCl (to induce the release of exosomes) for 6 hours in both cases (+ and −). The medium was ultracentrifuged to obtain exosome-enriched samples and was analysed by western blotting. (E) Quantification of the levels of full-length p75 as determined by western blotting from the lysates of exosomes derived from sympathetic neurons (show in D). Relative intensity is shown, normalised to samples that were not treated with BDNF. Data show the mean±s.e.m.; $n=3$; $^{*}P<0.05$.

(Hanson and Cashikar, 2012). In addition, a subpopulation of MVBs does not fuse with lysosomes but, instead, fuses with the plasma membrane, releasing the ILVs to the extracellular space as exosomes. ILVs are particularly enriched in proteins belonging to the tetraspanin superfamily, including CD86, CD37, CD63, CD81 and CD82 (Escola et al., 1998).

CD63, the first tetraspanin discovered, is thought to be a marker of the ILVs from MVBs that fuse with the plasma membrane for exosomal release, and several results suggest that CD63 participates in endocytic pathways to generate populations of MVBs with potentially distinct properties (Simons and Raposo, 2009). For example, CD63 has been shown to interact and traffic with the LMP1 protein, which is released in exosomes, defining two different populations of MVBs (Pols and Klumperman, 2009; van Niel et al., 2011). Recently, it was shown that CD63 participates in the ILV sorting of PMEL, a component of melanosomes in melanocytes, in an ESCRT-independent manner, regulating PMEL processing (van Niel et al., 2011). Therefore, we suggest that p75 defines a specific subset of MVBs that are different from the MVBs found in the endolysosomal system in neuronal cells, because p75 does not localise to late endosomes and confocal microscopy demonstrated that a proportion of the internalised p75 was found in CD63-positive organelles. In addition, when cells were treated with ligand to promote p75 endocytosis, there were more p75-positive exosomes in the culture medium of sympathetic neurons after KCl-mediated depolarisation. This result indicated that these extracellular vesicles were of endosomal origin and not the type of extracellular vesicles that originate from the plasma membrane. Indeed, the p75-positive extracellular vesicles were of a size reported for exosomes in both PC12 cells and sympathetic neurons (between 60 and 100 nm; supplementary material Fig. S4) (Simons and Raposo, 2009).

There is evidence that MVBs regulate signalling (Dobrowolski and De Robertis, 2012; Taelman et al., 2010). For example, the sequestration of GSK3β in MVBs is required for the activation of Wnt signalling (Taelman et al., 2010). By contrast, the sequestration of β-catenin and its release into exosomes

downregulates Wnt signalling in HEK 293T cells (Taelman et al., 2010). Additionally, the exosomal secretion of LMP1 decreases the constitutive activation of NFκB induced by this oncoprotein in tumour cells (Verweij et al., 2011). Indeed, the role of exosomes as immune-modulators and regulators of cancer progression is extensively documented (Vlassov et al., 2012). Furthermore, our recent publication indicates that Schwann cells release exosomes containing p75 and that these vesicles enhanced axonal regeneration (Lopez-Verrilli et al., 2013).

Further experiments are required in order to understand the physiological role of p75 exosomal release and the signalling role of $p75^{+}CD63^{+}$ MVBs in the cell bodies and axons of neurons.

MATERIALS AND METHODS

Reagents

NGF, BDNF and the MC192 mouse monoclonal anti-p75ECD were obtained from Alomone Labs (Jerusalem, Israel). Dynasore, aphidicolin, mouse anti-FLAG antibodies, 20-nm gold-labelled Protein A (P9785) and Mowiol 4-88 were obtained from Sigma (St Louis, MO). Hibernate-E medium without Phenol Red was obtained from BrainBits (Springfield, IL). Qdot–streptavidin-655, Hoechst 33258 and all Alexa-Fluor-labelled proteins and molecules were obtained from Molecular Probes (Eugene, OR). Protease-free bovine serum albumin (BSA) and ChromePure human IgG Fc fragment were obtained from Jackson ImmunoResearch (West Grove, PA). Antibodies against Rab5A, β-tubulin, Na^{+}/K^{+} ATPase β1 and CD63 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against μ2, β2 and the recombinant human TrkB–Fc chimera were obtained from BD Bioscience (Franklin Lakes, NJ). The rabbit antibody against the p75 intracellular domain (anti-p75ICD) was obtained from Upstate-Millipore (Billerica, MA). The rabbit anti-Lamp2 antibody was obtained from Zymed Laboratories (San Francisco, CA). The mouse monoclonal anti-Rab5 antibody was obtained from Abcam (Cambridge, UK). The goat anti-mouse and anti-rabbit IgGs conjugated to peroxidase were obtained from BioRad (Hercules, CA). Goat $F(ab')_2$ anti-mouse-IgG labelled with 10-nm gold particles was obtained from BB International (Cardiff, UK). The GeneTailor site-directed mutagenesis system kit, EZ-link biotin-PEO-amine, Slide-A Lyzer mini dialysis units and protein-G–Sepharose beads were obtained from Pierce (Rockford, IL). The proteinase inhibitor cocktail was obtained from Roche. siRNAs, SiGLO Green and Dharmafect 1 and 3 were obtained from Thermo Scientific (Rockford, IL). The Formvar-carbon-coated 300-mesh grids used for electron microscopy (01753-F) were obtained from Ted Pella (Redding, CA).

DNA constructs

The rat p75 gene was mutated by using the GeneTailor site-directed mutagenesis system kit, with the pcDNA3-p75-HA plasmid as the template and the primers 5′-AAGGGTGATGGCAACGCCGCCAGTG-CGCCCCCCTGACCAAGCG-3′ and 5′-CGCTTGGTCAGGGGGGCG-CTACTGGCGGCGTTGCCATCACCCTT-3. The sequence of WT p75 L^{336}YSSL (p75-WT), located in the p75 intracellular domain, was replaced with the sequence A^{336}ASSA (p75-YXXθ). The cDNAs for Rab5-WT–GFP and the constitutively active Rab5 mutant (Rab5-CA–GFP) were kindly provided by Victor Faúndez (Emory University, Atlanta, GA). The cDNA for Rab7-WT–GFP was kindly provided by Alex Kruttgen (Aachen University, Aachen, Germany). The cDNA for Rab11-WT–GFP was kindly provided by Rejji Kuruvilla (John Hopkins University, Baltimore, MD).

Cell culture and transfection

PC12 cells were grown and cultured for experiments as described previously (Bronfman et al., 2003). Sympathetic neurons were dissected from the superior cervical ganglia of Sprague-Dawley rats at postnatal day one, as described previously (Vaillant et al., 1999). The PC12 cells were transfected by using Lipofectamine 2000 according the manufacturer's instructions, and sympathetic neurons were transfected by electroporation using the O-03 programme of an Amaxa Nucleofector device. In all cases, the experiments were conducted 24 hours after transfection. PC12 cells and sympathetic neurons were serum-starved in DMEM supplemented with 25 mM HEPES and 1 mg/ml of BSA (incubation medium).

Conjugation of anti-p75ECD with Qdots gold or Alexa dye

The anti-p75ECD antibody was biotinylated using the EZ Link Micro PEO4 Biotinylation kit according to the manufacturer's instructions. 200 nM of biotinylated anti-p75ECD was incubated with 200 nM streptavidin–Qdots-655 in a final volume of 30 μl of DMEM (lacking Phenol Red) at 4℃ for 30 minutes. To conjugate anti-p75ECD with gold, 200 nM anti-p75ECD was incubated with 200 nM $F(ab')_2$ anti-mouse-IgG labelled with 10-nm gold particles [$F(ab')_2$–gold] that was previously dialysed in a Slide-A Lyzer mini dialysis unit (Pierce) to eliminate sodium azide. Anti-p75ECD was labelled with an Alexa dye according to the manufacturer's instructions.

Electron microscopy (EM) of internalised p75

PC12 cells and sympathetic neurons were starved for 1 hour in incubation medium and then incubated with anti-p75ECD–gold (0.1 μg/ml) in incubation medium supplemented with 150 ng/ml BDNF for the sympathetic neurons and 100 ng/ml NGF for PC12 cells at 37℃. As a control for the specificity of the anti-p75ECD–gold internalisation, the cells were incubated only with the $F(ab')_2$–gold. The cells were fixed for 2 hours by immersion in 3% glutaraldehyde, 0.05% picric acid, 0.05 M cacodylate buffer, pH 7.4. The cells were rinsed in the same buffer and immersed in 1% OsO_4 in 0.05 M cacodylate buffer, pH 7.4, for 30 minutes. Later, the cells were dehydrated with a graded ethanol series and infiltrated with Epon. Ultrathin sections (50–80 nm) were obtained by using a Reichert UM-2 ultramicrotome and then were incubated with 2% uranyl acetate as a contrast dye. The grids were examined using a Phillips Tecnai 12 electron microscope operated at 80 kV.

Immunoendocytosis of p75 for fluorescence microscopy

PC12 cells and sympathetic neurons were starved for 1 hour in incubation medium and then incubated with 3 μg/ml anti-p75ECD or 10 μg/ml anti-HA for 1 hour at 4℃ (or for 120 minutes at 4℃ for neurons). The cells were then incubated with 100 ng/ml NGF (or 150 ng/ml BDNF for neurons) and 10 μg/ml Trf–Alexa-Fluor-568 (or 60 μg/ml Trf–Alexa-Fluor-568 for neurons) for different times at 37℃, washed and fixed in 3% paraformaldehyde for 10 minutes, followed by a 10-minute incubation with 0.15 M glycine, pH 7.4. For the p75 and Trf colocalisation assays, immunoendocytosis was performed using anti-p75ECD–Alexa-Fluor-488. Immunofluorescence was performed as described previously (Bronfman et al., 2003). The antibody concentrations were as follows: anti-Lamp2, 1.25 μg/ml; anti-Rab5, 5 μg/ml; anti-HA, 5μg/ml and anti-CD63, 1 μg/ml. The secondary antibodies were diluted 1:400.

To inhibit dynamin, PC12 cells and sympathetic neurons were serum-starved for 1 hour at 37℃ and then incubated with 80 μM dynasore in PBS plus 4.5 g/l glucose for 30 minutes (PC12) or 1 hour (sympathetic neurons) at 37℃, followed by immunoendocytosis of p75 or internalisation of Trf–Alexa-Fluor-568 in the presence of 80 μM dynasore in PBS plus 4.5 g/l glucose (Kirchhausen et al., 2008; Macia et al., 2006).

The labelled cells were examined by using a Zeiss LSM Pascal 5 (including a triple laser module Arg 458/488/514 nm, He-Ne 543 nm, Carl Zeiss, Thornwood, NY) connected to an inverted microscope (Axiovert 2000) with an apochromat 63×/1.4 oil differential interference contrast (DIC) objective. The ImageJ programme was used to quantify the cell-associated fluorescence. The total cellular fluorescence was calculated after subtracting the non-specific fluorescence from images of untreated cells obtained with the same illumination and exposure conditions. Internalised anti-p75ECD, anti-HA or anti-Trf were expressed as the ratio of the internalised receptor (intracellular fluorescence) versus the cell-surface-associated receptor (cell-surface-associated fluorescence) or expressed as a percentage of internalised fluorescence (intracellular p75), considering 100% to be the total cell-associated fluorescence

(internalised plus cell-surface fluorescence). An anti-p75ECD antibody at 4℃ was used to label the plasma membrane. The value of internalisation at time 0 was defined as the fluorescence value obtained when the cells were treated with anti-p75ECD, anti-HA or Trf at 4℃ for 90 minutes (PC12 cells) or 120 minutes (sympathetic neurons). This value was subtracted from the internalisation values obtained from treatment at 37℃.

For quantitative determination of the colocalisation of two or three labels, confocal images were obtained using the parameters in the range of the Nyquist sampling criteria. The images were subjected to deconvolution using the MetaMorph programme (v 6.1r0). With this programme, the images were separated in each colour channel and then a 2D deconvolution was performed. The nearest neighbour option was chosen for this purpose. After deconvolution, the colour channels were merged. The deconvoluted merged images were opened in the ImageJ programme, and the JaCoP plugin was used to calculate the Manders' coefficients to obtain the percentage colocalisation. To identify true colocalisation events, we performed Van Steensel's approach (cross-correlation function, CCF) and tested the results for Gaussian fitting (Bolte and Cordelières, 2006). See supplementary material Fig. S3 for the details about the evaluation of the colocalisation of the three proteins.

Receptor-recycling assay

PC12 cells were transfected with a cDNA driving the expression of the lipoprotein E receptor 2 amino-tagged with HA (ApoER2–HA). The cells were starved for 1 hour with incubation medium and then a recycling assay was performed. To calculate the proportion of ApoER2 receptors that had recycled back to the plasma membrane, the equation $[(A-B)/A]\times100$ was applied, where '*A*' was the cell-associated fluorescence after ApoER2 internalisation. To obtain this value, the cells were incubated with an anti-HA–Alexa-Fluor-488 antibody for 1 hour at 37℃ to induce internalisation. Under these conditions, all of the labelled ApoER2–HA was internalised (Cuitino et al., 2005; Fuentealba et al., 2007). '*B*' represents the cell-associated fluorescence after ApoER2 internalisation and quenching of the recycling receptor. To obtain this value, after immunoendocytosis, the cells were incubated with an anti-Alexa-Fluor-488 antibody (20 µg/ml) for 1 hour at 37℃ to block the signal of the labelled receptor that recycled back to the plasma membrane.

To calculate the proportion of p75 receptors that had recycled back to the plasma membrane, the equation $[(B-C)/A]*100$ was applied. '*A*' represents the cell-associated fluorescence after p75 internalisation. To obtain this value, the cells were incubated with an anti-p75ECD–Alexa-Fluor-488 antibody for 90 minutes at 4℃. Then, internalisation was induced at 37℃ for 90 minutes in the presence of 100 ng/ml NGF. '*B*' represents the cell-associated fluorescence after quenching the cell-surface-associated fluorescence. To obtain this value, after immunoendocytosis, the cells were incubated for 90 minutes with an anti-Alexa-Fluor-488 antibody (20 g/ml) at 4℃. '*C*' is the cell-associated fluorescence after quenching the recycling p75 receptor at 37℃. To obtain this value, after immunoendocytosis, the cells were incubated for 90 minutes at 37℃ in incubation medium plus anti-Alexa-Fluor-488 (20 µg/ml).

Immunoprecipitation and western blotting

PC12 cells were serum-starved for 90 minutes at 37℃ and incubated with 100 ng/ml NGF at 37℃. The cells were then washed and treated with lysis solution (10 mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol, 1% NP40 and a protease inhibitor cocktail). The cells were centrifuged at 21,500 ***g*** for 15 minutes at 4℃. An aliquot of 1,500 µg of protein was pre-cleared with an 80-µl suspension (50%) of Protein-G–Sepharose beads for 1 hour at 4℃. The beads were removed and the supernatant was incubated with 6 µg of anti-p75ECD overnight; 80 µl of Protein-G–Sepharose was then added for 2 hours at 4℃. The proteins were eluted for 10 minutes at 60℃ with SDS-PAGE sample buffer. The eluted proteins were loaded onto a 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane. Antibodies against the following proteins were used for western blotting: p75ICD (1 µg/ml); β-tubulin (1 µg/ml); β2 (0.05 µg/ml); µ2 (1 µg/ml); CD63 (0.4 µg/ml); Rab5 (1 µg/ml); Na^+/K^+ ATPase (0.2 µg/ml) and β-COP (1:1,000). The secondary antibody was goat anti-rabbit-IgG or anti-mouse-IgG conjugated to peroxidase (1:10,000).

RNA Interference

The sequences of the siRNA used to downregulate the µ2 levels were µAP2A-5′-GAUCAAGCGCAUGGCAGGCAU-3′ and µAP2B-5′-AAGUGGAUGCCUUUCGCGUCA-3′, and the control siRNA used was siGenome non-targeting siRNA pool #1. DharmaFECT-3 was used to incorporate the siRNA into the sympathetic neurons according to the manufacturer's instructions, with a 3:1 ratio of siRNA:siGLO Green.

Real-time microscopy

PC12 cells were serum-starved for 1 hour and then incubated with 1 µg/ml anti-p75ECD–Qdots in incubation medium at 4℃ for 90 minutes. The cells were then treated with 100 ng/ml NGF in Hibernate-E medium and observed by using an inverted microscope (Olympus IX7I) equipped with a thermo-regulated stage and a Qicam Fast 1394 Qimaging digital camera, which was connected to a computer with Image-pro express software (v 6.3.0.531).

To evaluate whether the time of residence of the endocytosed p75 in the Rab5, Rab11 and CD63 organelles was different, we performed two-colour live-cell imaging of PC12 cells transfected with Rab5–GFP, Rab11–GFP or CD63–GFP. The cells were serum starved in incubation medium for 60 minutes and then incubated with 3 µg/ml anti-p75ECD–Qdots for 120 minutes at 4℃. The cells were then incubated with 100 ng/ml NGF and imaged for timeframes of 5 minutes between 0 and 25 minutes after NGF addition. The images were captured at 37℃ using a Leica DMI6000b inverted microscope equipped with 63× glycerine-immersion lens, high velocity emission and excitation filters and an iXon 887 EMCCD camera (Andor, Tokyo, Japan), which was connected to a computer running LAS AF software. To quantify the residence time, the images were subjected to deconvolution algorithms, digital amplification and Gaussian filters in ImageJ software. The endosomes in focus with observable p75 were then selected and we quantified the number of p75 particles arriving to the selected endosome and the proportion of them leaving.

Exosome purification

To prepare an exosome-enriched sample, 10-cm Petri dishes of PC12 cells at 90% confluence (or 4 wells of a 12-well plate of sympathetic neurons) were used. The cells were serum starved for 60 minutes at 37℃ and incubated with 150 ng/ml BDNF for 2 hours at 37℃ in the case of PC12 cells or 4 hours in the case of sympathetic neurons. When the sympathetic neurons were incubated without BDNF, TrkB–Fc (400 ng/ml) was added to block the endogenous BDNF; when the neurons were incubated with BDNF, a control IgG Fc (400 ng/ml) was used. To induce the release of exosomes, the cells were stimulated with a buffer containing 30 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 140 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 0.7% glucose and 15 mM HEPES pH 7.4 (exosome release buffer) at 37℃ for 30 minutes (PC12 cells) or for 6 hours (sympathetic neurons). Afterwards, the exosome release buffer that was added to cells was collected and subjected to differential centrifugation. First, the cell medium was centrifuged at 2500 ***g*** for 5 minutes and the resulting supernatant was centrifuged at 300 ***g*** for 10 minutes. Then, the supernatant was centrifuged at 2000 ***g*** for 10 minutes, followed by centrifugation at 10,000 ***g*** for 30 minutes. This final supernatant was ultracentrifuged at 100,000 ***g*** for 70 minutes in a Hitachi WX series Himac CP80WX ultracentrifuge with a P55ST2 rotor. The exosome sample was resuspended in PBS and 5× lysis buffer (50 mM Tris-HCl pH 8, 750 mM NaCl, 5% IGEPAL, 50% glycerol and protease inhibitor cocktail) was added. The sample was sonicated in a water bath for 5 minutes and centrifuged at 18,000 ***g*** for 5 minutes. The supernatant was analysed by western blotting.

To analyse the presence of p75 in exosomes by electron microscopy, the cells were treated as described above for western blotting analysis and, to induce the release of exosomes, the cells were then stimulated with exosome release buffer at 37℃ for 4 hours (PC12 cells) or for 6 hours (sympathetic neurons). The exosome release buffer added to cells was subjected to differential centrifugation as described above. After ultracentrifugation, the exosome samples were resuspended in PBS and then paraformaldehyde was added to a final concentration of 2%. After an overnight fixation step at 4℃, 5 µl of sample was placed on a

300-mesh grid, washed with PBS and then incubated with 1% fish gelatin in PBS for 10 minutes at room temperature. The grid was incubated with 20-nm gold-labelled Protein A, further incubated with 1% glutaraldehyde for 5 minutes, washed with water, incubated with 2% uranyl acetate for 1.5 minutes and, finally, dried. Grids that had intact exosomes were examined by using a Phillips Tecnai 12 electron microscope operated at 80 kV.

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Competing interests
The authors declare no competing interests.

Author contributions
C.A.E. performed most of the experiments and wrote the paper. O.M.L. performed the two-colour life cell imaging and performed and quantified part of the co-localization experiments. C.G. performed and quantified part of the co-localization experiments. J.I.P. performed and quantified part of the co-localization experiments. M.A.L.-V. helped with the exosomes purification. C.C. performed the experiments with the CA Rab5 mutant. L.L. performed and quantified part of the co-localization experiments. U.S. performed p75 internalization experiments in sympathetic neurons. C.R. performed the two-colour life cell imaging. A.G. designed research. M.-P.M. designed research and helped with editing the paper. B.C. designed research and helped with editing the paper. F.A.C. designed research, contributed to electron microscopy and helped with editing the paper. F.C.B. designed research and wrote the paper.

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Supplementary material
Supplementary material available online at
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