Cellular/Molecular

Nuclear Translocation of the p75 Neurotrophin Receptor Cytoplasmic Domain in Response to Neurotrophin Binding

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The intracellular domain of the p75 neurotrophin receptor (p75^{ICD}) can be released by γ-secretase in response to the previous activation of α-secretase by phorbol esters. However, ligand-dependent release of p75^{ICD} has yet to be described. We show here that nerve growth factor can induce the release of p75^{ICD} and facilitate its translocation to the nucleus in a γ-secretase-dependent manner. This effect was observed in RN22 schwannoma cells cultured under serum-free conditions, as well as in Schwann cells, and it was mimicked by other neurotrophins, such as brain-derived neurotrophic factor or neurotrophin-3. Unlike other known examples of regulated intramembrane proteolysis, ligand-dependent release of p75^{ICD} did not need the previous activation of α-secretase. These results suggest that nuclear translocation of p75^{ICD} may represent a novel neurotrophin-mediated signaling pathway.

Key words: α-secretase; γ-secretase; p75^{NTR}; neurotrophin; nuclear translocation; regulated intramembrane proteolysis

Introduction

The neurotrophin family is composed of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5. These molecules act as ligands for the Trk neurotrophic receptor tyrosine kinases, as well as for the common p75 neurotrophic receptor (p75^{NTR}), a member of the tumor necrosis factor (TNF) receptor family (López-Sánchez and Frade, 2002). In recent years, a number of signaling pathways have been described that mediate the response of ligand binding to p75^{NTR} (López-Sánchez and Frade, 2002). More recently, the activation of α-secretase by phorbol esters was shown to provoke the release of the intracellular domain of p75^{NTR} (p75^{ICD}) in a number of cell lines, including RN22 schwannoma cells (RN22-SCs) (Jung et al., 2003; Kanning et al., 2003). However, evidence for ligand-mediated intramembrane cleavage of p75^{NTR} has yet to be presented. In this article, we show that endogenously expressed p75^{ICD} can be released and translocated to the nucleus in response to both NGF and other neurotrophins. Moreover, it seems that the release of p75^{ICD} is independent of metalloproteinase-dependent shedding of the p75^{NTR} ectodomain.

Materials and Methods

Materials. All of the chemical reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. The rabbit polyclonal antisera (9992) against p75^{ICD} and against the extracellular domain of p75^{NTR} (p75^{ECD}) (9651; diluted 1:800 for immunocytochemistry; diluted 1:2000 for Western blots) and the affinity-purified antiserum against the C terminus of SC-1 (used at 4 μg/ml) were kindly provided by Moses Chao (New York University, New York, NY). The anti-human p75^{ICD} polyclonal antiserum (diluted 1:500; Promega, Madison, WI) also revealed nuclear staining in response to NGF (data not shown). The anti-neurotrophin receptor homolog 2 (NRH2) antiserum (kindly provided by Phil Barker, McGill University, Montreal, Québec, Canada) was diluted 1:800 (immunocytochemistry), diluted 1:5000 (immunodepletion), or diluted 1:10,000 (Western blotting). The amyloid precursor protein (APP)-specific monoclonal antibody 22C11 (Chemicon, Hampshire, UK) was used at 5 μg/ml. The γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) was used at 1 μM, the metalloproteinase inhibitor TNF-α protease inhibitor-2 (TAPI-2) was used at 20 μM, and the metalloproteinase inhibitor GM 6001 was used at 10 μM (all purchased from Calbiochem, La Jolla, CA). PMA was used at 100 ng/ml. Human recombinant NGF-β, BDNF, and NT3 were stored at 10 ng/μl in PBS/0.5% bovine serum albumin and were used at 100 ng/ml unless otherwise stated. TrkB receptor bodies (human TrkB/Fc chimera; R & D Systems, Abingdon, UK) were used at 1 μg/ml.

Reverse transcription-PCR. Amplification of cDNAs derived from RN22-SCs, postnatal day 2 (P2) rat brain, or P2 rat cortex was performed using standard procedures. The PCR primers used were as follows: *GAPDH* (base pairs 502–521, 1101–1120; GenBank accession number X02231), *TrkB* (base pairs 852–871, 1569–1588; GenBank accession number M55291), *TrkC* (base pairs 266–285, 766–785; GenBank accession number L03813), *NGF* (base pairs 655–674, 973–992; GenBank accession number M36589), *BDNF* (base pairs 564–583, 745–764; GenBank accession number M61178), and *NT3* (base pairs 773–792, 1032–1051; GenBank accession number NM_031073). *TrkB/TrkC* primers are specific for both full-length and truncated forms.

Cell culture. RN22-SCs were maintained at 37°C in DMEM/10% FCS (Invitrogen, Carlsbad, CA). For most experiments, RN22-SCs (10,000 cells/cm^2) were initially cultured for 24 h in DMEM, whereas RN22-SCs expressing human APP were cultured in DMEM/10% FCS. Schwann cells from P2 mouse sciatic nerves were isolated as described by Chan et al. (2000), and they were cultured (10,000 cells/cm^2) for 15 h in DMEM containing 2 μM forskolin and bovine pituitary extract (BPE) diluted

1:100. Cell death was determined using the *In Situ* Cell Death Detection kit (Hoffman-La Roche, Basel, Switzerland).

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde (15 min). Immunostaining was performed following a protocol described previously (López-Sánchez et al., 2005). Nuclei were counterstained with 1 μg/ml propidium iodide or bisbenzimide.

Cell fractionation. All of the steps were performed at 4°C. Cells were lysed in cytoplasmic buffer (CB) (10 mM HEPES, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.075% Triton X-100, 1 mM DL-dithiothreitol, and 1× protease inhibitor mixture) and homogenized by passing them through a 22 ga needle. The nuclei were spun down at 325 × *g* for 4 min, and the supernatant was isolated (cytoplasmic fraction). The pellets were washed with CB without detergent and extracted in nuclear buffer (20 mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 25% glycerol, and 1× protease inhibitor mixture). NRH2 immunodepletion of nuclear fractions was performed in 50 mM Tris-HCl, pH 8.0, 168 mM NaCl, 0.6 mM MgCl$_2$, 0.08 mM EDTA, 10% glycerol, and 0.1% Triton X-100. Removal of anti-NRH2 was performed with protein G-agarose beads diluted 1:25 (Santa Cruz Biotechnology, Santa Cruz, CA). Conditioned media (7.5 ml) were concentrated using Centriprep YM-30 (Millipore, Bedford, MA). Total extracts were obtained by cell solubilization in Laemli's buffer.

Western blot. Total extracts (equivalent to 200,000 cells), nuclear and cytoplasmic extracts (15 μg of protein), or concentrated conditioned media (equivalent to 330 μl of original culture media) were separated by SDS-PAGE on 11% acrylamide gels under nonreducing conditions and transferred to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). APP-conditioned media (20 μl) were separated on 8% acrylamide gels. Membranes were blocked in 5% nonfat dried milk in PBS with 0.1% Tween 20 (PBTw) for 1 h and incubated overnight at 4°C with the antisera in blocking buffer. After being washed five times in PBTw, the membranes were incubated with goat anti-rabbit HRP-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:50,000 in blocking buffer (1 h) and washed as above. Protein bands were visualized using ECL (Amersham Biosciences, Piscataway, NJ). The films were scanned, and the relative optical density (ROD) of the bands was determined using the Analytical Imaging Station (AIS) 4.0 (Imaging Research, St. Catharines, Ontario, Canada). ROD values were normalized to the value of the protein bands from the control cultures.

Cell counting. Cells were examined by confocal microscopy to define the subcellular localization of the immunostaining using a Leica (Nussloch, Germany) TCF-4D confocal setup. Cell counting was performed with a Nikon (Melville, NY) E80i microscope with phase contrast and epifluorescence illumination. In other experiments, randomly selected fields containing cells counterstained with bisbenzimide were digitalized using a DXM1200F camera (Nikon), and the ROD for p75^{ICD} immunostaining was measured with AIS 4.0. The average ROD value of the cytoplasm defined the threshold that was used under all of the experimental conditions to identify those cells with stronger nuclear labeling for p75^{ICD}. This approach yielded results similar to those obtained by direct visual counting (data not shown). On average, 500 cells were analyzed per coverslip.

Statistical analysis. Quantitative data are shown as the mean ± SEM from *n* independent experiments. Statistical differences were analyzed using the Student's *t* test.
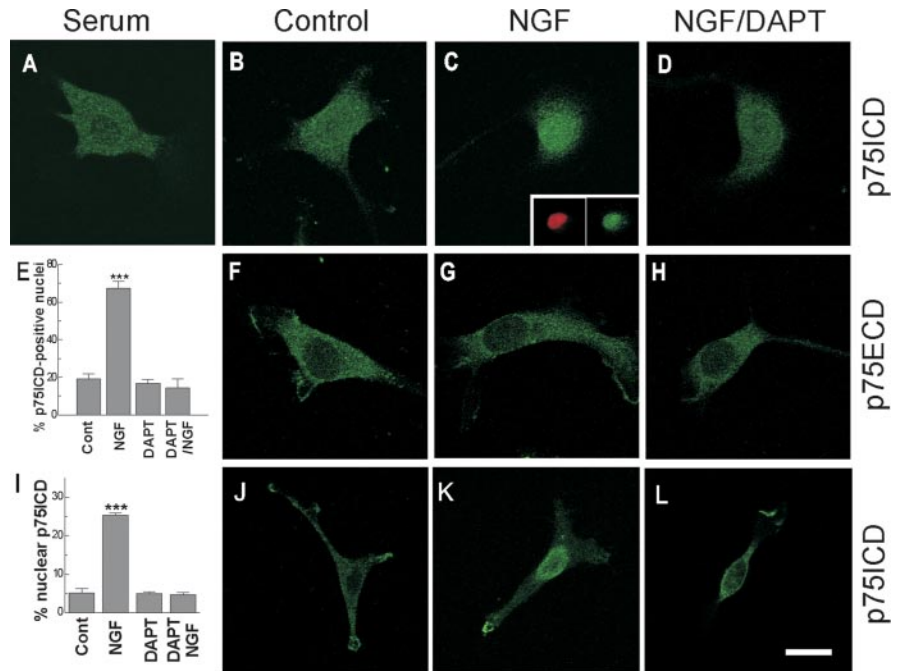

Figure 1. NGF induces nuclear translocation of p75^{ICD} as revealed by immunocytochemistry. *A–H*, RN22-SCs cultured in the presence (*A*) or absence (*B–H*) of FCS were treated for 1 h with vehicle (*B, F*), NGF (*C, G*), DAPT, or both (*D, H*) and then were immunostained for p75^{ICD} (*A–D*) or p75^{ECD} (*F–H*). *B*, Weak nuclear p75^{ICD} immunoreactivity was observed in most cells after removal of FCS. However, exposure to NGF increased the proportion of nuclei containing strong nuclear p75^{ICD} staining (*C, E*), an effect that was inhibited by DAPT (*D, E*). *C*, Inset, Propidium iodide-labeled nucleus (red) with strong p75^{ICD} immunoreactivity (green). *F–H*, p75^{ECD} was never observed in the cell nucleus. *I–L*, Schwann cells cultured in the presence of FCS, forskolin, and BPE (*I–L*) were treated for 2 h with vehicle (*J*), NGF (*K*), DAPT, or both (*L*). The proportion of nuclei containing high levels of p75^{ICD} increased in the presence of NGF (*I, K*), whereas exposure to DAPT prevented this effect of NGF (*I, L*). ***$p < 0.001$. Scale bar: *A–D*, *F–H*, 14 μm; *J–L*, 10 μm. Cont, Control. Error bars represent SEM.

Results

NGF induces nuclear translocation of p75^{ICD} in serum-free cultured RN22-SCs

The SC line RN22 lacks the NGF-specific neurotrophic receptor TrkA but contains high levels of p75^{NTR} (Gentry et al., 2000). The cellular distribution of p75^{ICD} was determined in these cells by immunocytochemistry, and laser-scanning confocal microscopy confirmed that, in the presence of FCS, p75^{ICD} did not accumulate in the nucleus (Fig. 1*A*) (Kanning et al., 2003). However, in the absence of serum, the intensity of p75^{ICD} labeling in the majority of nuclei was equivalent to or less than that in the cytoplasm (Fig. 1*B*). In the presence of NGF, a considerable accumulation of p75^{ICD} occurred in ~70% of nuclei, in contrast to control cells, in which only ~20% of the nuclei were strongly labeled for p75^{ICD} (Fig. 1*C,E*). The increase in the proportion of nuclei strongly labeled for p75^{ICD} was observed 10 min after the addition of NGF (Fig. 2*A*) and lasted for at least 24 h (data not shown). The half-maximal effect of NGF was attained after 2–3 min. The proportion of nuclei strongly labeled for p75^{ICD} increased in a concentration-dependent manner, with ~5–10 ng/ml NGF producing a half-maximal effect (Fig. 2*B*). Exposure to DAPT prevented the increase in the proportion of nuclei that accumulated high levels of p75^{ICD} in response to NGF (Fig. 1*D,E*). Hence, the ligand-dependent release of p75^{ICD} seems to require γ-secretase activity. As expected, p75^{ECD} immunolabeling was absent from the nucleus, regardless of the presence or absence of NGF or DAPT (Fig. 1*F–H*).

To confirm that the p75^{ICD} does indeed accumulate in the nucleus in response to NGF, nuclear and cytoplasmic extracts
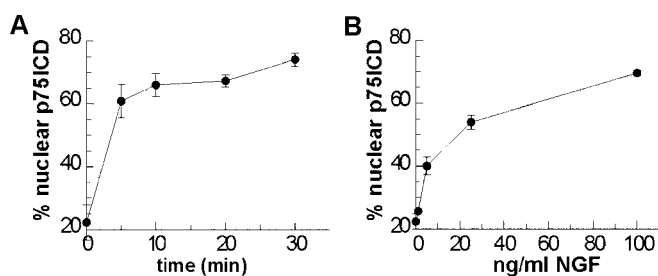
Figure 2. Time course and dose–response of the NGF-induced nuclear translocation of p75^{ICD}. *A*, Percentage of RN22-SC nuclei with strong p75^{ICD} immunoreactivity when cultured in the presence of NGF for the time periods indicated. *B*, Percentage of RN22-SC nuclei with strong p75^{ICD} immunoreactivity in cells cultured for 24 h in the presence of the concentrations of NGF indicated. Error bars represent SEM.

were subjected to Western blotting with a p75^{ICD}-specific antiserum. A 25 kDa band, characterized previously as p75^{ICD} (Jung et al., 2003; Kanning et al., 2003), was enriched in nuclear extracts from RN22-SCs exposed to NGF for 2 h ($n = 2$) (Fig. 3A). In the presence of the γ-secretase inhibitor DAPT, the levels of this protein diminished to those of control cells. Additional protein bands of 28, 33, and 37 kDa were observed in these extracts, but they remained at a similar intensity under all of the conditions (Fig. 3A). These bands are likely to represent NRH2 moieties that cross-react with the anti-p75^{ICD} antiserum because they were also detected with the NRH2-specific antiserum ($n = 2$) (Fig. 3A). Immunodepletion with this anti-NRH2 antiserum allowed us to unambiguously identify the 25 kDa band corresponding to p75^{ICD} in the nuclear extracts from RN22-SCs (Fig. 3A). Densitometric analysis demonstrated that this band was significantly increased in the NGF-treated cultures, an increase that was prevented by DAPT [control, 1; NGF, 9.63 $\pm$ 1.88 ($p < 0.01$); DAPT, 0.74 $\pm$ 0.25; DAPT/NGF, 1.57 $\pm$ 0.10; $n = 3$].

In the cytoplasmic fractions, the p75^{ICD}-specific antiserum only detected the full-length p75^{NTR} protein (Fig. 3A). Interestingly, the intensity of the full-length protein was reduced in the fraction obtained from the NGF-treated cells, in accordance with the cleavage and nuclear translocation of p75^{ICD}, and this decrease was blocked in the presence of DAPT [control, 1; NGF, 0.45 $\pm$ 0.01 ($p < 0.001$); DAPT, 1.24 $\pm$ 0.03; DAPT/NGF, 1.14 $\pm$ 0.02; $n = 2$]. No higher-mobility bands corresponding to the p75^{NTR} C-terminal domain were observed in the cytoplasmic fraction, even in the presence of DAPT. Thus, the release of p75^{ICD} in response to NGF does not seem to require the previous shedding of the ectodomain attributable to α-secretase activity. Alternatively, NGF might induce α-secretase cleavage and the release of the p75^{NTR} extracellular domain, but immunoblotting may not be sufficiently sensitive to detect the C-terminal fragment in the presence of DAPT.

To discriminate between these two alternatives, total cell extracts were subjected to Western blotting with the p75^{ICD}-specific antiserum (Fig. 3B). This analysis confirmed that NGF induces the accumulation of a faint 25 kDa band, and this effect is blocked by DAPT. No high-mobility bands specific for the p75^{NTR} C-terminal domain were observed in extracts from cells treated with NGF and DAPT, thus indicating that the release of p75^{ICD} does not seem to require two cleavage steps.

To study further whether the activation of α-secretase is necessary for the release of p75^{ICD} provoked by NGF, culture media conditioned by RN22-SCs were analyzed in Western blots using the anti-p75^{ECD} antiserum (Fig. 3C). A protein of 55 kDa was identified in medium conditioned by NGF-treated cells but not

in medium from control cells. This protein was slightly larger than the 50 kDa p75^{NTR} ectodomain released by α-secretase in response to PMA in the presence of serum ($n = 2$) (Weskamp et al., 2004) (Fig. 3C) and could represent the complete p75^{NTR} ectodomain cleaved by γ-secretase. In agreement with this idea, the intensity of this band in the medium conditioned by cells treated with NGF was dramatically reduced by the presence of DAPT [NGF, 1; DAPT/NGF, 0.08 $\pm$ 0.06 ($p < 0.05$); $n = 3$] (Fig. 3C). These data strongly suggest that the interaction of NGF with p75^{NTR} results in γ-secretase cleavage of p75^{NTR} without the previous activation of α-secretase. Moreover, it seems that matrix metalloproteinases are not involved in the γ-secretase cleavage of p75^{NTR}. In the presence of TAPI-2, an inhibitor of matrix metalloproteinases that prevents PMA-dependent shedding of p75^{NTR} (Jung et al., 2003; Kanning et al., 2003; Weskamp et al., 2004), the proportion of RN22-SC nuclei strongly labeled for p75^{ICD} when visualized by immunocytochemistry still increased in response to NGF [control, 22.18 $\pm$ 6.09%; NGF, 69.42 $\pm$ 1.57% ($p < 0.005$); TAPI-2, 24.49 $\pm$ 3.09%; TAPI-2/NGF, 70.48 $\pm$ 1.96% ($p < 0.001$); $n = 3$]. Likewise, the release of the p75^{NTR} ectodomain in response to NGF was not affected by the presence of TAPI-2 (NGF, 1; TAPI-2/NGF, 0.88 $\pm$ 0.12; $n = 2$; nonsignificant) (Fig. 3D). The activity of TAPI-2 under these conditions was confirmed by its ability to prevent APP shedding from RN22-SCs expressing human APP [control, 1; TAPI-2, 0.18 $\pm$ 0.03 ($p < 0.005$); $n = 2$] (Fig. 3D). The metalloproteinase inhibitor GM 6001 also failed to prevent the accumulation of p75^{ICD} in the nuclei of the RN22-SCs in response to NGF [control, 28.90 $\pm$ 8.80%; NGF, 73.21 $\pm$ 6.71% ($p < 0.005$); GM 6001, 20.19 $\pm$ 5.16%; GM 6001/NGF, 85.24 $\pm$ 0.69% ($p < 0.001$); $n = 4$].

To determine whether p75^{ECD} can also accumulate in the cytoplasm in response to NGF, the cytoplasmic fractions of RN22-SCs cultured with different combinations of NGF and DAPT were analyzed by Western blot using the p75^{ECD}-specific antiserum. These cytoplasmic fractions did not contain the 55 kDa p75^{ECD} but rather the full-length p75^{NTR}, which was less intense in the extracts obtained from NGF-treated cells than in control cells [control, 1; NGF, 0.34 $\pm$ 0.03 ($p < 0.005$); DAPT, 0.97 $\pm$ 0.15; DAPT/NGF, 1.17 $\pm$ 0.05; $n = 2$] (Fig. 3C). A 28 kDa protein band was also observed after all of the treatments, which may be attributable to nonspecific binding, or it may correspond to a degradation product of p75^{ECD}. This band might account for the strong staining for p75^{ECD} observed in the cytoplasm of NGF-treated cells (Fig. 1G).

NGF induces nuclear translocation of p75^{ICD} in Schwann cells
We determined whether the nuclear accumulation of p75^{ICD} in response to NGF might occur in nontransformed cells. In Schwann cells derived from P2 mice grown in DMEM supplemented with FCS, p75^{ICD} did not accumulate in the nuclei (data not shown). However, in the presence of forskolin and BPE, ~5% of the nuclei contained p75^{ICD} (Fig. 1I). Moreover, in the presence of NGF, the proportion of p75^{ICD}-immunoreactive nuclei increased to ~25%, (Fig. 1I,K). The effect of NGF on the nuclear accumulation of p75^{ICD} was inhibited by the addition of DAPT (Fig. 1I,L). Hence, the activation of p75^{NTR} by NGF seems to provoke regulated intramembrane proteolysis (RIP) and the nuclear translocation of p75^{ICD} in primary cell cultures.

BDNF and NT3 also induce nuclear translocation of p75^{ICD}
The possibility that other neurotrophins could induce nuclear accumulation of p75^{ICD} was also evaluated in RN22-SCs. Be-

cause these cells do not express either *TrkB* or *TrkC* (Fig. 4*F*), any interference of these BDNF- and NT3-specific tyrosine kinase receptors with the signaling of p75^{NTR} was avoided. Both BDNF and NT3 increased the proportion of cells showing strong nuclear p75^{ICD} immunoreactivity in a manner similar to NGF (Fig. 4*A–D*). These data indicate that all of the neurotrophins have a similar capacity to induce p75^{ICD} release and nuclear translocation.

We tested whether RN22-SCs cultured in the absence of serum express NGF, BDNF, or NT3. BDNF expression could be detected by reverse transcription (RT)-PCR in cDNAs derived from these cells (Fig. 4*E*). This suggests that the presence of endogenous BDNF in the culture medium during the 24 h period before the treatment with NGF could be responsible for the 20% of nuclei strongly stained with the anti-p75^{ICD} antiserum under control conditions (Fig. 1*E*). Indeed, the addition of DAPT at the time of serum withdrawal strongly reduced the background levels of cells with strong nuclear p75^{ICD} immunoreactivity [$1.17 \pm 0.19\%$ ($p < 0.001$); $n = 4$]. Blockage of endogenous BDNF with the addition of TrkB receptor bodies at the time of serum withdrawal (1 μg/ml) also reduced the proportion of nuclei with high levels of p75^{ICD} immunoreactivity [$8.69 \pm 0.21\%$ ($p < 0.005$); $n = 4$]. This indicates that, during the period in which the RN22-SCs are initially cultured in the absence of serum, p75^{ICD} accumulates in the nucleus in response to ligand-mediated, γ-secretase-dependent release of p75^{ICD}. This effect seems to be mediated at least partially by BDNF.

Discussion

In this study, we present evidence that the activation of p75^{NTR} with NGF promoted the release of p75^{ICD} via the activity of γ-secretase. We show that the increased accumulation of p75^{ICD} in the nuclei of RN22-SCs or Schwann cells in the presence of NGF was impaired by the γ-secretase inhibitor DAPT. Furthermore, the detection of a p75^{ICD}-specific protein band in the nuclear and total cell extracts from RN22-SCs treated with NGF was also inhibited by DAPT. Finally, Western blot analysis indicated that the levels of full-length p75^{NTR} were reduced in the cytoplasmic and total cell extracts of RN22-SCs treated with NGF, suggesting that p75^{ICD} is released after neurotrophin binding. This effect was also reversed by DAPT.

p75^{ICD} accumulation in the nucleus was dependent on the concentration of NGF and was completed in <10 min. This rapid activation of γ-secretase has also been seen in other RIP paradigms (Ray et al., 1999). Furthermore, the rapid response of p75^{NTR} is not unique because the association of TRAF6 (TNF receptor-associated factor 6) with p75^{NTR} occurs 1 min after NGF treatment and also reaches a maximum after 10 min (Khursigara et al., 1999). In contrast, internalization of p75^{NTR} in response to neurotrophins is much slower, taking ~45 min (Bron-
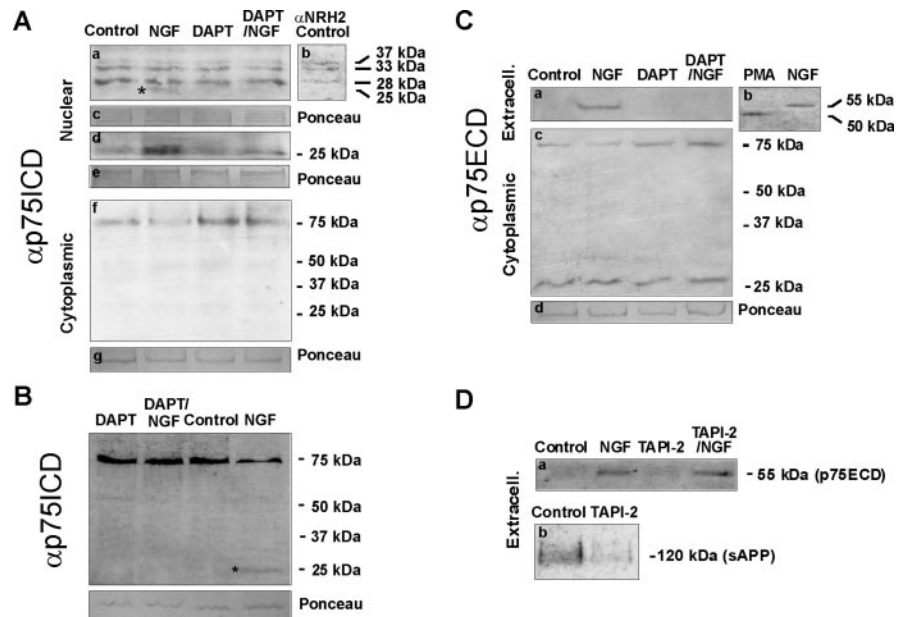

Figure 3. NGF induces p75^{ECD} shedding and nuclear accumulation of p75^{ICD} independent of metalloproteinases. *Aa*, Nuclear extracts (15 μg) from RN22-SCs treated for 2 h with vehicle (Control), NGF, DAPT, or both (DAPT/NGF). The p75^{ICD} was observed as a 25 kDa band in the NGF-treated cells (asterisk), an effect that was prevented by DAPT. *Ab*, The 28, 33, and 37 kDa bands observed in these extracts were also recognized by the NRH2-specific antiserum. *Ad*, The p75^{ICD}-specific band was clearly observed in NRH2-immunodepleted nuclear extracts (15 μg) after a long exposure of the membranes, and its intensity augmented in the presence of NGF. *Af*, The anti-p75^{ICD} antiserum recognized a p75^{NTR}-specific band in the cytoplasmic extracts (15 μg) of RN22-SCs. The intensity of this band was reduced in the presence of NGF. *B*, Total cell extracts from RN22-SCs treated for 2 h as above. The presence of NGF reduced the intensity of the p75^{ICD}-specific band ($59 \pm 7\%$ compared with control conditions; $n = 2$; $p < 0.05$) and induced the appearance of a faint band of 25 kDa (asterisk). Both of these effects were prevented by adding 1 μM DAPT. The C-terminal portion of p75^{NTR} was not observed after γ-secretase inhibition by DAPT in extracts from NGF-treated cells. *Ca*, The anti-p75^{ECD} antiserum recognized a single 55 kDa band in the culture medium conditioned for 24 h by cells treated with NGF. The intensity of this band was reduced in the presence of DAPT, and it was distinct from that released in response to PMA by RN22-SCs cultured in the presence of FCS (*Cb*). *Cc*, The anti-p75^{ECD} antiserum recognized a p75^{NTR}-specific band in cytoplasmic extracts (15 μg) from RN22-SCs, which was less intense in the presence of NGF. *Da*, The presence of TAPI-2 could not prevent the release of p75^{ECD} from cells cultured in the presence of NGF as detected with the anti-p75^{ECD} antiserum. However, TAPI-2 did prevent shedding of soluble APP in RN22-SCs expressing human APP (*Db*). See Results for quantification of the intensity of the bands and statistical analysis. The amount of nuclear or cytoplasmic protein on the membranes per lane was similar in all of the experiments as revealed by Ponceau S staining (*Ac, Ae, Ag, B, Cd*). Extracell., Extracellular.

fman et al., 2003). This suggests that the release of p75^{NTR} does not need a previous internalization step and that it can take place directly at the cell surface. It is unclear why neurotrophin-dependent RIP of p75^{NTR} was only observed when RN22-SCs were cultured in serum-free medium. Schwann cells grown in the presence of serum, forskolin, and BPE showed a similar, although less pronounced, effect in response to NGF. It is possible that protein kinase A (PKA), an indirect target of forskolin, might sensitize Schwann cells to undergo RIP in response to NGF. Indeed, PKA has been shown to be necessary for TNF receptor-1 shedding (Douvdevani et al., 1996).

The capacity of p75^{NTR} to translocate its intracellular domain to the nucleus in response to ligand activation resembles the pathway used by Notch or ErbB-4 to transduce intracellular signals (Ebinu and Yankner, 2002). Unlike these receptors, p75^{NTR} seems to undergo ligand-dependent RIP in the absence of α-secretase-dependent ectodomain shedding. This conclusion was based first on the failure to detect the C-terminal p75^{NTR} fragment derived from ectodomain shedding of the receptor in the cytoplasmic and total cell extracts from NGF-treated RN22-SCs cultured in the presence of DAPT. Moreover, the p75^{ECD} released in response to NGF as a 55 kDa fragment is larger than the p75^{NTR} ectodomain released by α-secretase (Weskamp et al.,
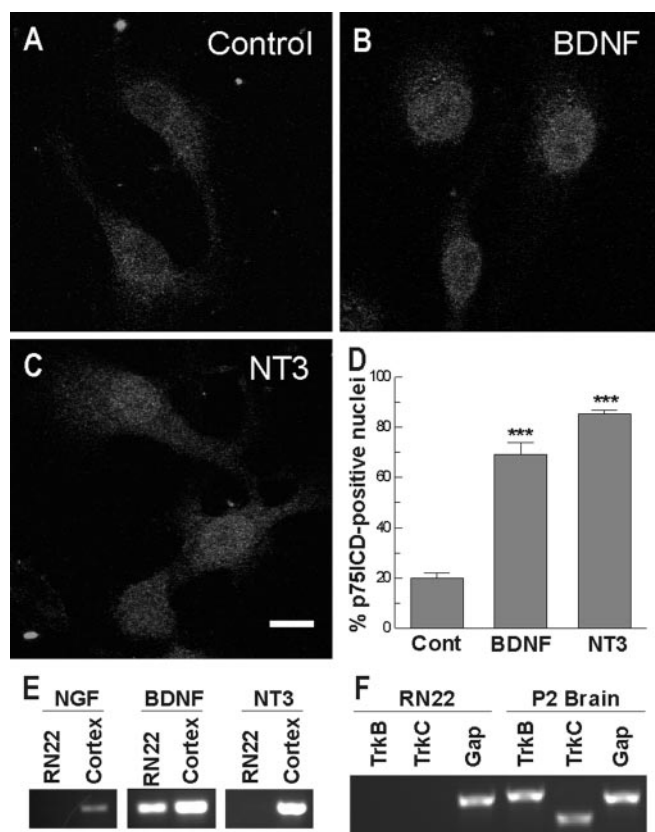
Figure 4. Both BDNF and NT3 also induce nuclear translocation of p75[ICD]. *B–D*, Treatment of RN22-SCs for 1 h with 100 ng/ml BDNF (*B*) or NT3 (*C*) increased the proportion of nuclei with strong p75[ICD] staining (*B–D*) when compared with control (Cont) cells (*A*, *D*). *E*, Expression of *NGF*, *BDNF*, and *NT3* was analyzed by RT-PCR (38 cycles) on mRNA from RN22-SCs (RN22) or P2 rat cortex (Cortex). *BDNF* expression could be detected in RN22-SCs. *F*, Expression of *TrkB*, *TrkC*, and *GAPDH* (Gap) was analyzed by RT-PCR (35 cycles) on total mRNA derived from RN22-SCs or P2 rat brain (P2 Brain). *TrkB*- and *TrkC*-specific transcripts could not be detected in RN22-SCs. In the absence of reverse transcription, no amplification was detected for any of these genes (data not shown). ***$p < 0.001$. Scale bar, 10 μm. Error bars represent SEM.

2004; this study), and blocking γ-secretase activity prevented the NGF-dependent release of this 55 kDa ectodomain fragment. Finally, the metalloproteinase inhibitors TAPI-2 and GM 6001 were both unable to reduce the proportion of nuclei with high levels of p75[ICD] in RN22-SCs treated with NGF, and TAPI-2 could not prevent the release of the 55 kDa ectodomain fragment. This novel behavior of p75[NTR] suggests that neurotrophin binding provokes conformational changes in its transmembrane domain necessary for γ-secretase cleavage.

In RN22-SCs grown with FCS, p75[ICD] was only observed in cell extracts in the presence of proteasome inhibitors (Kanning et al., 2003). Here, endogenously expressed p75[ICD] was detected in FCS-depleted RN22-SCs without the need for proteasome inhibitors. One explanation for the stability of p75[ICD] in the absence of FCS is that it may interact with proteins that stabilize it, as occurs with Notch and APP (Kimberly et al., 2001). One such protein that interacts with p75[ICD] is Schwann cell factor 1 (SC1), which is located in the nucleus in the absence of FCS (Chittka and Chao, 1999). Indeed, we detected nuclear SC1 in RN22-SCs cultured in

the absence of serum (data not shown), consistent with the possibility that SC1 stabilizes p75[ICD] in the nucleus of these cells.

The analysis of the nuclear fraction by Western blotting with the anti-p75[ICD]-specific antiserum identified the presence of 28, 33, and 37 kDa bands, whose intensity was not affected by the experimental manipulations. These bands were probably the result of a cross-reaction with NRH2 because they were detected by a well characterized anti-NRH2 antiserum (Paul et al., 2004) Moreover, they were absent in the NRH2-immunodepleted nuclear fractions analyzed with the anti-p75[ICD] antiserum. For unknown reasons, the NRH2-specific bands were not detected by the p75[ICD] antiserum in total cell extracts, as described previously by Murray et al. (2004).

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