Cellular/Molecular

Detection of $p75^{NTR}$ Trimers: Implications for Receptor Stoichiometry and Activation

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The p75 neurotrophin receptor ($p75^{NTR}$) is a multifunctional receptor that participates in many critical processes in the nervous system, ranging from apoptosis to synaptic plasticity and morphological events. It is a member of the tumor necrosis factor receptor (TNFR) superfamily, whose members undergo trimeric oligomerization. Interestingly, $p75^{NTR}$ interacts with dimeric ligands (i.e., proneurotrophins or mature neurotrophins), but several of the intracellular adaptors that mediate $p75^{NTR}$ signaling are trimeric (i.e., TNFR-associated factor 6 or TRAF6). Consequently, the active receptor signaling unit remains uncertain. To identify the functional receptor complex, we evaluated its oligomerization *in vitro* and in mice brain tissues using a combination of biochemical techniques. We found that the most abundant homotypic arrangement for $p75^{NTR}$ is a trimer and that monomers and trimers coexist at the cell surface. Interestingly, trimers are not required for ligand-independent or ligand-dependent $p75^{NTR}$ activation in a growth cone retraction functional assay. However, monomers are capable of inducing acute morphological effects in neurons. We propose that $p75^{NTR}$ activation is regulated by its oligomerization status and its levels of expression. These results indicate that the oligomeric state of $p75^{NTR}$ confers differential responses and offers an explanation for the diverse and contradictory actions of this receptor in the nervous system.

Key words: $p75^{NTR}$; proNGF; signaling unit; stoichiometry; TNF receptor; trimerization

Significance Statement

The p75 neurotrophin receptor ($p75^{NTR}$) regulates a wide range of cellular functions, including apoptosis, neuronal processes remodeling, and synaptic plasticity. The goal of our work was to inquire whether oligomers of the receptor are required for function. Here we report that $p75^{NTR}$ predominantly assembles as a trimer, similar to other tumor necrosis factor receptors. Interestingly, monomers and trimers coexist at the cell surface, but trimers are not required for $p75^{NTR}$ activation in a functional assay. However, monomers are capable of inducing acute morphological effects in neurons. Identification of the oligomerization state of $p75^{NTR}$ begins to provide insights to the mechanisms of signal initiation of this noncatalytic receptor, as well as to develop therapeutic interventions to diminish its activity.

Introduction

The p75 neurotrophin receptor ($p75^{NTR}$) regulates a wide range of cellular functions, including apoptosis during development and following injury, neuronal processes remodeling, and synaptic plasticity (Ibáñez and Simi, 2012). Although initially identified as a nerve growth factor (NGF) receptor (Johnson et al., 1986; Radeke et al., 1987), $p75^{NTR}$ can interact with the three additional neurotrophins (BDNF, neurotrophin-3, and neurotrophin-4) and with pro-neurotrophins (proNTs), including proNGF and proBDNF (Hempstead, 2014). Moreover, $p75^{NTR}$ can engage tropomyosin receptor kinase (Trk) to cooperate in NTs-induced neuronal survival and differentiation (Chao, 2003), sortilin or SorCS2 (sortilin-related VPS10 domain containing receptor 2) to trigger proNT-induced cell death and growth cone retraction (Nykjaer et al., 2004; Deinhardt et al., 2011), and the

Received Feb. 11, 2015; revised July 14, 2015; accepted July 22, 2015.

Author contributions: A.A., M.V.C., and B.L.H. designed research; A.A. performed research; P.A.B. and M.V.C. contributed unpublished reagents/analytic tools; A.A. and B.L.H. analyzed data; A.A., P.A.B., M.V.C., and B.L.H. wrote the paper.

This work was supported by National Institutes of Health Grants NS21072, AG025970, and HD23315 to M.V.C., and Grants NS030687, NS070173, and NS064114 to B.L.H., Canadian Institutes for Health Research Grant 325420 to P.A.B., the IBRO Return Home Fellowship to A.A., and the ISN-CAEN Return Home Award to A.A. We thank Dr. Brian Pierchala (University of Michigan) for the kind gift of the *p75*$^{NTRflx/flx}$ mice.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.0591-15.2015

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Nogo receptor to mediate axonal growth inhibitory effects of myelin (Wang et al., 2002). Other transmembrane proteins, such as death receptor 6 (Hu et al., 2013) and neurotrophin receptor homolog 2 (Kim and Hempstead, 2009; Vilar et al., 2014), have been reported to cooperate with $p75^{NTR}$ to mediate diverse activities. Despite the long list of functions described for $p75^{NTR}$, its mechanism of activation and signal initiation remains unclear.

$p75^{NTR}$ is the founding member of the tumor necrosis factor receptor (TNFR) superfamily, a group of receptors characterized by a tandem of cysteine-rich-domains (CRDs) in their extracellular domains (ECDs) and trimeric oligomerization (Locksley et al., 2001). $p75^{NTR}$ has four CRDs and contains a death domain in the intracellular domain (ICD) (Roux and Barker, 2002). The ICD does not have intrinsic enzymatic activity, and signaling is thought to occur through interaction of the ICD with cytoplasmic adaptor proteins, as has been found for many of TNFR superfamily members (Li et al., 2013), and potentially in the nucleus (Parkhurst et al., 2010). Most of the TNFRs bind to trimeric ligands, which favor the interaction of their ICD with adaptor proteins, such as TNFR-associated factors (TRAFs) (Li et al., 2013). Unlike other TNFRs, $p75^{NTR}$ binds neurotrophin and proneurotrophins ligands, which exist as dimers (Kolbeck et al., 1994). However, the $p75^{NTR}$ ICD can interact with cytoplasmic proteins, such as TRAF6, which have trimeric symmetry (Ye et al., 2002). How $p75^{NTR}$ engages dimeric ligands and trimeric adaptors and the active signaling stoichiometry are still outstanding questions. It has been proposed that $p75^{NTR}$ homodimerizes by covalent and noncovalent interactions and that this assembly is required for intracellular adaptor recruitment and activation (Vilar et al., 2009b). However, a variety of molecular masses have been described for $p75^{NTR}$ oligomers since its discovery (Puma et al., 1983; Grob et al., 1985; Langevin et al., 2002; Yaar et al., 2002; Sykes et al., 2012), leading to confusion in the field. Consequently, we decided to reevaluate $p75^{NTR}$ oligomerization with a combination of biochemical techniques. Here we report that $p75^{NTR}$ predominantly assembles as a trimer, similar to other TNFRs. Interestingly, monomers and trimers coexist at the cell surface, but trimers are not required for ligand-independent and ligand-dependent $p75^{NTR}$ activation as assessed by a growth cone retraction assay.

Materials and Methods

Animals and cell line. C57BL/6 mice, *$p75^{NTRflx/flx}$* in a C57BL/6 background (kindly provided by Dr. Brian Pierchala) (Bogenmann et al., 2011) and *$p75^{NTRflx/flx}$* EIIA-Cre crossed mice of either sex were maintained with a 12 h light–dark cycle and with free access to water and food. Animal care was in accordance with Weill Medical College of Cornell University Institutional Animal Care and Use Committee. HEK293 cells (ATCC) were grown in DMEM supplemented with 10% FBS (Invitrogen) and penicillin–streptomycin. HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions, and lysed 48 h after. PC12 rat pheochromocytoma cells (ATCC) were grown in RPMI 1640 (Invitrogen) supplemented with 10% horse serum, 5% FBS, and penicillin–streptomycin. PC12 cells were differentiated for 7 d in RPMI 1640 medium supplemented with 1% horse serum and 50 ng/ml NGF (Harlan).

Plasmids. The substitutions of the transmembrane cysteine for alanine in the human $p75^{NTR}$ sequence (C256A-$p75^{NTR}$) or in the HA-tagged rat $p75^{NTR}$ sequence (C257A-$p75^{NTR}$), and the glycine 265 to isoleucine (G265I-$p75^{NTR}$) in the human $p75^{NTR}$ sequence were introduced by PCR using the QuikChange II Site-Directed Mutagenesis Kit (Agilent) following the manufacturer's instructions. Constructs with deletions on the rat $p75^{NTR}$, including ΔICD (deleted intracellular domain, amino acids 275–425), ΔECD (deleted extracellular domain, amino acids 30–250), ΔCRDs (deleted cysteine-rich domains 1–4, amino acids 30–236) were previously generated (Zampieri et al., 2005). Constructs with deletions on the rat $p75^{NTR}$, including ΔCRD1 (deleted cysteine-rich domain 1, amino acids 32–66) and ΔCRD2–4 (deleted cysteine-rich domains 2–4, only expressing the CRD1 after the stalk region, amino acids 71–191) were generated using overlap PCR.

SDS-PAGE and Western blot. Dissected mice cortex or hippocampus, DRG neurons in culture, PC12 pheochromocytoma cells, or transfected HEK293 cells were lysed in 60 mM octyl β-D-glucopyranoside (Sigma), 1% Triton X-100 (Sigma), and 10 mM iodoacetamide (Sigma) in Tris-buffered saline, pH 7.4, supplemented with protease inhibitor mixture (Sigma). Protein concentration was determined by Bradford (Bio-Rad). Samples were prepared by mixing equal volumes of lysates (30–50 μg total protein) and 2× sample buffer (250 mM Tris, pH 6.8, 25% glycerol, 5% SDS, 0.05% bromophenol blue). For nonreducing conditions, lysates were incubated with the sample buffer at 20°C–25°C for 20 min before loading into the gel. For reducing conditions, lysates were incubated with the sample buffer containing β-mercaptoethanol (βME) or DTT for 5 min at 95°C. Proteins were resolved in large (17 × 17 cm) Tris/HCl polyacrylamide gels with SDS (SDS-PAGE). After separation, proteins were transferred to a 0.45 μm PVDF membrane (Millipore), and the membranes were blocked with 5% milk (Bio-Rad) in Tris-buffered saline, pH 7.4, with 0.2% Tween 20 (Bio-Rad). Membranes were incubated with the following combination of antibodies: (1) antibody specific for the ICD of $p75^{NTR}$ (1:10,000, 1 h at 20°C–25°C, Covance, catalog #PRB-602C), followed by anti-rabbit HRP secondary antibody (1:10,000, 1 h at 20°C–25°C, Calbiochem-Millipore); and (2) antibody specific for the ECD of $p75^{NTR}$ (1:1000, 16 h at 4°C, R&D Systems, catalog #AF367), followed by anti-goat HRP secondary antibody (1:7000, 1 h at 20°C–25°C, Calbiochem-Millipore). To detect endogenous $p75^{NTR}$ in cortical lysates, the Covance $p75^{NTR}$ ICD antibody was used at a 1:2000 concentration for 2 h at 20°C–25°C, followed by anti-rabbit HRP secondary antibody (1:5000, 1 h at 20°C–25°C, Calbiochem-Millipore). Proteins were detected by enhanced chemiluminescence (GE Healthcare) and digitalized for analysis.

Enzymatic deglycosylation. The deglycosylation kit was purchased from ProZyme; 5 μl of 5× Tris reaction buffer provided by the manufacturer was added to 20 μl of lysates containing 50 μg of protein (without any other detergent or denaturing agent). The mixture was incubated with 1 μl of either N-glycanase (to remove N-linked oligosaccharides), sialidase A (to remove sialic acid from glycoproteins), O-glycanase (to remove O-linked glycosylations), or the three together for 1 h at 37°C. Samples were subsequently incubated with sample buffer containing 3% βME for 5 min at 95°C before loading in the gel.

Electroelution. To electroelute $p75^{NTR}$ oligomers from the gel, the same sample (lysate + sample buffer) was loaded in two lanes separated by a prestrained molecular weight marker lane in a large-size gel. After running the electrophoresis, the gel was divided in two by cutting in the middle of the molecular weight marker and leaving one of the sample lanes in each half. One half of the gel was kept in water, and the other half was rapidly transferred, and the Western blot was blocked and developed using antibodies specific for the $p75^{NTR}$ ICD. Subsequently, the gel that was kept in water was aligned on top of the developed film by overlapping the prestained molecular weight marker, and the region of the gel overlapping with the developed oligomers was excised with a clean scalpel. The gel slice was inserted into a dialysis Eppendorf tube (D-Tube Dialyzer Mini, MWCO 12–14 kDa, Novagen) together with 80 μl of running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The tube was positioned fully immersed into running buffer in a horizontal electrophoresis chamber perpendicular to the electric field to permit electric current to pass through the tube. Elution of the proteins from the gel took place after application of 120 V for 3 h. Afterward, the current was inverted for 2 min to release the protein from the membrane. The protein was recovered from the tube with a pipette and combined with sample buffer with reducing agents and heated 5′ at 95°C before loading it in a gel.

Surface biotinylation and surface protein detection. HEK293 cells 48 h after transfection were washed with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 8.0 (PBS^{+}). Sulfo-NHS-LC-Biotin (Thermo Scientific)

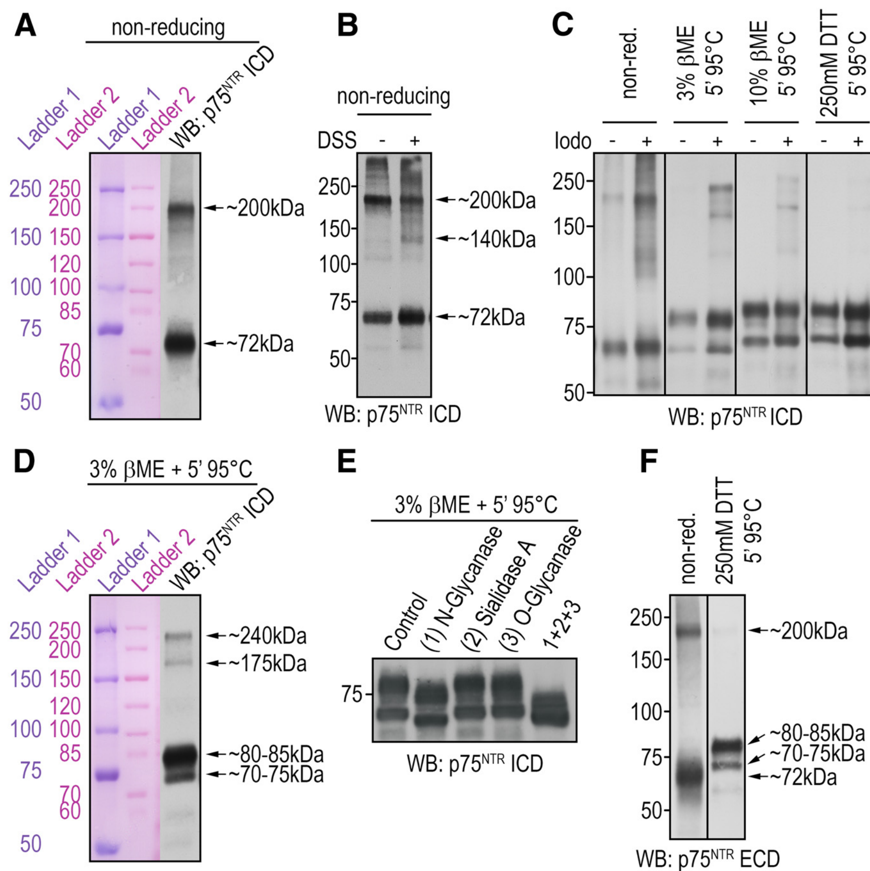

Figure 1. The most abundant p75^{NTR} oligomer is a trimer. Expression of p75^{NTR} in transfected HEK293 cells detected by nonreducing (non-red.) or reducing SDS-PAGE using antibodies specific for the ICD (***A***–***D***) or ECD (***F***) of p75^{NTR}. Iodoacetamide was used in the lysis buffer to inhibit disulfide bond formation during cell lysis, and its inclusion enhanced p75^{NTR} detection. ***A***, In nonreducing conditions, p75^{NTR} migrated as a ~72 kDa monomer and a ~200 kDa oligomer, suggesting trimerization of the receptor. ***B***, In nonreducing conditions, chemical crosslinking allowed the detection of p75^{NTR} species consistent with dimers resolved at ~140 kDa (~2 times the molecular mass of the ~72 kDa monomer in this condition). ***C***, Reducing conditions shifted the molecular weight of the monomer to ~80–85 kDa, and a second band of ~70–75 kDa was detected. The 250 mM DTT and 5 min at 95°C reduced the oligomers to monomers. ***D***, After the addition of βME and 5 min at 95°C, p75^{NTR} oligomers were detected at ~240 kDa and ~175 kDa, strongly suggesting a trimer and dimer, respectively (~2 or 3 times the molecular mass of the ~80–85 kDa monomer in this condition). ***E***, Identification of the ~80–85 kDa and the ~70–75 kDa p75^{NTR}-positive bands found in reducing conditions. p75^{NTR} has one asparagine-linked glycosylation in the first cysteine-rich domain and several O-linked glycosylations in the ECD juxtamembrane region. Therefore, we tested whether the bands correspond to differentially glycosylated isoforms. Lysates from p75^{NTR}-transfected HEK293 cells were deglycosylated with N-glycanase, sialidase A, O-glycanase, or with the three enzymes together. N-glycanase reduced the molecular mass of both bands, suggesting that both are N-linked glycosylated. Neither sialidase A nor O-glycanase affected p75^{NTR} molecular mass. However, sequential deglycosylation using the three enzymes together shifted the molecular weights as follows: (1) the higher band shifted more than with N-glycanase alone and started to collapse on top of the lower band, confirming that this isoform is N- and O-linked glycosylated; and (2) the lower band was resolved at the same molecular mass as after the N-glycanase, suggesting that this isoform is only N-linked glycosylated but is missing O-linked oligosaccharides. Overall, the molecular mass shifts were partial after deglycosylation. This may be due to iodoacetamide affecting the enzymatic activity and/or on the partial reducing efficacy of βME in the experimental conditions used, which may only incompletely unfold the ECD and consequently partially expose the oligosaccharides for enzymatic removal. ***F***, Antibodies specific for the p75^{NTR} ECD displayed the same pattern of bands as the ICD antibody in nonreducing and DTT reducing conditions.

was freshly dissolved at a 0.5 mg/ml concentration in PBS$^+$ and added to the cells for 20 min at 4°C. The reaction was quenched with 30 mM Tris for 10 min; the cells were washed with PBS$^+$ and lysed as described above. The lysates (500 μg total protein) were precleared with protein A/G agarose (Thermo Scientific), immunoprecipitated using an antibody against the ECD of p75^{NTR} (3 μg, 2 h at 4°C, R&D Systems, catalog #AF367), followed by protein A/G agarose resin (Sigma) incubation. Beads were incubated with sample buffer (without reducing agents) at 20°C–25°C for 20 min before spinning and loading the supernatant into the gel, followed by SDS-PAGE. The membranes were incubated with streptavidin-HRP (1:1000, 1 h, 20°C–25°C, Jackson ImmunoResearch Laboratories) to detect the biotinylated proteins.

Induction of seizures. Twelve-week-old mice were treated with pilocarpine to induce seizures as described previously (VonDran et al., 2014). Briefly, mice were pretreated with 5 mg/kg methylscopolamine and 50 mg/kg phenytoin 30 min before the pilocarpine injection (300 mg/kg). After 2 h of severe seizures with prolonged loss of postural control or prolonged tonus, mice were treated with diazepam (10 mg/kg). Animals were killed 3 d after seizure for biochemical analysis as described in SDS-PAGE and Western blot.

DRG cultures. DRG neurons were isolated from E14 C57BL/6 mouse embryos. Neurons were dissociated with trypsin (Invitrogen), plated on poly-D-lysine (Sigma)/laminin (Sigma)/collagen (BD Biosciences)-coated dishes, and grown first in MEM containing D-glucose, 10% FBS, glutamax, 5-fluoro-2′-deoxyuridine, and 50 ng/ml NGF (Harlan), and then followed by neurobasal medium (Invitrogen) containing B27 (Invitrogen), glutamax (Invitrogen), penicillin–streptomycin, 5-fluoro-2′-deoxyuridine, and 50 ng/ml NGF (Harlan). Cultures were harvested at 8 d *in vitro* for biochemical analysis as described in SDS-PAGE and Western blot.

Crosslinking. HEK293 cells were washed 48 h after transfection with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 8 .0 (PBS$^+$), at 20°C–25°C. The crosslinker disuccinimidyl suberate (Thermo Scientific) was freshly dissolved in DMSO immediately before use and added to the cells at a 1 mM final concentration in PBS$^+$ for 20 min at 20°C–25°C. The reaction was quenched with 30 mM Tris for 10 min; the cells were washed with PBS$^+$ and lysed as described in the SDS-PAGE protocol.

Expression and purification of proNGF. Mouse proNGF was produced in insect cells using baculovirus for high-level protein expression following the Bac-to-Bac TOPO expression system (Invitrogen) instructions. Briefly, uncleavable proNGF sequence (^{120}KR^{121} to ^{120}AA^{121}) was cloned into the pFastBac plasmid to generate the recombinant bacmid. The bacmid was transfected in Sf9 cells using Cellfection II reagent to produce and amplify the baculovirus. Sf9 cells were maintained in Sf-900 II SFM media (Invitrogen). HighFive cells (Invitrogen) (maintained in Express Five SFM media; Invitrogen) were infected with the baculovirus to produce the recombinant proNGF, which was collected from the conditioned media 72 h after infection. The media was added with protease inhibitors (1 mM PMSF, 1 mM aprotinin, 0.5 mM leupeptin), centrifuged to remove cell debris, and frozen at −80°C until purification. ProNGF was purified using a nickel resin column (Ni-NTA, Invitrogen). The resulting protein was dialyzed in PBS, pH 7.4, and the sample purity was assessed by SDS-PAGE using Coomassie Blue and silver staining methods. The protein concentration was estimated by SDS-PAGE compared with known amounts of recombinant NGF (Harlan), and confirmed by the Bradford method. Finally, the recombinant protein was tested in a superior cervical ganglion neuron death assay for bioactivity as described previously (Nykjaer et al., 2004; Teng et al., 2005).

Growth cone retraction assay. Growth cone retraction assay was performed as described previously (Deinhardt et al., 2011; Anastasia et al., 2013). Primary hippocampal neurons were isolated from E17 C57BL/6 mouse embryos. Neurons were dissociated with 0.05% trypsin (Invitrogen) at 37°C for 15 min. For some experiments, hippocampal neurons were transfected with p75^{NTR}-HA or C257A-p75^{NTR}-HA constructs using Amaxa Nucleofector kit (Lonza). Cells plated on poly-D-lysine (Sigma)-coated dishes were grown in Neurobasal medium (Invitrogen) containing B27 (Invitrogen), glutamax (Invitrogen), and penicillin–streptomycin. Cultured neurons were washed with fresh culture media at day *in vitro* 1 (DIV1) and DIV3. Four hours after the last wash, cultures were washed again and immediately after they were treated with 20 ng/ml of proNGF for 30 min before fixation. Neurons were fixed with dry-ice-cold methanol for 10 min and washed with PBS. Coverslips were blocked with 10% normal donkey serum and 2% BSA in Tris-buffered saline for 30 min; incubated with mouse anti-actin (1:10,000, Sigma, clone AC-74) and goat anti-p75^{NTR} (1:750, R&D Systems) or rabbit anti-HA (1:750, Sigma). Primary antibodies were incubated for 30 min at 20°C–25°C, washed, followed by secondary antibodies (anti-mouse Alexa-488, anti-goat Alexa-568, anti-rabbit Alexa-568; 1:1000, Invitrogen) for 30 min at 20°C–25°C, Hoechst (1:10,000), and mounted with ProLong Gold (Invitrogen). Cells and growth cones were imaged with an epifluorescence Zeiss microscope and camera (Axio Obzerver.Z1 microscope equipped with an AxioCam MRm camera, Zeiss). Growth cone area (in μm^2) was analyzed using calibrated Fiji software (Schindelin et al., 2012).

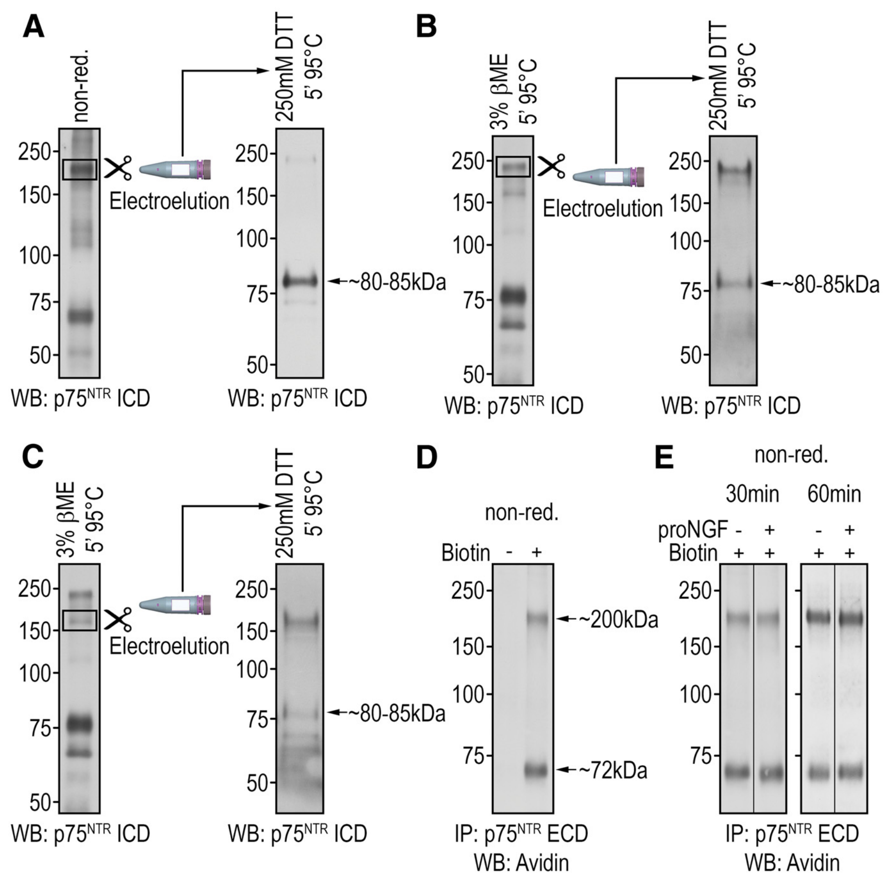

Figure 2. p75^{NTR} oligomers are formed by full-length monomers, and p75^{NTR} monomers and trimers coexist at the plasma membrane. ***A***, Electroelution of the ~200 kDa complex found in nonreducing conditions, followed by DTT reduction of the resolubilized protein, showed that the oligomers are formed by monomers of full-length p75^{NTR} (~80–85 kDa). ***B***, Electroelution of the ~240 kDa p75^{NTR} oligomer observed in the 3% βME + 5 min at 95°C condition. The resolubilized protein was further reduced with DTT and showed that the oligomer is formed by monomers of full-length p75^{NTR} (~80–85 kDa). ***C***, Electroelution of the ~175 kDa form observed in the 3% βME + 5 min at 95°C. The resolubilized protein was further reduced with DTT, and this showed that the oligomer is formed by monomers of full-length p75^{NTR} (~80–85 kDa). ***D***, p75^{NTR} oligomerization at the plasma membrane was studied in transfected and surface-biotinylated HEK293 cells (Biotin+). Monomers and trimers coexist at the cell surface. Biotin−, Transfected but not biotinylated cells. ***E***, Administration of 50 ng/ml of proNGF for 30 or 60 min did not affect the p75^{NTR} monomer/trimer ratio at the cell surface in transfected HEK293 cells.

Results

The most abundant p75^{NTR} oligomer is a trimer

The p75 neurotrophin receptor (p75^{NTR}) is a Type I transmembrane receptor that has a single asparagine-linked polysaccharide and several O-linked oligosaccharides in the ECD (Baldwin and Shooter, 1995). Its ECD has four CRDs, each containing three intrachain disulfide bridges, and the transmembrane domain encodes a cysteine that is capable of linking two receptors by a disulfide bridge (Roux and Barker, 2002; Vilar et al., 2009b). A variety of molecular masses have been described for p75^{NTR} oligomers giving a diversity of models and stoichiometries. To address the variability of p75^{NTR} oligomers, we used large (17 × 17 cm) polyacrylamide gels with SDS (SDS-PAGE) under nonreducing conditions to enhance protein resolution. These parameters permitted the separation of denatured p75^{NTR}, preserving disulfide bonds in the ECD and the transmembrane domain. Iodoacetamide was used in the lysis buffer to inhibit disulfide bond formation during cell rupture and protein solubilization. The large gel size permitted greater separation of the bands and facilitated assigning molecular weights with prestained and unstained protein standard markers. Under these conditions, monomeric p75^{NTR} was detected at ~72 kDa (Fig. 1*A*). Interestingly, the most prominent oligomer migrated at ~200 kDa (Fig. 1*A*), which is ~3 times the mass of a monomer, suggesting that p75^{NTR} may exist as a trimer. The p75^{NTR} species consistent with the molecular mass of dimers (~140 kDa) were occasionally observed in nonreducing gels. However, these complexes were consistently detected only after chemical crosslinking, suggesting that p75^{NTR} dimers are less abundant and less stable than the trimers (Fig. 1*B*).

In lysates prepared without iodoacetamide, exposure to βME or DTT reduced the high molecular weight oligomers to monomers (Fig. 1*C*). The inclusion of iodoacetamide enhanced p75^{NTR} detection, likely by preventing protein degradation due to its cysteine-peptidases inhibitor activity (Fig. 1*C*). Moreover, iodoacetamide partially blocked the βME-reducing effect by alkylation of its thiol groups (Fig. 1*C*). Therefore, in lysates prepared with iodoacetamide, high concentrations of DTT were required to reduce the oligomers (Fig. 1*C*). Interestingly, a sequential increase of the reducing conditions increased the molecular weight of the monomer and oligomers (Fig. 1*C*). For instance, in lysates exposed to 3% βME and 5 min at 95°C, monomers were resolved at ~80–85 kDa, and two different p75^{NTR} oligomeric bands migrated at ~175 kDa and ~240 kDa (Fig. 1*D*). The ~240 kDa form represents ~3 times the molecular mass of the monomeric receptor in this condition, consistent with

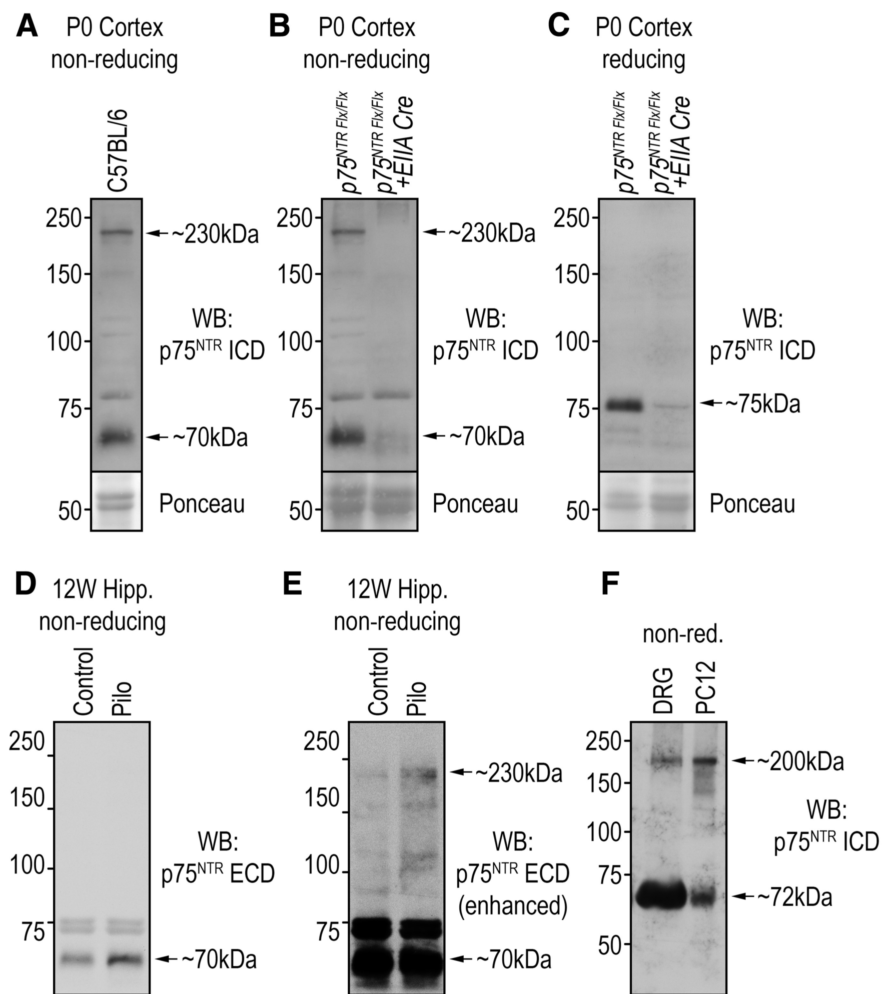

Figure 3. p75 NTR monomers and trimers are present in the mouse brain, DRGs in culture, and in PC12 cells. ***A–C***, Endogenous p75 NTR oligomerization was studied in postnatal day 0 (P0) mouse cortical lysates. ***A***, Nonreducing SDS-PAGE of C57BL/6 cortical lysates showed that p75 NTR monomers are resolved at ~70 kDa, whereas oligomers appeared at ~230 kDa, strongly supporting trimerization of the receptor. ***B***, ***C***, *p75$^{NTRflx/flx}$* conditional mice were used to study the specificity of the bands. ***B***, Recombination of the conditional alleles with Cre under the control of EIIa promoter results in loss of ~70 kDa and ~230 kDa p75 NTR bands observed in nonreducing conditions. ***C***, βME reduced the high molecular mass oligomers and shifted the molecular weight of the monomers to ~75 kDa. Ponceau red staining was used as the loading control. ***D***, ***E***, Endogenous p75 NTR oligomerization in the adult brain was studied in 12-week-old (12W) mouse hippocampal lysates, after loading 150 μg/lane in the SDS-PAGE, and using pilocarpine-induced seizures (Pilo) to increase p75 NTR endogenous levels. ***D***, Representative blot detecting p75 NTR by regular ECL. ***E***, The same blot shown in ***D*** developed using enhanced ECL signal was necessary to detect the ~230 kDa oligomers. ***F***, p75 NTR oligomerization in DRG neurons in culture and in PC12 pheochromocytoma cells.

p75 NTR trimerization. The ~175 kDa band observed in these reducing conditions has approximately twice the molecular mass of monomeric p75 NTR, suggesting that trimers may be reduced to intermediate dimers and monomers. Under βME- or DTT-reducing conditions, a second p75 NTR band of ~70–75 kDa was observed (Fig. 1*C*,*D*). Enzymatic deglycosylation of the lysates demonstrated that the ~70–75 kDa species represented an underglycosylated isoform of p75 NTR (Fig. 1*E*).

We compared the pattern of p75 NTR-positive bands detected using antibodies against the ICD (Fig. 1*A–D*) and ECD (Fig. 1*F*) of p75 NTR. The similarity of molecular weights detected with these antibodies (~200 kDa and ~72 kDa in nonreducing conditions; ~80–85 kDa and ~70–75 kDa in reducing conditions) strongly suggests that oligomers are formed by full-length monomers. To confirm this, the ~200 kDa oligomer observed in nonreducing conditions was electroeluted from the gel, and the resolubilized protein was reduced to full-length monomers with DTT (Fig. 2*A*). Similarly, electroelution of the ~240 kDa (Fig. 2*B*) and ~175 kDa (Fig. 2*C*) oligomers resolved in the 3% βME-reducing condition, confirming that these complexes are formed by monomers of full-length p75 NTR (~80–85 kDa).

We assessed p75 NTR oligomerization at the plasma membrane using cell surface biotinylation. Transfected HEK293 cell lysates were immunoprecitated with p75 NTR antibodies followed by separation of the proteins in nonreducing large gels, and biotinylated proteins were detected using streptavidin-HRP. We found that p75 NTR monomers and trimers were present at the plasma membrane in the absence of exogenous ligands (Fig. 2*D*). Moreover, the administration of 50 ng/ml of the dimeric ligand proNGF for 30 min and 60 min (Fig. 2*E*) did not affect the p75 NTR monomer/trimer ratio at the cell surface, and dimers were still undetectable at the plasma membrane.

To study the oligomerization of endogenous p75 NTR, we examined early postnatal mouse cortices using nonreducing SDS-PAGE. In C57BL/6 cortex lysates, the p75 NTR monomer migrated at ~70 kDa, and the most prominent oligomer was detected at ~230 kDa, suggesting a trimer isoform (Fig. 3*A*). To confirm that these bands represent endogenous p75 NTR species, cortical lysates were prepared from *p75$^{NTRflx/flx}$* EIIA-Cre mice (kindly provided by Dr. Brian Pierchala) (Bogenmann et al., 2011). The EIIa Cre line expresses the cre recombinase in many tissues in the early mouse embryo (Lakso et al., 1996). The ~70 kDa and ~230 kDa bands were absent in cortical lysates prepared from *p75$^{NTRflx/flx}$* EIIA-Cre animals (Fig. 3*B*). Treatment of cortical lysates with βME reduced the high molecular mass oligomers and shifted the molecular weight of the monomers to ~75 kDa (Fig. 3*C*). These results indicated that p75 NTR coexists as monomers and trimers in the perinatal mouse brain. Furthermore, the oligomerization of endogenous p75 NTR in the adult brain was studied in 12-week-old mice hippocampal lysates using nonreducing SDS-PAGE. Because p75 NTR expression is very low in the healthy adult brain, we loaded 150 μg/lane of total protein in the SDS-PAGE, and we also examined mouse hippocampal lysates after pilocarpine-induced seizures, a condition that results in an increase in the expression of the endogenous receptor (Roux et al., 1999). p75 NTR monomers were detected at ~70 kDa (Fig. 3*D*,*E*), whereas the most abundant oligomer resolved at ~230 kDa was only detected after significant enhancement of the ECL signal (Fig. 3*E*). Endogenous p75 NTR oligomers consistent with the molecular mass of trimers were also detected (at varying monomer/trimer ratios) in DRG primary neuronal cultures and in rat pheochromocytoma PC12 cells (Fig. 3*F*).

The cysteine 256 in the transmembrane domain and the extracellular domain are required for trimerization

To map the p75^{NTR} domains involved in trimerization, we used a series of point mutations of the receptor. These mutants were expressed in HEK293 cells, followed by separation of the proteins in large nonreducing gels. As the cysteine in the transmembrane domain of p75^{NTR} has been shown previously to be involved in oligomerization (Vilar et al., 2009b), we generated a point mutation that replaced the cysteine with alanine (C256A-p75^{NTR}) in the human sequence. Interestingly, this substitution completely prevented trimerization (Fig. 4*A*,*B*), but chemical crosslinking of the C256A-p75^{NTR}-expressing cells revealed that this mutant was able to form ∼140 kDa dimers (approximately double the mass of the ∼72 kDa monomer) (Fig. 4*A*,*B*). These C256A-p75^{NTR} dimers were only observed after covalent crosslinking, suggesting that these complexes are less stable than trimers of wild-type p75^{NTR}. It has been suggested that the self-association motif ^{261}AVVVG^{265} in the transmembrane domain contributes to p75^{NTR} oligomerization (Vilar et al., 2009b; Sykes et al., 2012). However, p75^{NTR} with a G265I mutation in the self-association sequence did not alter the receptor trimerization (Fig. 4*C*).

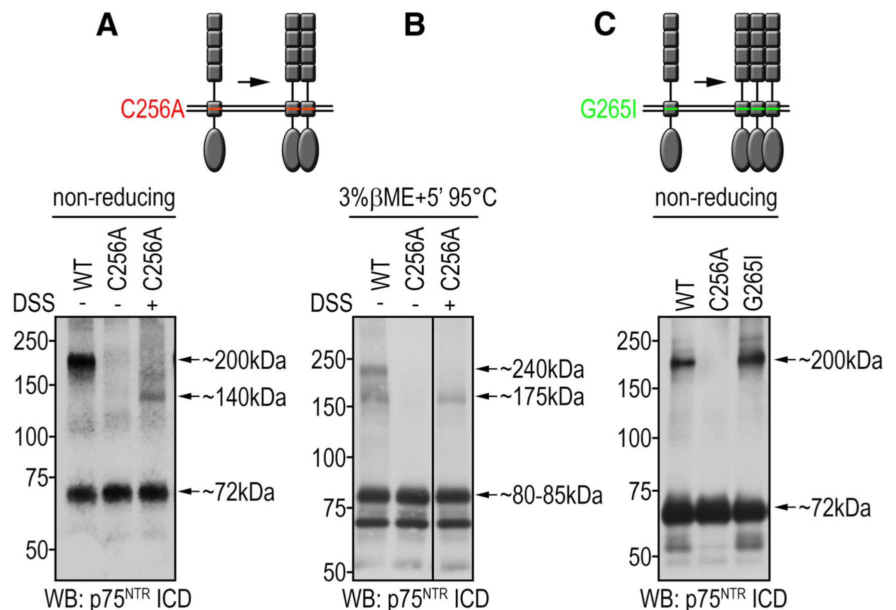

Figure 4. The cysteine 256 in the transmembrane domain is required for trimerization. Representative images of nonreducing and reducing (3% βME and 5 min at 95°C) SDS-PAGE displaying HEK293 cells transfected with p75^{NTR} point mutations. A graphic representation of p75^{NTR} mutations and the oligomers found for each one are displayed on top of each image. Wild-type p75^{NTR} is shown as "WT" (in nonreducing conditions, monomers run at ∼72 kDa and trimers at ∼200 kDa; in reducing conditions, monomers run at ∼80–85 kDa, dimers at ∼175 kDa, and trimers at ∼240 kDa). ***A***, ***B***, Mutation of the cysteine 256 in the transmembrane domain of human p75^{NTR} to alanine (C256A) abrogated trimerization. The third lane of both images displays C256A-p75^{NTR}-transfected HEK293 cells followed by chemical crosslinking with disuccinimidyl suberate (DSS). ***A***, In nonreducing conditions, chemical crosslinking allowed the detection of C256A-p75^{NTR} dimers at ∼140 kDa (∼2 times the molecular mass of the ∼72 kDa monomer in this condition). ***B***, In reducing conditions, the chemical crosslinking revealed C256A-p75^{NTR} dimers at ∼175 kDa (∼2 times the molecular mass of the ∼80–85 kDa monomer in this condition). ***C***, Mutation of the glycine 265 in the transmembrane domain to isoleucine (G265I) did not affect trimerization of p75^{NTR}.

To further dissect the domains of p75^{NTR} required for oligomerization, we expressed a series of deletions of p75^{NTR} in HEK293 cells and assessed oligomerization in large nonreducing gels. Deletion of p75^{NTR} ICD did not affect the trimerization as we detected ∼150 kDa species of ∼50 kDa monomers (Fig. 5*A*). In contrast, constructs lacking the entire extracellular domain (ΔECD) or the four cysteine-rich domains (ΔCRDs) showed monomeric species running at ∼25 kDa and ∼28 kDa but did not generate trimers of predicted ∼75 kDa and ∼84 kDa (Fig. 5*B*). Interestingly, the ΔECD and ΔCRDs p75^{NTR} mutants displayed ∼50 kDa and ∼55 kDa species, indicating that they dimerize (Fig. 5*B*). These species were reduced to monomers with the addition of βME (Fig. 5*C*), suggesting that disulfide bonds, most likely through the C256, are involved in their assembly.

The N-terminal cysteine-rich domain 1 (CRD1) of many TNFRs, such as FasR, TNFR1, and TNFR2, has been shown to be essential for the formation of homotypic, ligand-independent receptor trimers (Chan et al., 2000; Chan, 2007). Consequently, the CRD1 of these receptors has been called the preligand assembly domain (PLAD). To determine whether the CRD1 serves a similar function in p75^{NTR}, we generated a CRD1-deleted p75^{NTR} (ΔCRD1) and expressed it in HEK293 cells. The ΔCRD1 mutant (with monomers resolved at ∼60 kDa) exhibited a diminution in the proportion of trimers and an enrichment in the amount of dimers compared with wild-type p75^{NTR}, but the effect was partial (Fig. 5*D*,*E*). We also produced p75^{NTR} lacking CRD2 to CRD4 and thus expressing the CRD1 in the ECD (with monomers resolved at ∼40 kDa). This mutant was capable of forming dimers (∼80 kDa) but not trimers (with predicted molecular weight of ∼120 kDa) (Fig. 5*F*). These results suggest that the CRD1 contributes to some degree to promote multimerization as a PLAD domain but is not sufficient to promote trimerization, and that all four CRDs contribute to p75^{NTR} trimerization.

To summarize, the data collectively suggest that (1) cysteine 256 in p75^{NTR} transmembrane domain is sufficient to promote dimerization through a disulfide bond as it has been previously demonstrated (Vilar et al., 2009b); (2) the four CRDs are also sufficient to induce dimerization of the receptor; and (3) interestingly, both the C256 and the CRDs are required to produce stable trimers.

p75^{NTR} trimerization is not required for proNGF-induced growth cone retraction

A robust functional effect of proNGF is to induce p75^{NTR}-dependent hippocampal neuron growth cone retraction within minutes (Deinhardt et al., 2011). We reproduced these results in neurons expressing endogenous p75^{NTR} (Fig. 6*A*). We used this growth cone retraction assay to test whether trimerization is required for this effect, by transfecting hippocampal neurons with wild-type p75^{NTR} or C257A-p75^{NTR} (substitution of the transmembrane cysteine for alanine in the rat sequence). The C257A-p75^{NTR} construct is defective in trimer formation (Fig. 4*A*,*B*). Interestingly, the mere expression of wild-type p75^{NTR} resulted in ligand-independent growth cone retraction in the absence of exogenous proNGF (Fig. 6*B*). However, the expression of the C257A-p75^{NTR} mutant in hippocampal neurons produced significantly more growth cone retraction compared with the wild-type p75^{NTR}, in the absence of exogenous ligands (Fig. 6*B*). This result suggests that trimers may be a less active form in promoting

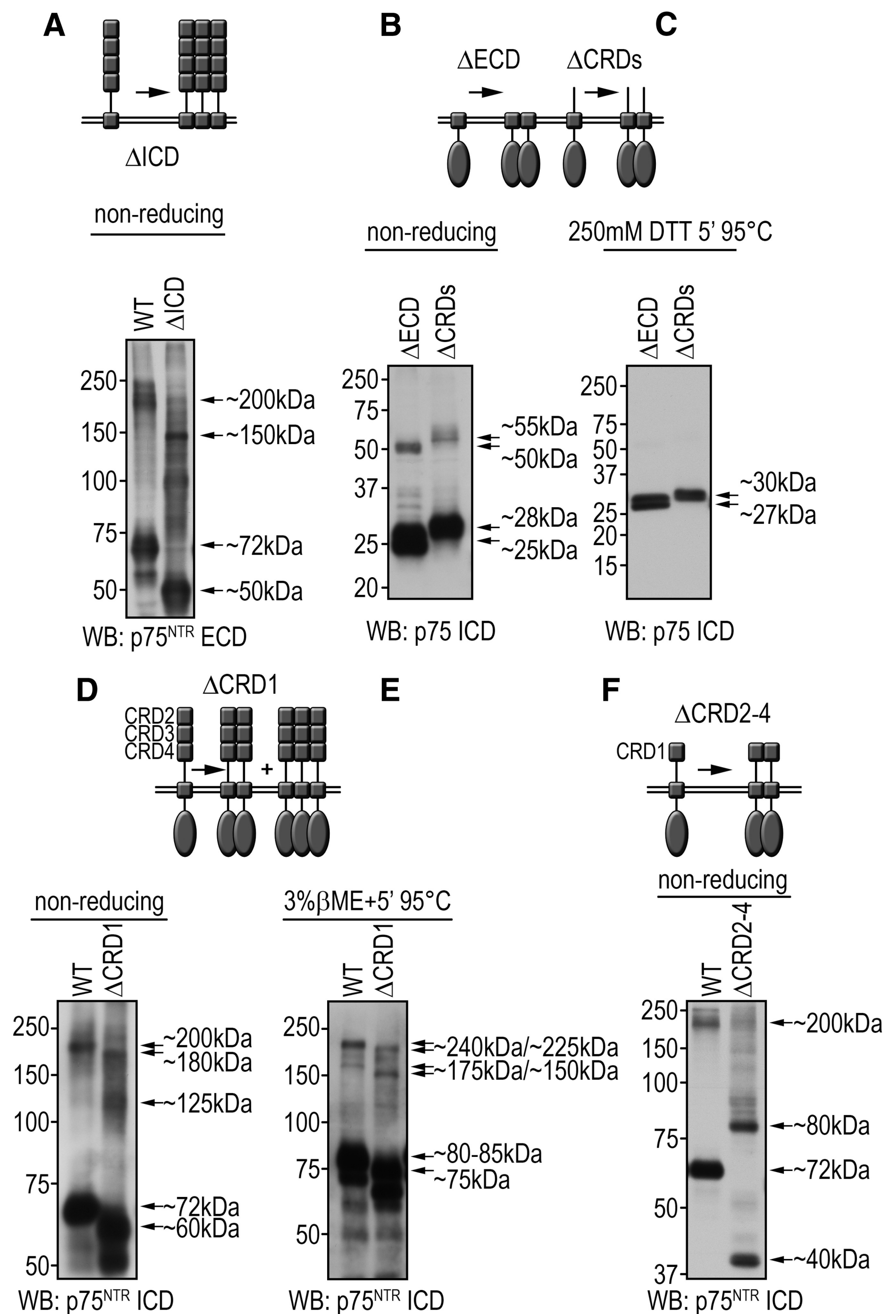

Figure 5. The cysteine-rich domains are required for trimerization. Representative images of nonreducing and reducing (3% βME and 5 min at 95°C) SDS-PAGE displaying HEK293 cells transfected with p75 NTR deletion mutants. A graphic representation of p75 NTR deletions and the oligomers found for each one are displayed on top of each image. Wild-type p75 NTR is shown as "WT" (in nonreducing conditions, monomers run at ~72 kDa and trimers at ~200 kDa; in reducing conditions, monomers run at ~80–85 kDa, dimers at ~175 kDa, and trimers at ~240 kDa). ***A***, Deletion of the intracellular domain (ΔICD) did not affect trimerization (ΔICD monomer: ~50 kDa; ΔICD trimer: ~150 kDa). ***B***, ***C***, Deletion of the extracellular domain (ΔECD) or the four cysteine-rich domains (ΔCRDs) prevented trimerization, but dimers were detected. ***B***, In nonreducing conditions, ΔECD monomers were detected at ~25 kDa, ΔECD dimers at ~50 kDa, ΔCRDs monomers at ~28 kDa, and ΔCRDs dimers at ~55 kDa. ***C***, In reducing conditions, ΔECD monomers were detected at ~27 kDa and ΔCRDs monomers at ~30 kDa. ***D***, ***E***, Deletion of the cysteine-rich domain 1 (ΔCRD1) partially prevented trimerization and increased the proportion of dimers. ***D***, In nonreducing conditions, ΔCRD1 monomers were detected at ~60 kDa, dimers at ~125 kDa, and trimers at ~180 kDa. ***E***, In reducing conditions, ΔCRD1 monomers were detected at ~75 kDa, dimers at ~150 kDa, and trimers at ~225 kDa. ***F***, Deletion of the cysteine-rich domains 2–4 (ΔCRD2–4) prevented trimerization, but dimers were still detected (ΔCRD2–4 monomer: ~40 kDa, ΔCRD2–4 dimer: ~80 kDa).

ligand-independent p75 NTR activation. Moreover, proNGF administration induced additional growth retraction in both wild-type p75 NTR and C257A-p75 NTR-transfected hippocampal neurons (Fig. 6*B*,*C*). These results suggest that trimerization is not required for p75 NTR activation in growth cone retraction and that monomers and/or dimers are sufficient for acute morphological effects. Interestingly, the expression of ΔCRD1 in hippocampal cultures, which showed significantly augmented dimerization (Fig. 5*D*,*E*), resulted in similar ligand-independent growth cone retraction compared with the wild-type p75 NTR (Fig. 6*B*). The ΔCRD1 mutant did not bind to NGF (data not shown), consistent with published structural data describing that one of the sites of contact surface between NGF or proNGF and p75 NTR is in the CRD1-CRD2 junction of the receptor (He and Garcia, 2004; Feng et al., 2010). Therefore, proNGF administration to ΔCRD1-transfected hippocampal neurons did not result in additional growth cone retraction (Fig. 6*B*).

Discussion

The goal of this work is to clarify the oligomerization state of p75 NTR and to inquire whether oligomers of the receptor are required for function. As p75 NTR is a member of the TNFR superfamily, whose members generally assemble as trimers (Chan et al., 2000; Chan, 2007), we sought to determine whether this was also the case for p75 NTR (alternatively known as TNFR16). Oligomerization of p75 NTR has been previously studied, and several different results have been found. For instance, chemical cross-linking experiments of p75 NTR and iodinated NGF (^{125}I-NGF) have documented a 200, 210, 220, or 225 kDa complex on human melanoma A875 cells, rat pheochromocytoma PC12 cells, Schwann cells, and heterologous cells expressing p75 NTR (Grob et al., 1983; Ross et al., 1984; DiStefano and Johnson, 1988; Jing et al., 1992; Mahadeo et al., 1994; Murray et al., 2004). Characterization of p75 NTR in the absence of ligands revealed variations in the high molecular weights species from 200 to 240 kDa (Puma et al., 1983; Langevin et al., 2002), to 150–200 kDa (Grob et al., 1985), and 220–230 kDa (Yaar et al., 2002). These oligomers have been variously labeled as dimers or trimers. More recently, it has been reported that p75 NTR forms disulfide-linked dimers in the absence of ligands (Vilar et al., 2009b). However, in these studies, the monomers appear to have lower, and the dimers higher, molecular masses compared with their predicted values of 75 kDa (monomer) and 150 kDa (dimer). Given these controversies, we evaluated p75 NTR high molecular weight stoichiometry by using large (17 × 17 cm) polyacrylamide gels, which permits extensive separation of the bands and greater

accuracy in the measurement of molecular masses. We used nonreducing SDS-PAGE, chemical crosslinking, and a variety of biochemical approaches to demonstrate that main oligomer of p75^{NTR} was resolved at ~200 kDa, which is ~3 times the mass of a monomer (~72 kDa), strongly suggesting that p75^{NTR} assembles as trimers in the absence of ligand. Further confirmation came from the detection of similar molecular species of endogenous p75^{NTR} in the murine brain, DRG primary neuronal cultures, and PC12 pheochromocytoma cells. This ~200 kDa assembly is slightly lower than the predicted molecular weight of three p75^{NTR} monomers (~216 kDa), possibly due to a more compact structure, but it is significantly larger than the expected mass of two monomers (~144 kDa). Therefore, we propose that p75^{NTR} is not an exception to the TNFR superfamily, but that it also self-associates with trimeric symmetry. Our experiments do not completely exclude the possibility that the ~200 kDa band might represent a heterocomplex with another endogenous protein. However, it is improbable that an endogenously expressed protein in HEK293 is as abundant as the expressed p75^{NTR} and that it happens to have a similar molecular mass of ~75 kDa.

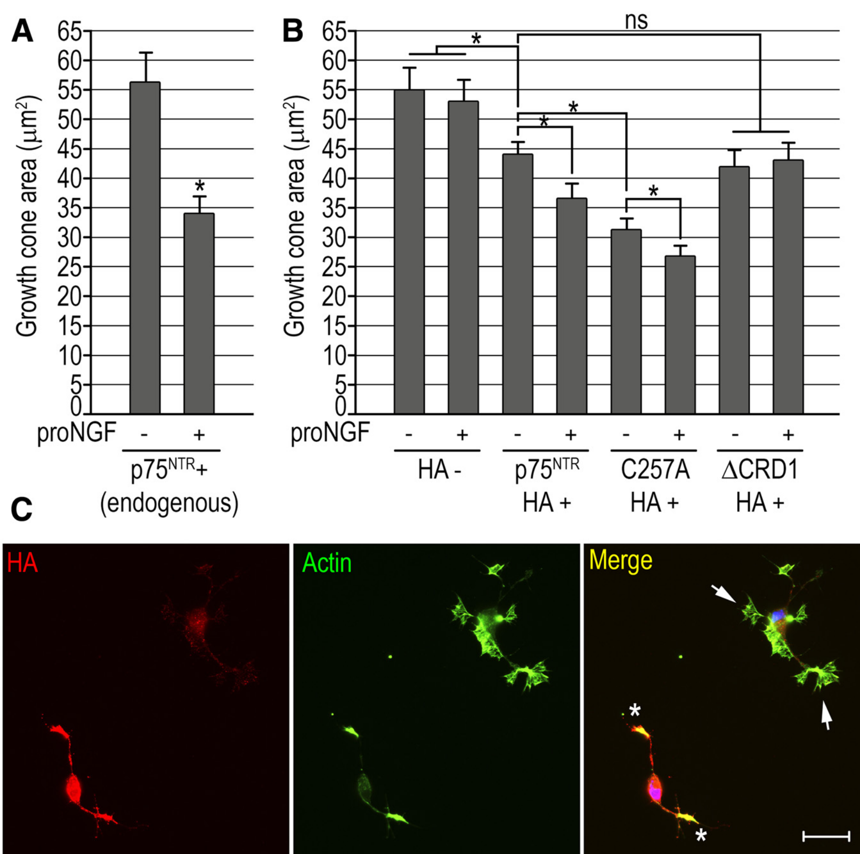

Figure 6. p75^{NTR} trimerization is not required for proNGF-induced growth cone retraction. ***A***, E17-dissociated hippocampal neurons were treated with proNGF (20 ng/ml) for 30 min, fixed, and stained for p75^{NTR} and actin. ProNGF induced growth cone retraction in endogenously p75^{NTR}-expressing hippocampal neurons. Quantification of growth cone area (μm^2) was performed to determine growth cone retraction. Error bars indicate mean ± SEM. ***B***, E17-dissociated hippocampal neurons were transfected with rat wild-type p75^{NTR}-HA, C257A-HA, or ΔCRD1-HA constructs. At DIV3, cultures were treated with proNGF for 30 min, fixed, and stained for HA and actin. Quantification of growth cone area (μm^2) to assess growth cone retraction in transfected p75^{NTR}-HA, C257A-HA, or ΔCRD1-HA-positive neurons, and HA-negative (HA−) neurons of the same cultures. Error bars indicate mean ± SEM. Statistical comparisons were made by Kruskal–Wallis nonparametric analysis of the variance test. $n = 3$ independent experiments; at least 2 coverslips per experiment per condition were analyzed. *$p < 0.05$. ns, Nonsignificant. ***C***, Representative image of a p75^{NTR}-HA-positive and a p75^{NTR}-HA-negative neuron after proNGF treatment. Arrows indicate expanded growth cones. * indicates retracted growth cones. Scale bar, 20 μm.

We found that both the highly conserved cysteine in the transmembrane domain and the four cysteine-rich domains are required for p75^{NTR} trimer formation and stability. Our results are consistent with a report that showed that p75^{NTR} oligomers require the transmembrane cysteine for stability (Vilar et al., 2009b); however, this study suggested that the most abundant oligomers were dimers. Other TNFR superfamily members, such as TNFRI, TNFRII, and Fas receptor, which also assemble as trimers, also have transmembrane cysteines. But previous studies have not determined whether those cysteine residues are required for their trimerization. The N-terminal cysteine-rich domain 1 (CRD1) in the extracellular domain of TNFRs is required for the formation of trimers, and for this reason it has been named the PLAD for preligand assembly domain (Chan et al., 2000; Chan, 2007). The CRD1 of p75^{NTR} promotes trimerization, thus functions as a PLAD domain, although all four CRDs are required for trimerization of the receptor.

Analysis of p75^{NTR} oligomerization using a membrane-permeable crosslinker revealed that dimers are present in low levels (~140 kDa in nonreducing conditions and ~175 kDa in reducing conditions), but these dimers were not detected at the cell surface. Thus, we hypothesize that the dimers present intracellularly might function as intermediate oligomers in the assembly of trimers, which are the unique multimer found at the cell surface. Evaluation of p75^{NTR} oligomers in partial reducing conditions achieved by a combination of reducing agents and iodoacetamide (which partially blocks the reducing effect), showed that the ~175 kDa p75^{NTR} species is consistent with the molecular mass of dimers, as monomers in these conditions migrated at ~80–85 kDa. Reducing agents can cleave the transmembrane cysteine-cysteine bond (Vilar et al., 2009b) and also reduce (some of) the 12 cysteine bridges found on the CRDs, unfolding their rigid structure (He and Garcia, 2004). Therefore, we reasoned that partial reducing conditions may disassemble p75^{NTR} trimers in part by cleavage of the transmembrane disulfide bond and also by unfolding the CRDs. Interestingly, the C256A-p75^{NTR}, the ΔCRDs, or the ΔCRD2–4 mutations exhibited impaired trimerization but contained sufficient structural domains to promote dimerization of the receptor. The ΔCRDs dimers were reduced with βME, suggesting that disulfide bonds are involved in their assembly. As the intracellular cysteines are in a reducing environment unable to support disulfide bridges, our results suggest that the ΔCRDs dimers may be formed by an inter-receptor disulfide using the cysteine in the transmembrane domain. These results are consistent with a model in which p75^{NTR} trimers might be formed by a C256-C256 disulfide bridge and CRDs-CRDs interactions.

Our results indicating that the four CRDs promote dimerization are in agreement with structural data of the recombinant p75^{NTR} ECD, which have also shown dimeric symmetry. In the absence of ligands, the unglycosylated p75^{NTR} ECD has been shown to dimerize (He and Garcia, 2004; Gong et al., 2008). We believe that these structural studies failed to detect trimers of the receptor because the recombinant p75^{NTR} ectodomain used for these studies lacked the transmembrane domain containing the critical cysteine required for the trimeric assembly. This hypothesis is supported by studies of TNFR ECD (lacking their transmembrane cysteine) crystal structures, which showed dimeric instead of the well-established trimeric symmetry found in this receptor superfamily (Naismith et al., 1995; Kuester et al., 2011). Solution of the full-length p75^{NTR} crystal structure would be required to confirm our model, which proposes that C256-C256+CRDs-CRDs interactions are necessary for p75^{NTR} trimerization.

p75^{NTR} has been extensively studied; however, the molecular mechanisms that regulate activation is still unknown. It has been proposed that neurotrophins activate p75^{NTR} by inducing a conformational "snail-tong" movement exposing the death domains promoting the interaction of intracellular adaptors (Barker, 2009; Vilar et al., 2009b). As the most abundant oligomer of p75^{NTR} is a trimer, we assessed whether trimerization was required for function using a proNTs/p75^{NTR}-induced neuronal growth cone retraction assay. We found that expression of wild-type p75^{NTR} resulted in modest ligand-independent growth cone retraction, and further retraction was observed upon addition of proNGF. The expression of the C257A-p75^{NTR} mutant (trimer-deficient) resulted in significantly augmented growth cone retraction compared with the wild-type p75^{NTR} in the absence of exogenous ligands, and additional retraction was detected after proNGF administration. These results indicate that trimerization is not required to mediate growth cone retraction, and imply that trimers might sequester p75^{NTR} from an active form. ProNGF did not alter the oligomerization of p75^{NTR} at the cell surface, and dimers were still undetectable at the plasma membrane after the addition of the ligand. Therefore, we propose that p75^{NTR} monomers are sufficient for proNGF-induced acute morphological effects in neurons. However, we cannot exclude the possibility that ligands induce transient dimerization at the cell surface and that these dimers are undetectable by the methodology used. Our results are consistent with a study that showed that p75^{NTR}-induced apoptosis requires monomerization of the receptor and that oligomerization prevents this activity (Wang et al., 2000).

Overexpression of p75^{NTR} (Rabizadeh et al., 1993; Roux et al., 2001; Bhakar et al., 2003), oxidative stress, and disulfide-crosslinked oligomers (Vilar et al., 2009a; Kraemer et al., 2014) have been shown to induce ligand-independent activation of p75^{NTR}. We cannot exclude the possibility that endogenously secreted ligands may be responsible for activation of p75^{NTR}; however, the use of sparse cultures and frequent replacement of the culture media minimized the levels of ligand released to the media. The expression of ΔCRD1 (with enhanced dimer formation compared with wild-type p75^{NTR}) resulted in a similar degree of ligand-independent growth cone retraction compared with the wild-type p75^{NTR}. These results indicate that preformed dimers are not required for ligand-independent p75^{NTR} activation, suggesting that monomers are the active signaling unit in the absence of exogenous ligands.

Identification of the oligomerization state of p75^{NTR} begins to provide insights to the mechanisms of signal initiation of this noncatalytic receptor, as well as to develop therapeutic interventions to enhance or diminish its activity. Here we propose that p75^{NTR} activation is regulated by expression level to induce ligand-independent effects, and by its oligomerization status. We hypothesize that cellular conditions that favor p75^{NTR} trimerization may decrease p75^{NTR} activity, whereas monomer-favoring stimuli may activate the receptor, providing new targets to regulate p75^{NTR} activation.

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