Development/Plasticity/Repair

Death Domain Signaling by Disulfide-Linked Dimers of the p75 Neurotrophin Receptor Mediates Neuronal Death in the CNS

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The p75 neurotrophin receptor ($p75^{NTR}$) mediates neuronal death in response to neural insults by activating a caspase apoptotic pathway. The oligomeric state and activation mechanism that enable $p75^{NTR}$ to mediate these effects have recently been called into question. Here, we have investigated mutant mice lacking the $p75^{NTR}$ death domain (DD) or a highly conserved transmembrane (TM) cysteine residue (Cys^{259}) implicated in receptor dimerization and activation. Neuronal death induced by proneurotrophins or epileptic seizures was assessed and compared with responses in $p75^{NTR}$ knock-out mice and wild-type animals. Proneurotrophins induced apoptosis of cultured hippocampal and cortical neurons from wild-type mice, but mutant neurons lacking $p75^{NTR}$, only the $p75^{NTR}$ DD, or just Cys^{259} were all equally resistant to proneurotrophin-induced neuronal death. Homo-FRET anisotropy experiments demonstrated that both NGF and proNGF induce conformational changes in $p75^{NTR}$ that are dependent on the TM cysteine. *In vivo*, neuronal death induced by pilocarpine-mediated seizures was significantly reduced in the hippocampus and somatosensory, piriform, and entorhinal cortices of all three strains of $p75^{NTR}$ mutant mice. Interestingly, the levels of protection observed in mice lacking the DD or only Cys^{259} were identical to those of $p75^{NTR}$ knock-out mice even though the Cys^{259} mutant differed from the wild-type receptor in only one amino acid residue. We conclude that, both *in vitro* and *in vivo*, neuronal death induced by $p75^{NTR}$ requires the DD and TM Cys^{259}, supporting the physiological relevance of DD signaling by disulfide-linked dimers of $p75^{NTR}$ in the CNS.

Key words: apoptosis; disulfide bond; epilepsy; neurotrophins; seizures

Significance Statement

A detailed understanding of the physiological significance of distinct structural determinants in the p75 neurotrophin receptor ($p75^{NTR}$) is crucial for the identification of suitable drug targets in this receptor. We have tested the relevance of the $p75^{NTR}$ death domain (DD) and the highly conserved transmembrane residue Cys^{259} for the ability of $p75^{NTR}$ to induce apoptosis in neurons of the CNS using gene-targeted mutant mice. The physiological importance of these determinants had been contested in some recent *in vitro* studies. Our results indicate a requirement for DD signaling by disulfide-linked dimers of $p75^{NTR}$ for neuronal death induced by proneurotrophins and epileptic seizures. These new mouse models will be useful for clarifying different aspects of $p75^{NTR}$ physiology.

Introduction

In the adult nervous system, signaling pathways that are normally only functional during development often become reactivated after neural injury. Some of these pathways have neuroprotective or neuroregenerative functions and may represent a self-defense response to the ensuing damage. Other pathways, however, appear to amplify neural damage. As with inflammatory responses, induction of such pathways may have evolved as a mechanism for clearing damage produced after a lesion or insult to cellular ele-

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ments of the nervous system (Ibáñez and Simi, 2012). However, after severe insult, these pathways can do more damage than good. Expression of the p75 neurotrophin receptor (p75^{NTR}) increases markedly after neural injury in many of the same cell types that express p75^{NTR} during development and p75^{NTR} signaling can contribute to neuronal death, axonal degeneration, and dysfunction during injury and cellular stress (Ibáñez and Simi, 2012). Inhibition of p75^{NTR} signaling has therefore emerged as an attractive strategy for limiting neural damage in neurodegeneration and nerve injury. p75^{NTR} can interact with all members of the neurotrophin family, including mature neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) and their propeptide forms, such as proNGF and proBDNF, as well as other ligands unrelated to the neurotrophins, such as Nogo and β-amyloid (for review, see Underwood and Coulson, 2008). Proneurotrophins are potent inducers of neuronal death (Lee et al., 2001; Nykjaer and Willnow, 2012). Expression of proneurotrophins has been reported to be elevated in neurodegenerative conditions and after neural insults (Volosin et al., 2008; Iulita and Cuello, 2014).

The cytoplasmic domain of p75^{NTR} contains a C-terminal death domain (DD) similar to that found in other members of the tumor necrosis factor receptor superfamily (Liepinsh et al., 1997). The DD is linked to the transmembrane (TM) domain by a flexible juxtamembrane region. Studies in cultured primary neurons have implicated both the DD (Charalampopoulos et al., 2012) and the juxtamembrane domain (Coulson et al., 2000) in p75^{NTR}-mediated cell death. However, the necessity or sufficiency of these receptor domains for p75^{NTR}-mediated neuronal death *in vivo* is unclear.

Neurotrophins and proneurotrophins are dimeric ligands and form twofold symmetry complexes with dimers of the p75^{NTR} extracellular domain in x-ray crystal structures (Aurikko et al., 2005; Gong et al., 2008; Feng et al., 2010). In addition, we have shown recently that the p75^{NTR} DD can form low-affinity symmetric dimers in solution (Lin et al., 2015). In intact cells, p75^{NTR} can also form dimers in the absence of ligands through both covalent and noncovalent interactions. A highly conserved Cys residue in the p75^{NTR} TM domain stabilizes the formation of covalent p75^{NTR} dimers through disulfide bonding (Vilar et al., 2009; Sykes et al., 2012). FRET experiments have shown that the two DDs in the p75^{NTR} dimer are in close proximity to each other (high FRET state) and that NGF binding induces oscillations that result in a net decrease of the FRET signal, suggesting separation of intracellular domains upon ligand binding (Vilar et al., 2009). Disruption of this conformational change through mutation of the TM cysteine prevents p75^{NTR} signaling in response to neurotrophins (Vilar et al., 2009). A recent study has called into question the physiological significance of p75^{NTR} dimers and the conserved TM

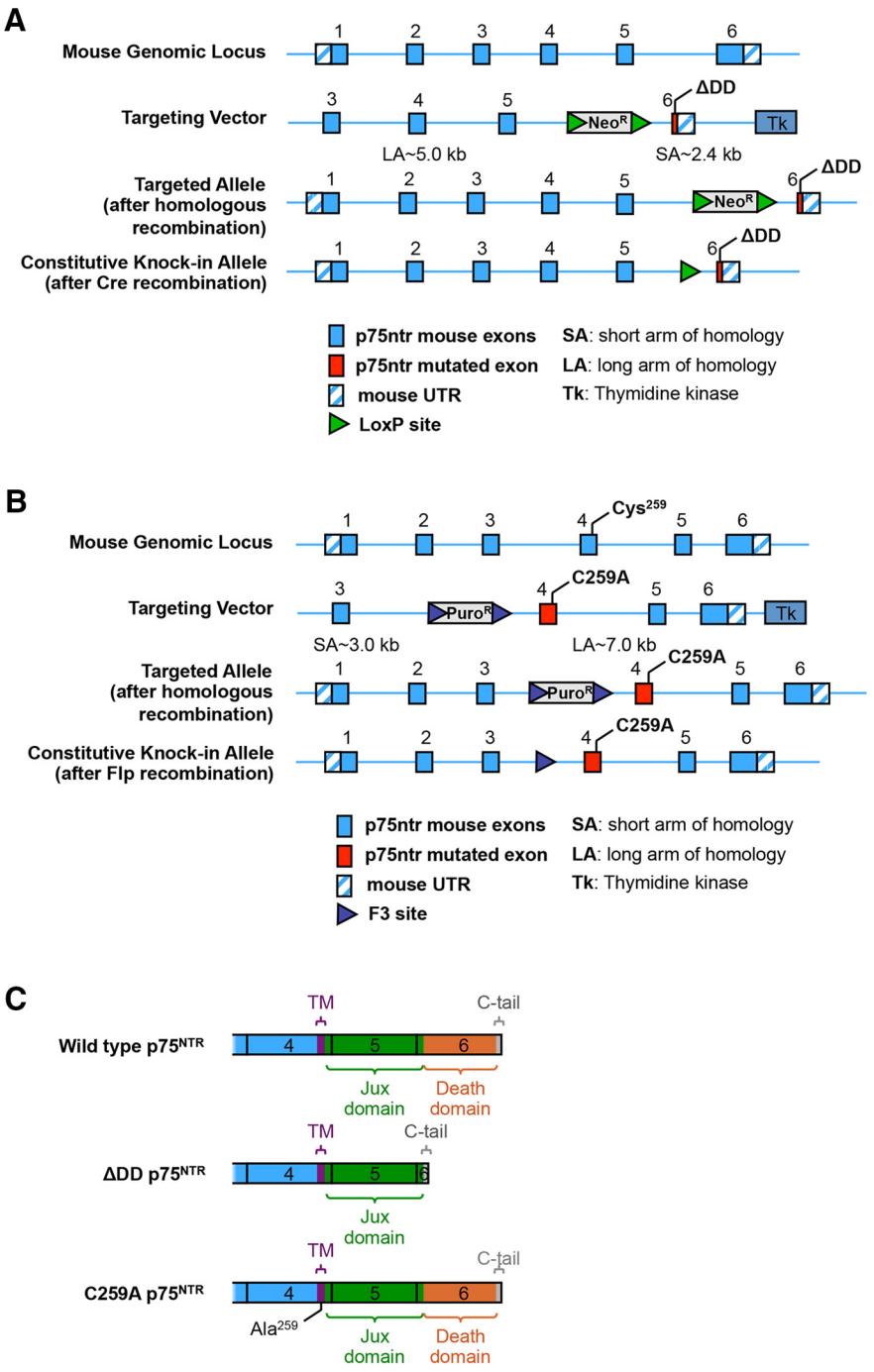

Figure 1. Generation of knock-in alleles of p75^{NTR} lacking the DD or TM cysteine Cys^{259}. ***A***, Schematic of the *p75ntr* locus (not to scale) with strategy for generation of the ΔDD mutant allele. ***B***, Schematic of the *p75ntr* locus (not to scale) with strategy for generation of the C259A mutant allele. ***C***, Schematic of C-terminal sequences in wild-type, ΔDD, and C259A p75^{NTR} proteins. Black lines outline the boundaries of exons 4, 5, and 6, denoted by their respective numbers. Sequences corresponding to the extracellular, TM, juxtamembrane (Jux), and DDs are colored in blue, purple, green, and orange, respectively. The C-terminal tail containing a putative PDZ-binding motif is in gray. The ΔDD protein ends in QGDTATSPV, where QGD corresponds to the end of the Jux domain and TATSPV to the C-terminal tail. For the C259A protein, the TGC (Cys) codon was changed to GCA (Ala). Black vertical lines mark the boundaries of exons 4, 5, and 6.

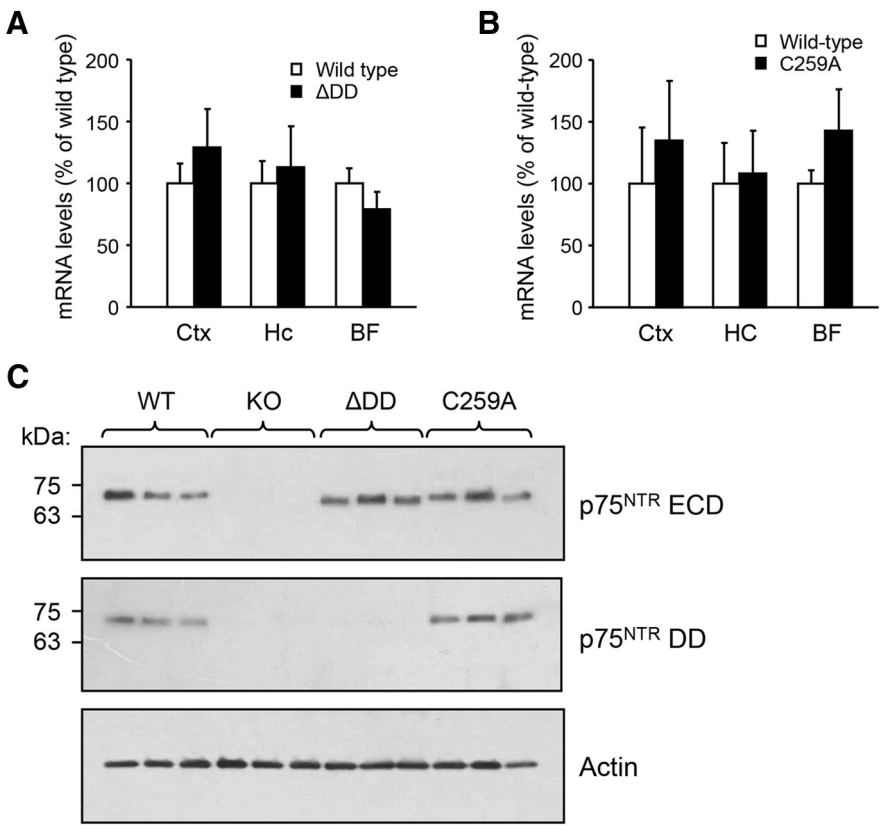

Figure 2. Expression of knock-in alleles of p75^{NTR} lacking the DD or TM cysteine Cys^{259}. ***A***, Expression of *p75ntr* mRNA in cortex (Ctx), hippocampus (Hc), and basal forebrain (BF) of p75^{NTR} wild-type and ΔDD adult mice as assessed by quantitative PCR. For each brain region, expression was normalized to actin mRNA levels. Error bars indicate average (relative to wild-type levels) ± SD of triplicate determinations ($n = 3$ different animals from each genotype). ***B***, Expression of *p75ntr* mRNA in Ctx, Hc, and BF of p75^{NTR} wild-type and C259A adult mice as assessed by quantitative PCR. For each brain region, expression was normalized to actin mRNA levels. Error bars indicate average (relative to wild-type levels) ± SD of triplicate determinations ($n = 3$ different animals from each genotype). ***C***, Expression of p75^{NTR} in cerebral cortex of 3-month-old wild-type (WT) knock-out (KO), ΔDD and C259A mice analyzed by Western blotting ($n = 3$). ECD, Antibody against extracellular domain; DD, antibody against DD. Ten micrograms of total protein extract was loaded in each lane. Reprobing with β-actin antibodies is shown as a loading control.

cysteine (Anastasia et al., 2015). Based on molecular weights estimated from SDS/PAGE gels and overexpression of p75^{NTR} constructs in cultured cells, those investigators argued that p75^{NTR} mainly exists as an inactive trimer and that neurotrophins induce biological activities through monomeric p75^{NTR} independently of the conserved TM cysteine.

Here, we report the generation of two new mouse models, lacking the p75^{NTR} DD or the conserved TM cysteine, respectively, generated by homologous recombination. We assessed neuronal death induced by proneurotrophin ligands in neuronal cultures or by epileptic seizures *in vivo* compared with p75^{NTR} knock-out and wild-type animals. Our results support the physiological relevance of DD signaling by dimers of p75^{NTR} in the CNS.

Materials and Methods

Animals. Mice were housed in a 12 h light/dark cycle and fed a standard chow diet. The following transgenic mouse lines were used: p75^{NTR} knock-out mice (Lee et al., 1994), p75^{NTR} ΔDD knock-in mice (this study), and p75^{NTR} C259A knock-in mice (this study). All mouse lines used in this study were backcrossed to the C57BL/6J background. Epilepsy studies were performed on male mice. For neuronal cultures, embryos of either sex were used. The ΔDD targeting vector was generated by A. Simi (Karolinska Institutet) using Sv129 genomic fragments from the *p75ntr* locus and transfected into Sv129 ES cells. The coding sequence of the ΔDD allele ends in QGDTATSPV, where QGD corresponds to the end of the Jux domain and TATSPV to the C-terminal tail. The C-terminal SPV motif has been proposed to interact with PDZ domains (Roux and Barker, 2002). Gene-targeted ΔDD mice were generated at the Karolinska Center for Transgene Technologies using standard methods. The C259A targeting vector was generated using BAC clones from the C57BL/6J RPCIB-731 BAC library and transfected into TaconicArtemis C57BL/6N Tac ES cell line. The TGC (Cys) codon was changed to GCA (Ala). Gene-targeted C259A mice were generated at TaconicArtemis using standard methods. Animal experiments were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

Tissue isolation, RNA preparation, and quantitative PCR. Total mRNA was isolated from cortex, hippocampus, and basal forebrain from mouse brains using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription using the Omniscript RT kit (Qiagen). Real-time PCR was conducted using the 7500 Real-Time PCR system (Applied Biosystems) with SYBR Green fluorescent probes. The following primer pairs were used: p75^{NTR}, 5′-GTTCTCCTGCCAGGACAAACAGAACAC-3′ and 5′-GCATTCGGCGTCAGCCCAGGGCGTGCA-3′; β-actin, 5′-GCTCTTTTCCAGCCTTCCTT-3′ and 5′-AGTACTTGCGCTCAGAGGA-3′. As a standard for assessment of copy number of PCR products, serial concentrations of each PCR fragment were amplified in the same manner. The amount of cDNA was calculated as the copy numbers in each reverse transcription product and normalized to β-actin values.

Western blotting. Mouse cerebral cortex was dissected, snap-frozen, and homogenized in RIPA lysis buffer supplemented with protease inhibitor mixture (Roche). Samples were centrifuged at 12,000 × *g* and the supernatants were used. The protein concentration was determined using the Pierce BCA protein assay kit. Proteins (20 μg) were applied to 8% SDS-polyacrylamide gel under reducing conditions and electrotransferred to a PVDF membrane. Immunoblots were performed using antibodies specific for p75^{NTR} extracellular domain (ECD) (1:2000, GT15057; Neuromics), p75^{NTR} DD (1:500, ab52987; Abcam), and β-actin (1:4000; A2668; Sigma-Aldrich). Immunoblots were developed using the SignalFire ECL reagent (Cell Signaling Technology) and exposed to x-ray films (Konica Minolta). x-ray films were digitally scanned and image analysis and quantification of band intensities were done with ImageJ.

Primary culture of hippocampal and cortical neurons and assessment of neuronal death. Primary neurons were isolated from mouse embryonic brain at embryonic day 17.5 (E17.5). The cortical or hippocampal tissue was dissected and dissociated by trypsin digestion and trituration in serum-free medium. Cells were counted by hemocytometer and seeded at a density of 8×10^4 cells per well in 24-well plates on poly-lysine-coated glass slides. They were maintained in Neurobasal medium supplemented with B27 (Invitrogen), Glutamax (Invitrogen), and penicillin/streptomycin at 37°C with 5% CO_2. After 3 d *in vitro*, cultures were treated for 12 h (for caspase-3) or 24 h (for propidium iodide) with 20 ng/ml proNGF or proBDNF (Alomone). Neuronal apoptosis was assessed by immunocytochemistry of activated caspase-3 and by staining with propidium iodide. For assessment of activated caspase-3, cells were fixed with acetone-methanol, permeabilized with 0.5% Triton X-100, and blocked in 10% normal donkey serum. Fixed cells were then incubated at 4°C overnight with anti-cleaved-caspase-3 (1:200; Cell Signaling

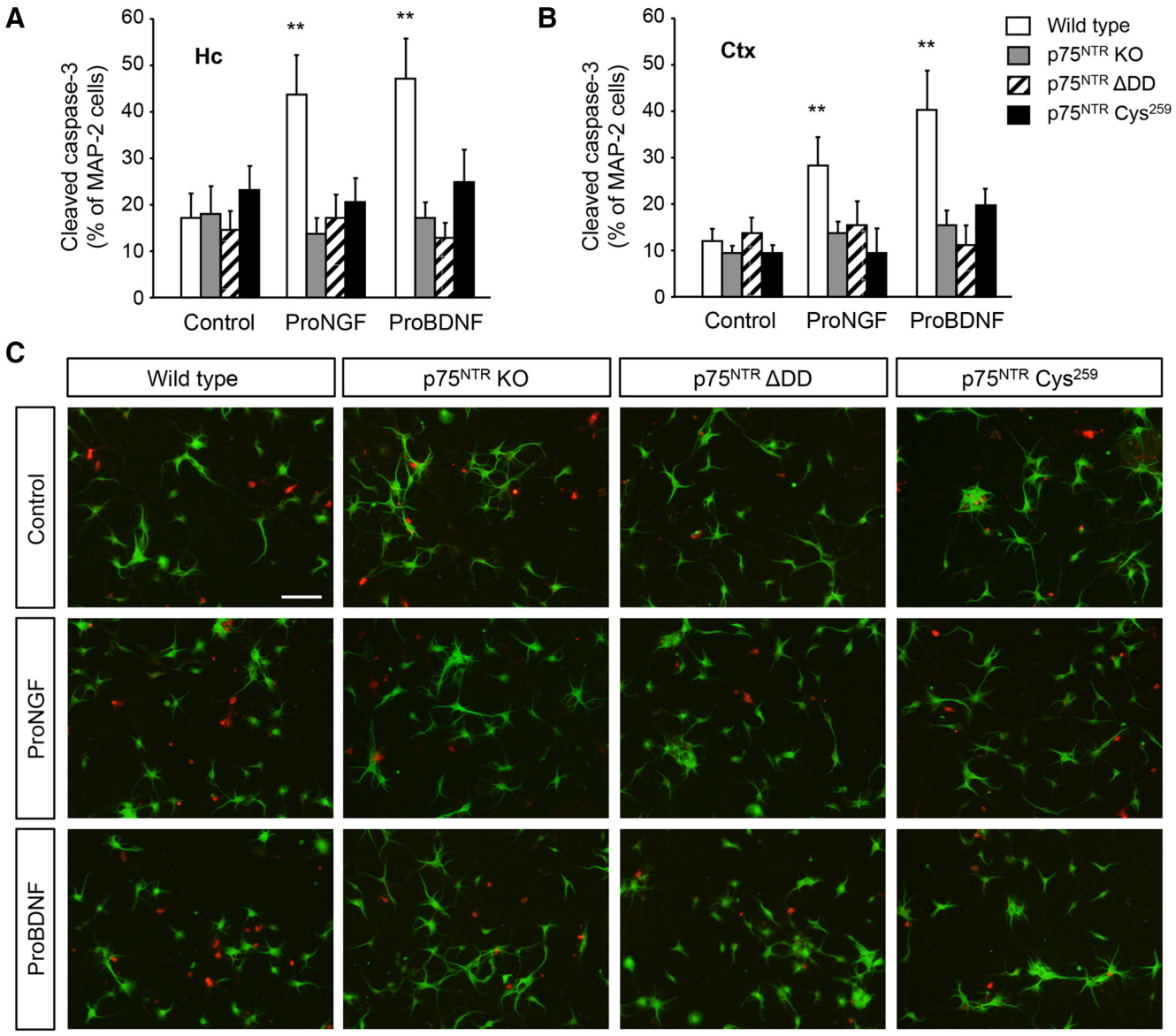

Figure 3. Induction of caspase-3 activation by proneurotrophins requires the p75 ^{NTR} DD and TM Cys ^{259}. ***A***, Activation of caspase-3 by 12 h treatment with proNGF or proBDNF in cultured embryonic hippocampal (Hc) neurons identified by MAP-2 staining, from p75 ^{NTR} wild-type, knock-out (KO), ΔDD, and C259A mice. Error bars indicate average ± SD of three independent determinations. $^{**}p < 0.01$ versus control. ***B***, Activation of caspase-3 by 12 h treatment with proNGF or proBDNF in cultured embryonic cortical (Ctx) neurons identified by MAP-2 staining, from p75 ^{NTR} wild-type, KO, ΔDD, and C259A mice. Error bars indicate average ± SD of three independent determinations. $^{**}p < 0.01$ versus control. ***C***, Representative photomicrographs of immunofluorescence staining for cleaved caspase-3 (red) and MAP-2 (green) in cultured cortical neurons. Scale bar, 50 μm.

Technology) and monoclonal anti-MAP-2 (1:4000; Abcam) antibodies, followed by incubation with fluorophore-conjugated secondary antibodies. Cleaved caspase-3-positive cells among the MAP2-positive cell population were counted in three random fields per well from triplicate wells at a 20× magnification. Evaluation of pyknotic nuclei was performed by propidium iodide staining in cultures that that had been treated for 24 h with 20 ng/ml proNGF. Cells were incubated with propidium iodide at a final concentration of 20 μg/ml for 30 min at 37°C before fixation in 4% PFA/4% sucrose, followed by overnight staining with anti-Tuj1 antibodies (1:1000; Sigma-Aldrich) and DAPI counterstaining. Cells showing pyknotic nuclei among the Tuj1-positive cell population were counted in 30 images per coverslip (≈250–600 neurons per coverslip) from triplicate wells at a 20× magnification. The experiments were performed two times with similar results. Approximately 95% of neurons stained with cleaved caspase-3 also stained with propidium iodide. Conversely, ~67% of neurons positive for propidium iodide also stained for cleaved caspase-3.

Homo-FRET anisotropy microscopy. Anisotropy microscopy was done as described previously (Vilar et al., 2009) in transiently transfected COS-7 cells. Images were acquired 24 h after transfection using a Nikon Eclipse Ti-E motorized inverted microscope equipped with an X-Cite LED illumination system. A linear dichroic polarizer (Meadowlark Optics) was placed in the illumination path of the microscope and two identical polarizers were placed in an external filter wheel at orientations parallel and perpendicular to the polarization of the excitation light, respectively. The fluorescence was collected via a CFI Plan Apochromat Lambda 40×, 0.95 numerical aperture air objective and parallel and polarized emission images were acquired sequentially on an Orca CCD camera (Hamamatsu Photonics). Data acquisition was controlled by the MetaMorph software (Molecular Devices). NGF, proNGF (both from Alomone Labs), or vehicle was added 3 min after the start of the time lapse at a concentration of 100 and 20 ng/ml, respectively. Anisotropy values were extracted from image stacks of 30 images acquired in both parallel and perpendicular emission modes every 30 s for a time period of 15 min after ligand addition. For each construct, 25–30 ROIs were mea-

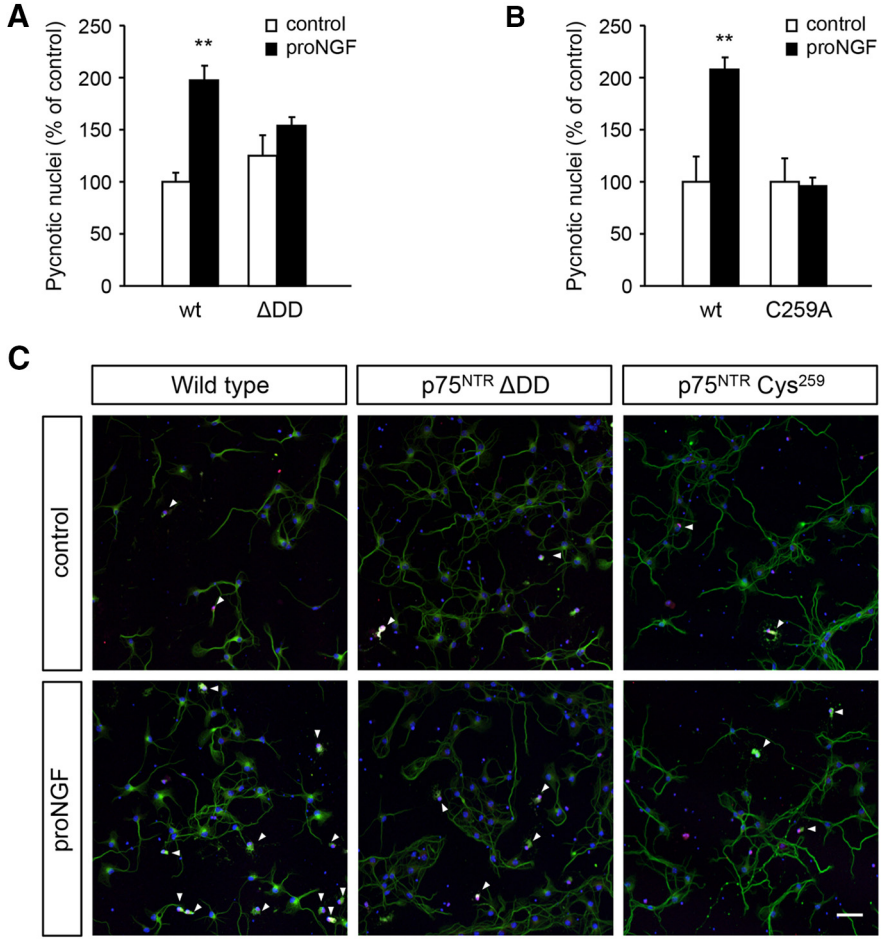

Figure 4. Neuronal death induced by proNGF requires p75 ^{NTR} DD and TM Cys ^{259}. ***A***, Pyknotic nuclei identified by propidium iodide staining after 24 h treatment with proNGF in cultured embryonic hippocampal neurons identified by Tuj1 staining and counterstained with DAPI, from p75 ^{NTR} wild-type and ΔDD mice. Error bars indicate average percentage of control ± SEM. $^{**}p < 0.01$ versus control ($n = 6$). ***B***, Pyknotic nuclei identified by propidium iodide staining after 24 h treatment with proNGF in cultured embryonic hippocampal neurons identified by Tuj1 staining and counterstained with DAPI from p75 ^{NTR} wild-type and C259A mice. Error bars indicate average percentage of control ± SEM. $^{**}p < 0.01$ versus control ($n = 3$). ***C***, Representative photomicrographs of pyknotic nuclei (red), denoted by arrowheads, and Tuj1 staining (green) in cultured hippocampal neurons counterstained with DAPI (blue). Scale bar, 50 μm.

sured in three independent transfections performed in duplicate. Fluorescence intensity and anisotropy images were calculated as described by Squire et al. (2004). Wild-type and C257A mutant cDNA constructs of rat p75 ^{NTR} were tagged at the C terminus with a monomeric version of EGFP (Clontech) carrying the A206K mutation that disrupts EGFP dimerization as described previously (Vilar et al., 2009).

Induction of epileptic seizures by pilocarpine injection. Treatment of adult mice began with injection of methyl-scopolamine (1 mg/kg, s.c.; Sigma-Aldrich) and phenytoin (50 mg/kg, s.c.; Sigma-Aldrich) to reduce peripheral muscarinic effects and mortality associated with tonic seizures, respectively. After 30 min, status epilepticus was induced by injection of pilocarpine (300 mg/kg, i.p.). Two hours later, status epilepticus was terminated by injection of diazepam (10 mg/kg, i.p.). Sham-control mice were treated exactly as the pilocarpine-treated animals except that the pilocarpine injection was replaced by saline injection.

Histological studies. Twenty-four hours after pilocarpine or saline injection, mice were anesthetized by pentobarbital and perfused transcardially with 4% paraformaldehyde followed by decapitation. Brains were harvested, postfixed by 4% paraformaldehyde, and cryoprotected with 20% sucrose. Coronal sections (30 μm) were cut in a cryostat and processed for TUNEL assay using a kit from Roche following the manufacturer's instructions and for immunohistochemistry with anti-NeuN antibody (1:200; Merck). Sections were taken between bregma 0.26 mm and −0.10 mm for somatosensory cortex and between bregma −1.94 and −2.18 mm for hippocampus, entorhinal cortex, and piriform cortex (Paxinos and Franklin, 2004). TUNEL-positive cells were counted in 3–5 consecutive sections (each corresponding to an area of ~0.25 mm^2) from each brain and averaged. A total of 10–13 animals were used per group, as indicated in Figure 5.

Statistical analyses. Statistically significant differences were assessed by two-way ANOVA followed by Student's *t* test (for cleaved caspase-3 and propidium iodide data) or Mann–Whitney *U* test (for TUNEL data).

Results

Generation and characterization of knock-in alleles of p75 ^{NTR} lacking the DD or TM cysteine Cys ^{259}

Alleles of the mouse *p75ntr* gene lacking sequences encoding the DD (ΔDD) or with an alanine substitution of TM residue Cys ^{259} (C259A) were generated by homologous recombination in embryonic stem cells (Fig. 1*A*,*B*). To generate the ΔDD allele, exon 6 sequences encoding the p75 ^{NTR} DD were removed from the targeting construct, leaving the juxtamembrane domain directly upstream of a short C-terminal tail (Fig. 1*C*) containing a putative PDZ-binding motif (Roux and Barker, 2002). p75 ^{NTR} mRNA expression levels in hippocampus, cerebral cortex, and basal forebrain of young adult mice were indistinguishable in ΔDD, C259A, and wild-type strains (Fig. 2*A*,*B*). In agreement with this, similar p75 ^{NTR} protein levels were detected in the cerebral cortex of ΔDD, C259A, and wild-type mice as assessed by Western blotting using an antibody directed toward the p75 ^{NTR} ECD (Fig. 2*C*). As expected, reprobing with antibodies directed toward the p75 ^{NTR} DD confirmed the absence of DD sequences in ΔDD mice (Fig. 2*C*, DD).

Neuronal death induced by proneurotrophins requires the p75 ^{NTR} DD and TM Cys ^{259}

Neuronal death induced by proneurotrophin ligands was assessed in cultures of hippocampal and cortical neurons isolated from E17.5 mouse embryos. Previous work showed that hippocampal neurons lacking p75 ^{NTR} are resistant to neuronal death induced by mature NGF (Troy et al., 2002). Using proNGF and proBDNF, we found that p75 ^{NTR} knock-out hippocampal and cortical neurons are also resistant to cell death induced by proneurotrophins as assessed by immunocytochemistry for activated caspase-3 (Fig. 3*A*–*C*). Interestingly, mutant neurons lacking either the p75 ^{NTR} DD or only TM Cys ^{259} were equally resistant to proneurotrophin-induced cell death as knock-out neurons (Fig. 3*A*–*C*). Cell death was also assessed by evaluation of pyknotic nuclei stained by propidium iodide in embryonic hippocampal neurons from ΔDD and C259A mutant mice after treatment with proNGF. Mutant neurons were resistant to induction of pyknotic nuclei by proNGF (Fig. 4*A*–*C*). These data indicate that both the p75 ^{NTR} DD and TM Cys ^{259} are required for neuronal death induced by proneurotrophins.

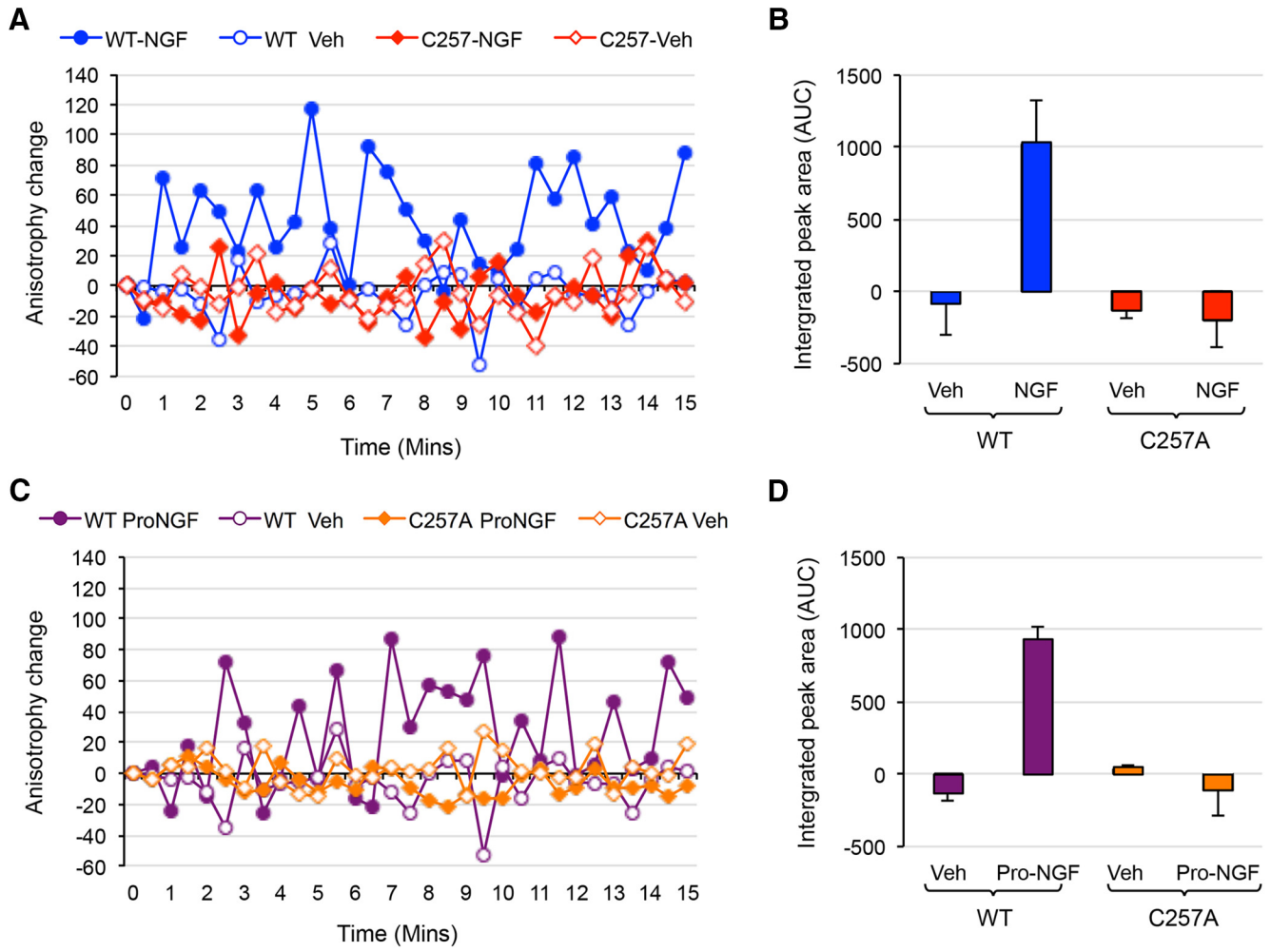

Figure 5. NGF and proNGF induce conformational changes in p75^{NTR} that are dependent on the conserved TM cysteine. ***A***, Representative experiment showing traces of average anisotropy change after addition of NGF or vehicle in cells expressing wild-type or C257A rat p75^{NTR}. Addition of NGF, but not vehicle, induced positive anisotropy oscillations above baseline (horizontal axis at 0) that were abolished in the C257A mutant. ***B***, Net anisotropy change over 15 min after addition of NGF or vehicle in cells expression wild-type or C257A p75^{NTR}. Results are expressed as average ± SD ($n = 3$ experiments; $n = 15–17$ cells examined per experiment). AUC, Area under curve. $^{**}p < 0.001$ versus vehicle. ***C***, Representative experiment showing traces of average anisotropy change after addition of proNGF or vehicle in cells expressing wild-type or C257A rat p75^{NTR}. Addition of proNGF induced positive anisotropy oscillations above baseline (horizontal axis at 0) that were abolished in the C257A mutant. ***D***, Net anisotropy change over 15 min after addition of proNGF or vehicle in cells of wild-type or C257A p75^{NTR}. Results are expressed as average ± SD ($n = 3$ experiments; $n = 15–17$ cells examined per experiment). $^{**}p < 0.001$ versus vehicle.

NGF and proNGF induce conformational changes in p75^{NTR} that are dependent on conserved TM cysteine residue

In our previous work, we showed that NGF induces a conformational change in p75^{NTR} that is dependent on TM Cys^{259} and results in the separation of the DDs in the p75^{NTR} dimer as measured by homo-FRET anisotropy (Vilar et al., 2009). Given the requirement of Cys^{259} for the effects of proNGF on neuronal death (Figs. 3, 4), we sought to determine whether this ligand also induces similar conformational changes in p75^{NTR}. Real-time homo-FRET anisotropy measurements of DD:DD interaction in response to NGF or proNGF were recorded in COS cells transfected with constructs expressing EGFP-tagged wild-type or C257A mutant rat p75^{NTR} as described previously (Vilar et al., 2009). (We note that mouse Cys^{259} corresponds to Cys^{257} in rat p75^{NTR}.) Application of NGF produced large oscillations of increased p75^{NTR} anisotropy at the cell membrane (Fig. 5*A*), resulting in a positive net change integrated over 15 min treatment compared with vehicle (Fig. 5*B*). Because FRET is inversely related to anisotropy, this indicates ligand-triggered separation of receptor intracellular domains (Vilar et al., 2009). Stimulation with proNGF produced quantitatively similar anisotropy changes in p75^{NTR} (Fig. 5*C*,*D*). Importantly, anisotropy changes in response to either ligand were abolished in the C257A p75^{NTR} mutant, indicating the requirement of the conserved TM cysteine for activation of p75^{NTR} in response to both mature NGF and proNGF.

Essential role of the p75^{NTR} DD and TM cysteine Cys^{259} in neuronal death induced by pilocarpine-mediated seizures

Epileptic seizures induce neuronal death in animal models and human epilepsy. In rodents, seizures elicit p75^{NTR}-mediated apoptosis of neurons in several brain areas, including hippocampus, cortex, and basal forebrain (Roux et al., 1999; Troy et al., 2002; Volosin et al., 2006; Unsain et al., 2008; Volosin et al., 2008; VonDran et al., 2014). Epileptic seizures induced by pilocarpine injection elicited apoptosis mainly in neurons, as assessed by overlap between TUNEL and NeuN in sections from the hippocampal formation (Fig. 6*A*). TUNEL levels in sham-operated animals were very low: between 0% and 5% of the those observed after pilocarpine treatment (data not shown). We assessed apoptosis 24 h after pilocarpine injection in

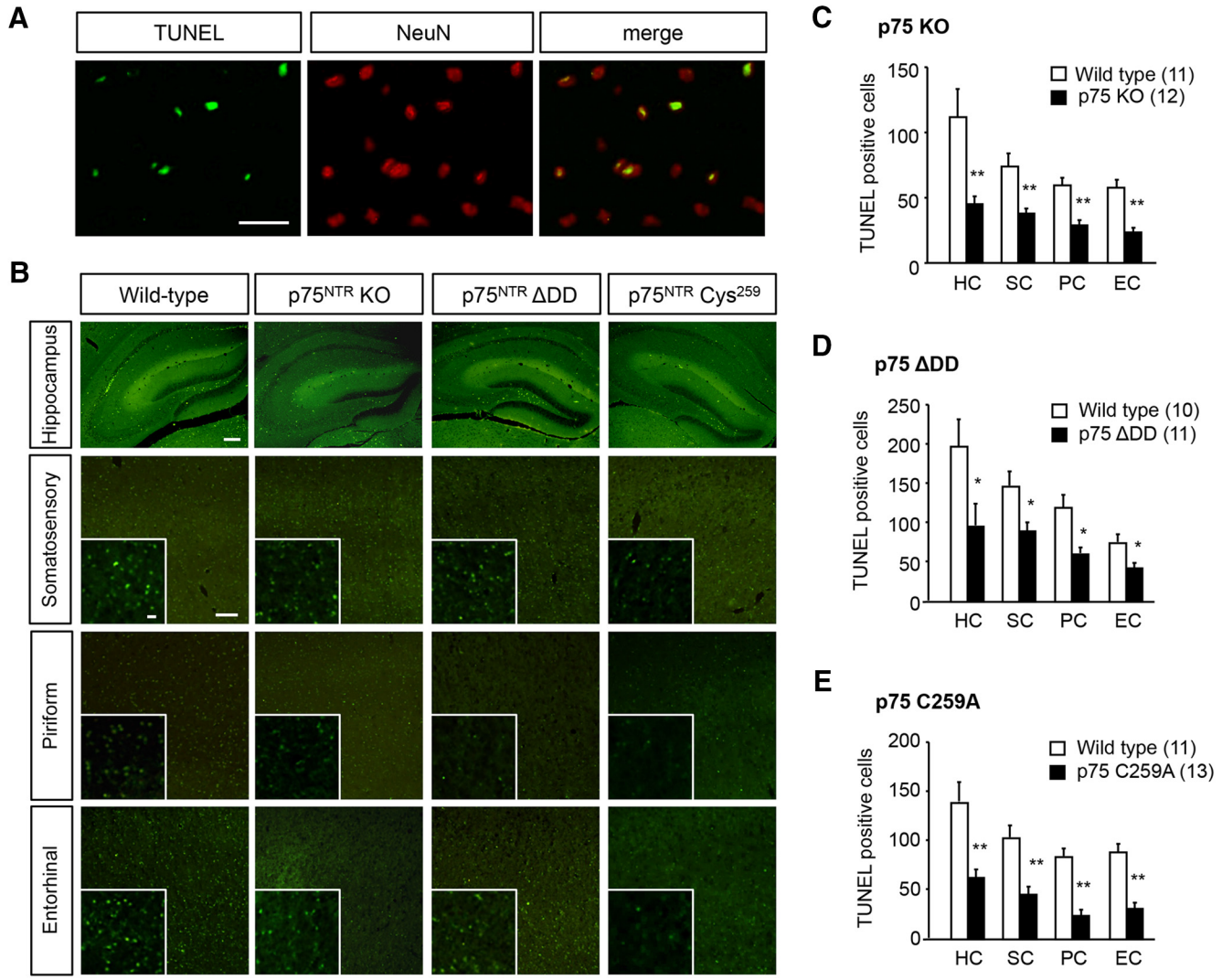

Figure 6. Essential role of the p75^{NTR} DD and TM cysteine Cys^{259} in cell death induced by pilocarpine-mediated seizures. ***A***, TUNEL staining (green) appears mainly on neurons identified by NeuN staining (red) in the hippocampal hilar region of adult wild-type mice 24 h after pilocarpine-induced seizures. Scale bar, 50 μm. ***B***, Representative photomicrographs of TUNEL staining (green) in hippocampus, somatosensory cortex, piriform cortex, and entorhinal cortex of wild-type, KO, ΔDD, and C259A mice. Scale bars, 100 μm, hippocampus; 50 μm, cortices (5 μm, insets). ***C***, TUNEL-positive cells in hippocampus (HC), somatosensory cortex (SC), piriform cortex (PC), and entorhinal cortex (EC) of wild-type and p75^{NTR} KO mice. Error bars indicate average ± SEM. The number of animals used in each group is indicated in brackets. $^{**}p < 0.01$ versus wild-type. ***D***, TUNEL-positive cells in HC, SC, PC, and EC of wild-type and p75^{NTR} ΔDD mice. Error bars indicate average ± SEM. The number of animals used in each group is indicated in brackets. $^{*}p < 0.05$ versus wild-type. ***E***, TUNEL-positive cells in HC, SC, PC, and EC of wild-type and p75^{NTR} C259A mice. Error bars indicate average ± SEM. The number of animals used in each group is indicated in brackets. $^{**}p < 0.01$ versus wild-type.

hippocampus as well as somatosensory, piriform, and entorhinal cortices of p75^{NTR} knock-out, ΔDD, and Cys^{259} mutant mice compared with wild-type controls (Fig. 6*B*). In agreement with previous studies (Troy et al., 2002), knock-out mice showed reduced seizure-induced apoptosis in hippocampus (Fig. 6*C*). Neuronal apoptosis was also reduced in all three cortical areas sampled in p75^{NTR} knock-out mice (Fig. 6*C*) compared with wild-type controls. Importantly, ΔDD and Cys^{259} mutant mice showed similar levels of protection to seizure-induced apoptosis as knock-out animals in all four brain areas investigated (Fig. 6*D*,*E*). Together, these data indicate that both the DD and TM Cys^{259} are required for neuronal death mediated by p75^{NTR} *in vivo*.

Discussion

While p75^{NTR} has emerged as an attractive therapeutic target for limiting neural damage in neurodegeneration and nerve injury, a detailed understanding of the physiological significance of its distinct structural determinants is crucial for the identification of suitable drug targets. Here, we have tested the relevance of the p75^{NTR} DD and the highly conserved TM residue Cys^{259} for the ability of p75^{NTR} to induce apoptosis in neurons of the CNS *in vitro* and *in vivo* using gene targeted mutant mice. The physiological importance of these determinants had been contested in some recent *in vitro* studies. Our results indicate that both the DD and TM Cys^{259} are required for neuronal death induced by p75^{NTR} and its ligands.

Being the most prominent domain within its intracellular region, the DD has long been suspected to play a critical role in p75^{NTR} physiology. Experiments in cultured cells devoid of p75^{NTR}, or derived from p75^{NTR} knock-out mice, have shown that p75^{NTR} constructs lacking the DD fail to rescue distinct p75^{NTR}-dependent functions, such as neurotrophin-induced apoptosis, which can otherwise be readily restored by wild-type constructs (Charalampopoulos et al., 2012). Before the present

study, however, the *in vivo* relevance of the p75^{NTR} DD for neuronal apoptosis had not been established. It should also be noted that other studies have implicated intracellular sequences distinct from the DD in cell death induced by p75^{NTR}. In particular, a short peptide in the juxtamembrane region upstream of the DD, otherwise known as the "Chopper" domain, was proposed to be necessary and sufficient to initiate neuronal death (Coulson et al., 1999, 2000). By introducing p75^{NTR} constructs into peripheral neurons, these researchers identified a deletion mutant lacking the Chopper domain that was unable to mediate apoptosis even if it contained a DD. In addition, a deletion construct lacking the DD but containing the Chopper sequence was still able to induce apoptosis. In the Chopper mutant, it is possible that lack of juxtamembrane sequences compromised the activation of the DD homodimer or its ability to interact with downstream signaling components of the apoptosis cascade. The mechanistic basis of the effects attributed to the Chopper domain is unclear as the cell death reported was ligand independent, brought about constitutively by p75^{NTR} overexpression. Moreover, as the neurons that were used are known to express p75^{NTR} endogenously at significant levels, it is uncertain whether the effects observed were dependent on endogenous p75^{NTR} expression. In this regard, it would be interesting to test whether sequences containing the Chopper domain are able to induce apoptosis in neurons from p75^{NTR} knock-out or ΔDD mice. Our present results demonstrate that, when expressed endogenously and at physiological levels, the p75^{NTR} DD is indeed required for ligand-induced apoptosis and seizure-induced cell death in neurons of the CNS. This indicates that other sequences in the p75^{NTR} intracellular domain are not sufficient for ligand-induced apoptosis in the absence of the DD.

The stoichiometry of p75^{NTR} has been debated for sometime. Studies using chemical cross-linking followed by denaturing gel electrophoresis have reported a main species of high molecular weight that has been attributed as receptor dimers or sometimes trimers. Species of even higher molecular weights were also observed in some studies. The use of chemical cross-linkers, particularly in whole cells or cell membranes, can lead to variable or artifactual results due to the unpredictable nature of the reaction as well as the possibility of spurious cross-linking to other cellular components. An early crystallography study of the extracellular domain of p75^{NTR} in complex with NGF reported a monomeric receptor bound to a dimer of NGF (He and Garcia, 2004). However, this was later shown to be an artifact of deglycosylation during sample preparation, and subsequent studies confirmed a dimeric arrangement in the complexes of the extracellular domain of p75^{NTR} with either NGF or neurotrophin-3 (Aurikko et al., 2005; Gong et al., 2008). In addition, we have recently solved several NMR structures of signaling complexes of the p75^{NTR} DD and showed that this domain forms low-affinity symmetric dimers in solution (Lin et al., 2015). Based mainly on evidence obtained from nonreducing gel electrophoresis, a recent study has argued that the main p75^{NTR} oligomer is a trimer (Anastasia et al., 2015). As it is well known, however, it is very difficult to determine molecular weights with any precision from the electrophoretic behavior of proteins in nonreducing gels. In particular, p75^{NTR} has a rich network of disulfide bonds in its four cysteine-rich extracellular domains. This aspect of its tertiary structure remains intact in nonreducing gels and is likely to result in anomalous retardation through the gel matrix. The authors of this study found that a p75^{NTR} construct with a mutation in the conserved TM cysteine failed to form "trimers" in nonreducing gels (Anastasia et al., 2015), a result that agrees with our earlier work showing that such mutant receptor is indeed monomeric in nonreducing gels (Vilar et al., 2009). However, the mechanisms by which a disulfide bond, which can link two but not three subunits, may induce a trimeric oligomer remained unclear. The same study also reported that overexpression of p75^{NTR} in wild-type hippocampal neurons increased the incidence of growth cone collapse and that this activity was maintained in the p75^{NTR} cysteine mutant. The researchers interpreted this as evidence for the biological activity of p75^{NTR} monomers. As discussed above, there is always a danger in overinterpreting ligand-independent activities of overexpressed receptors. Also in this case, the presence of endogenous wild-type p75^{NTR} in the transfected neurons might have influenced the results. Our present study has shown that in the absence of the TM cysteine i) neither mature NGF nor proNGF can induce conformational changes in p75^{NTR} intracellular domains, and ii) p75^{NTR} is unable to propagate apoptotic signals in response to neurotrophins in cultured neurons as well as in the epileptic brain. On the other hand, as we have shown previously, the lack of the TM cysteine does not affect the ability of p75^{NTR} to regulate the RhoA pathway in response to myelin-derived ligands (Vilar et al., 2009). Thus, it remains possible that the effects of p75^{NTR} overexpression on growth cone collapse, if confirmed *in vivo*, may require a different mechanism or structural determinants.

In conclusion, our results indicate a requirement for DD signaling by disulfide-linked dimers of p75^{NTR} for neuronal death induced by proneurotrophins and epileptic seizures. The new mouse models reported in this study will be useful to clarify the roles of the DD and TM Cys^{259} in other aspects of p75^{NTR} physiology.

Note added in proof. While this paper was in press, Vilar and colleagues reported the NMR structure of the transmembrane domain of p75NTR showing it to be a dimer, not a trimer. Nadezhdin KD, García-Carpio I, Goncharuk SA, Mineev KS, Arseniev AS, Vilar M (2016) Structural basis of p75 transmembrane domain dimerization. J Biol Chem., doi/10.1074/jbc.M116.723585.

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