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Fast-diffusing p75NTR monomers support apoptosis and growth cone collapse by neurotrophin ligands

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The p75 neurotrophin (NT) receptor (p75NTR) plays a crucial role in balancing survival-versus-death decisions in the nervous system. Yet, despite 2 decades of structural and biochemical studies, a comprehensive, accepted model for p75NTR activation by NT ligands is still missing. Here, we present a single-molecule study of membrane p75NTR in living cells, demonstrating that the vast majority of receptors are monomers before and after NT activation. Interestingly, the stoichiometry and diffusion properties of the wild-type (wt) p75NTR are almost identical to those of a receptor mutant lacking residues previously believed to induce oligomerization. The wt p75NTR and mutated (mut) p75NTR differ in their partitioning in cholesterol-rich membrane regions upon nerve growth factor (NGF) stimulation: We argue that this is the origin of the ability of wt p75NTR, but not of mut p75NTR, to mediate immature NT (proNT)-induced apoptosis. Both p75NTR forms support proNT-induced growth cone retraction: We show that receptor surface accumulation is the driving force for cone collapse. Overall, our data unveil the multifaceted activity of the p75NTR monomer and let us provide a coherent interpretative frame of existing conflicting data in the literature.

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Here, we directly and quantitatively assess p75NTR oligomerization status in the plasma membrane of living cells by means of single-molecule fluorescence microscopy with a minimally invasive strategy (21, 22); this relies on insertion of a short peptide tag in the p75NTR protein and its labeling with 1:1 stoichiometry (23, 24). This method also allows imaging the receptor with small organic dyes, which, unlike the more cumbersome Quantum dots (Qdot), permits one to retrieve the receptor oligomeric state from their fluorescent intensity (21). We show that membrane p75NTR is mostly a fast-diffusing monomer, regardless of NT stimulation. Its self-interactions are far too transient to result in substantial receptor di- or trimerization; importantly, they do not depend on Cys256 or on other residues previously suggested to play a role in receptor oligomerization. We also gather evidence on why the p75NTR C256A mutant does not elicit NT-dependent apoptosis (18) but is functional for growth cone collapse signaling (20). By solving this apparent contradiction, we revisit here p75NTR function, in the conceptual framework of a versatile monomeric receptor.

Results

Expression, Validation, and Membrane Fluorolabeling of Human p75NTR Constructs. We previously demonstrated that p75NTR can be enzymatically labeled with small chemical probes (e.g., biotin, organic fluorophores) in a site-specific way, inserting 12 amino acid-long tags of the ACP/PCP family at its N terminus (23). This method also allows imaging the receptor with small organic dyes, which, unlike the more cumbersome Quantum dots (Qdot), permits one to retrieve the receptor oligomeric state from their fluorescent intensity (21). We show that membrane p75NTR is mostly a fast-diffusing monomer, regardless of NT stimulation. Its self-interactions are far too transient to result in substantial receptor di- or trimerization; importantly, they do not depend on Cys256 or on other residues previously suggested to play a role in receptor oligomerization. We also gather evidence on why the p75NTR C256A mutant does not elicit NT-dependent apoptosis (18) but is functional for growth cone collapse signaling (20). By solving this apparent contradiction, we revisit here p75NTR function, in the conceptual framework of a versatile monomeric receptor.

Expression, Validation, and Membrane Fluorolabeling of Human p75NTR Constructs. We previously demonstrated that p75NTR can be enzymatically labeled with small chemical probes (e.g., biotin, organic fluorophores) in a site-specific way, inserting 12 amino acid-long tags of the ACP/PCP family at its N terminus (23). This allows a covalent modification with 1:1 stoichiometry (i.e., 1 probe per p75NTR molecule) of the receptors exposed on the surface of living cells (24, 25). Here, we used S6-p75NTR (Fig. L4) for its better labeling performance when compared with A1-p75NTR (SI Appendix, Fig. S1). We show that S6-p75NTR correctly localizes on the plasma membrane, as well as on intracellular structures like the nuclear envelope, similar to endogenous p75NTR expressed by PC12 cells (26) (Fig 1B). Like endogenous p75NTR (SI Appendix, Fig. S2), S6-p75NTR undergoes palmitoylation (Fig 1C), an important posttranslational modification for p75NTR death domain signaling capability (27). The p75NTR was recently reported to localize asymmetrically in neurons, helping in specifying the future axon (28); accordingly, biotinylated S6-p75NTR receptors preferentially localize in neurites and growth cones in developing hippocampal neurons (Fig. LD). Furthermore, S6-p75NTR-EGFP was able to induce growth cone collapse in the same neurons upon proNGF administration (Fig. 1E), as reported for previous p75NTR constructs devoid of chemical tags (29). Finally, proBDNF binding to p75NTR was recently reported to increase the number of apoptotic neurons (18) (Fig. IF, Top). By transducing S6-p75NTR in cortical neurons from p75NTR knockout (KO) mice (30) with an inducible lentiviral vector (24), we found that it was able to recapitulate proBDNF-induced apoptosis (Fig. IF, Middle and Bottom). The lower percentage of apoptotic neurons, with respect to that raised by endogenous p75NTR (31), is likely due to the presence of untransduced neurons.

Overall, our data demonstrate that the S6-tagged construct of human p75NTR retains the properties of endogenous p75NTR. We therefore employed it to visualize the p75NTR membrane pool in living cells and to describe its behavior in a direct, unperturbed physiological way.

p75NTR Single Molecules Diffuse as Monomers in the Cell Membrane. We first sought to investigate the membrane p75NTR diffusive properties and oligomerization state in living cells. To this end, we expressed S6-p75NTR in neuroblastoma SK-N-BE(2) cells, a line that conceivably models a neuronal membrane with the advantage of lacking endogenous p75NTR and TrkA at both messenger RNA and protein levels (31). Once labeled with Abberior635P dye (SI Appendix, Fig. S3), S6-p75NTR imaged with total internal reflection fluorescence (TIRF) microscopy appears as a carpet of spots decorating the cell basal membrane,
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Fig. 2. Membrane dynamics of p75NTR molecules in the cell membrane. (A) Expression of S6-p75NTR regulated by doxycycline (doxy). TIRF images of Abbrevior635P-labeled p75NTR receptors in SK-N-BE(2) cells show the dependence of the number of receptors per area (blue scale) on doxycycline concentration in the medium (black values below). (Scale bar, 5 µm.) (B) TIRF image of S6-p75NTR labeled with Abbrevior635P; superimposed trajectories are shown in blue. (Scale bar, 5 µm.) (C) S6-tagged constructs. The wt p75NTR is as in Fig. 1A; mut p75NTR bears mutations C256A and G256I, and lacks residues 221 to 246 encompassing the O-stalk domain in the JM region; and dim p75NTR has residues 213 to 251 of the JM portion replaced with the O-stalk domain in the JM region; and Callegari et al. (25), revealed that wt p75NTR and mut p75NTR are indistinguishable, while dim p75NTR is significantly slower, showing a distribution shifted toward D values compatible with what is expected for dimers (35) and a somewhat higher low-D tail (Fig. 2D); these data suggest the absence of stable dimers for both wt p75NTR and mut p75NTR. To identify possible transient p75NTR dimers, we analyzed the dynamic association/dissociation of spots during their trajectories (merge and split [M&S] events, shown in Movies S4 and S5 and schematized in Fig. 2F), as previously done by Kasai and Kusumi (36). The number of M&S events per membrane area was significantly lower for dim p75NTR than for wt p75NTR and mut p75NTR; the latter 2, instead, did not differ significantly (Fig. 2E). This shows that the 3 p75NTR constructs display an observable dynamic equilibrium between monomers and dimers, but while wt p75NTR has association/dissociation kinetics similar to mut p75NTR, dim p75NTR has either a higher dimerization probability or a lower separation rate. We also quantified the mean duration of transient dimerization (Td) events (orange in Fig. 2F), i.e., the trajectory segments between a merge event and a split event. The distribution of average Td lifetime demonstrated that while wt p75NTR and mut p75NTR display dimerization events equally peaked between 0.4 and 0.5 s, those of dim p75NTR peak at 0.6 and 0.8 s (Fig. 2G). These results allow us to determine that wt p75NTR does not form stable dimers or higher oligomers in the living cell membrane. Because of the transient nature of p75NTR dimers, as well as of photobleaching during tracking, analysis of the average intensity of the trajectories in live cells could not give an unambiguous answer on the stoichiometry (SI Appendix, Fig. S5). Therefore, we analyzed the intensity step photobleaching profile of isolated p75NTR/Abbrevior635P spots in fixed cells (yellow boxes in Fig. 3A). For each spot, we quantified 1) the number of photobleaching steps (red arrows in Fig. 3B) and 2) the mean intensity before bleaching (IPRE; green lines in Fig. 3B). Both constitute a direct measure of the number of molecules in a spot for receptor membrane oligomerization (37). The vast majority of analyzed wt p75NTR and mut p75NTR spots are monomers; that is, they display 1 photobleaching step (about 77% for both species; Fig. 3C). Conversely, the NGF-stimulated S6-TrkA construct displays a significantly higher proportion of dimers and oligomers (26) (SI Appendix, Fig. S6A). Importantly, the majority of dim p75NTR spots showed a 2-step photobleaching profile (55%; Fig. 3C), and monomers were reduced to 35%. Only dim p75NTR displayed a sizeable amount of spots with 3 and 4 photobleaching steps. IPRE distributions obtained for the 3 p75NTR variants confirmed the bleaching step analysis (SI Appendix, Fig. S6B).

We underline that some mut p75NTR apparent dimers were detected (about 20% of the analyzed spots) similar to wt p75NTR. This proves the lack of a relationship with the TM residues previously indicated as driving receptor dimerization (17). Overall,
our experiments distinguish between the diffusivity of monomers and dimers, and challenge the existence of stable p75NTR dimers in the membrane of live cells.

**wt p75NTR and mut p75NTR Display Different Membrane Partitioning in Response to NGF Stimulation.** We next compared wt p75NTR and mut p75NTR membrane diffusivity following NT stimulation, to see if this might impact the oligomeric state of the receptor. Also, we aimed at identifying a possible molecular basis, an alternative to the lack of oligomerization, as the source of the impaired apoptotic signaling of mut p75NTR following NT stimulation (17, 18).

We measured D values from trajectories of wt p75NTR and mut p75NTR with or without 15 min of NGF stimulation. The diffusivity of both constructs upon NGF treatment remained significantly higher than that of the dim p75NTR construct (Fig. S4). This suggests that NGF does not induce dimerization of either construct. These data are consistent with the absence of fluorescence resonance energy transfer (FRET) or homo-FRET changes following NT addition in cells expressing fluorescent p75NTR constructs (17, 38). However, NGF elicits a small but statistically significant shift of D distributions of the 2 constructs in opposite directions (i.e., it slightly slows down wt p75NTR, while it speeds up mut p75NTR). Similarly, a slowing down of wt p75NTR and mut p75NTR movements across membrane areas with different composition. In this scenario, lipid rafts may represent the discriminating factor, as they could confine and alter transiently the diffusivity of membrane proteins (39). Cholesterol plays a key structural role in lipid rafts, and the association of proteins with lipid rafts can be detected experimentally by testing cholesterol-dependent, confined diffusion (40). Consistently with this, p75NTR mobility was observed to depend on the cholesterol content in the plasma membrane (41), and we measured a clear anticorrelation between its D value and membrane cholesterol in 6 cellular models (Fig. 4B and SI Appendix, Fig. S8). The fastest p75NTR diffusivity is observed in cortical neurons, which display the lowest membrane cholesterol levels of our survey; SK-N-BE(2) cells have a cholesterol content similar to neurons, thus validating the choice of this model system to study p75NTR membrane dynamics.

**Fig. 3.** p75NTR is predominantly a monomer in the cell membrane. (A) TIRF image showing receptor spots on the surface of fixed cells (yellow squares represent analyzed spots; analyzed cells had 0.2 to 0.5 spot per square micrometer). (Scale bar, 1 μm) (B) Typical intensity profile traces of a monomer (Top), dimer (Middle), and trimer (Bottom) showing the parameters considered in the calculation. Im (green line) is the particle average intensity before the first bleaching step, red arrows point to single photobleaching steps, and the gray line represents background intensity. a.u., arbitrary units. (C) Photobleaching steps per trace for wt p75NTR, mut p75NTR, and dim p75NTR.

**Fig. 4.** Membrane cholesterol regulates p75NTR diffusivity and response to NGF. (A) Box-plot for D values from trajectories of wt p75NTR or mut p75NTR in SK-N-BE(2) cells in resting conditions (black) and up to 15 min after NGF administration (gray); the distribution for dim p75NTR (blue) is also shown. Boxes represent 25th to 75th percentiles, lines represent medians, and dashed lines represent means. ***P < 0.001, 1-way ANOVA (with Bonferroni comparison of means). (B) Plot of membrane cholesterol (CHO) content (mean intensity ± SEM of filipin III-stained cells) versus D of p75NTR single molecules (peak ± full width at half maximum of its distribution as in SI Appendix, Fig. S6C) in 6 different cell models. Cholesterol content is normalized to SK-N-BE(2) results. The black line indicates linear fit. HEK, human embryonic kidney 293 cells. (C) TIRF images of filipin III-stained SK-N-BE(2) cells exhibiting modulation of cholesterol levels (quantified as mean intensity ± SEM) with mevastatin (meva., green) or soluble cholesterol (chole., red). *P < 0.05 and ***P < 0.001, 1-way ANOVA. (Scale bar, 10 μm) (D) Same graph as in A, obtained for NGF-stimulated wt and mut S6-p75NTR trajectories in SK-N-BE(2) cells (gray), treated with mevastatin (green) and cholesterol (red). ***P < 0.001, Kruskal–Wallis test (with Dunn’s means comparison). (E) Outline of the colocalization experiment with TIRF images of cholera toxin B subunit (CT-B)-stained GM1 (green) and p75NTR single molecules (red). (Scale bar, 5 μm; Inset, 1 μm) (F) Quantification of wt/mut p75NTR and CT-B colocalization in the absence or presence of NGF. Box-plots show median and 25th to 75th percentiles of average p75NTR intensity inside CT-B–stained domains (I_{CT-B} over average p75NTR intensity within the whole cell I_{total} = p75NTR). Whiskers indicate Tukey intervals, and red circles represent individual data. *P = 0.016, 1-way ANOVA (with Bonferroni comparison of means). ns, not significant at the 0.05 level.
Given that raft domains are crucial for apoptotic signaling via p75NTR (42), we considered that differential residency of wt p75NTR and mut p75NTR upon NT binding in these areas might explain not only the D changes observed (Fig. 4A) but also their different signaling abilities. To test this hypothesis, we first monitored NGF-driven diffusivity of the 2 receptor forms following up- or down-regulation of membrane cholesterol in SK-N-BE(2) cells (Fig. 4C). While increasing membrane cholesterol slows down both receptor forms, removal of membrane cholesterol has 2 opposite outcomes: wt p75NTR is accelerated, while mut p75NTR slowed (Fig. 4D). Since the effect of cholesterol depletion on lateral diffusion depends on the composition of the membrane regions explored by the membrane receptors (43), these data suggest that wt p75NTR and mut p75NTR partition into different membrane areas after NGF binding. These relocalizations most probably occur in a very dynamic way, given the small diffusivity changes involved (Fig. 4A and SI Appendix, Fig. S7).

Indeed, biochemical isolation of raft domains showed wt p75NTR localizing in both raft and nonraft areas, in the absence and presence of NGF (44, 45). Thus, to capture transient raft occupancy, we imaged lipid rafts and p75NTR simultaneously, after 15 min of NGF stimulation, by dual-color TIRF microscopy (Fig. 4E), similar to the method used by Plaum et al. (46). Rafts were visualized by cross-linking membrane ganglioside GM1 with a fluorescent cholera toxin B subunit (47). NGF stimulation significantly increases the localization of wt p75NTR in GM1 regions, but not that of mut p75NTR (Fig. 4F).

We conclude that p75NTR translocates to lipid rafts upon NGF binding, and mut p75NTR has a reduced residency in cholesterol-rich membrane microdomains upon NGF binding compared with the wt counterpart. Notably, in the absence of competing Trk receptors, both NTs and proNTs induce coherent effects on p75NTR in terms of both membrane diffusivity (Fig. 4A and SI Appendix, Fig. S7) and biological activity; for instance, proBDNF induces apoptosis via p75NTR (18), but mut p75NTR also mediates apoptosis in retinal neurons by NGF (48) and in sympathetic neurons by BDNF (49).

Membrane Cholesterol Regulates p75NTR Apoptotic Signaling. Following previous observations, we tested if membrane cholesterol levels also affected NT-dependent apoptotic signaling via p75NTR; here, C256 TM residue was shown to play a crucial role (17, 18). The neurons with invagination of methyl-bromo-cyclodextrin (MβCD) strongly decreases membrane cholesterol, as measured by filipin staining, while loading neurons with cholesterol has the opposite effect (Fig. 5A and B). The proBDNF-induced apoptosis was abolished in cholesterol-depleted cortical neurons from wt mice, while it was slightly increased upon cholesterol overload (Fig. 5C). Cholesterol modulation was also applied to wt p75NTR and mut p75NTR (Fig. 5 D–F) transduced in p75NTR KO mouse neurons and induced with 0.05 μg/mL doxycycline, a concentration not leading to overexpression (Fig. 2A). The p75NTR KO neurons were not responsive to proBDNF; wt p75NTR, but not mut p75NTR, restored proapoptotic signaling (Fig. 5D). When neurons were treated with mevastatin/MβCD, wt p75NTR lost its ability to induce apoptosis, confirming our results on wt neurons (Fig. 5 C and E). Conversely, cholesterol administration boosted proapoptotic signaling of wt p75NTR (Fig. 5 F and G); surprisingly, the same treatment also conferred apoptotic capability to mut p75NTR (Fig. 5 F and G). This was not an effect of the combination of proBDNF and cholesterol load: Untransduced neurons from p75NTR KO mice were not responsive in these conditions (Fig. 5F).

From these results, we conclude that the inability of mut p75NTR to induce apoptosis (18) (Fig. 5D) is due to its poorer occupancy of cholesterol-rich membrane regions when compared with wt p75NTR, rather than to impaired signaling of the protein per se. Accordingly, under membrane-saturating conditions obtained inducing p75NTR expression with 1 μg/mL doxycycline (Fig. 2A), both mut p75NTR and wt p75NTR were able to induce apoptosis (SI Appendix, Fig. S9). These findings, along with those obtained in SK-N-BE(2) cells (Fig. 4), show that NT binding regulates the partitioning of p75NTR in and out of lipid rafts, thereby regulating its ability to induce apoptosis.

Surface-Exposed p75NTR Mediates Growth Cone Collapse in the Presence and Absence of proNGF. Growth cone retraction caused by overexpression of both wt p75NTR and a C256A p75NTR mutant was reported to occur upon proNGF administration in developing neurons (20). Hence, p75NTR collapse action may not necessarily depend on receptor partitioning in cholesterol-rich regions, unlike apoptotic signaling (Fig. 5). We therefore investigated the mechanisms of axonal growth regulation by p75NTR. We found that endogenous levels of p75NTR can also regulate axon branching. Axonal arbors of CA3 neurons projecting into the CA1 region are significantly more ramified and occupy larger areas in p75NTR KO mice than in wt mice (SI Appendix, Fig. S10 A and B). This is reflected in a higher number of synaptic boutons (SI Appendix, Fig. S10C), consistent with previous observations of p75NTR KO animals showing increased dendritic complexity (50). Neuronal cultures of the same animals recapitulated this result (Fig. 6 A and B), with p75NTR KO axons being longer (Fig. 6C) and displaying an increased number of branch points (Fig. 6D) and lateral growth cones per length unit (Fig. 6E) with respect to wt axons. Importantly, transient expression of wt p75NTR, or mut p75NTR constructs in p75NTR KO neurons completely rescued the phenotype observed in wt neurons (Fig. 6 C–E). In agreement with previous data (20), expression of either wt p75NTR or mut p75NTR in hippocampal neurons leads to growth cone collapse in response to proNGF (SI Appendix, Fig. S11 A and B). Overall, this suggests that the regulation of axonal complexity and proNT-dependent collapse of growth cones share a common mechanism regulated by p75NTR independently on Cys256 and other residues mutated or missing in mut p75NTR (Fig. 2C).

To gain further insight into this mechanism, we monitored the membrane pool of S6-p75NTR-EGFP during collapse by biotinylating the receptors on the cell surface before proNGF incubation, and detecting receptors still present on the plasma membrane with streptavidin-Qdot after proNGF incubation for 30 min; EGFP fluorescence marked the total content of p75NTR (Fig. 7A). The proNGF caused a dramatic increase in the membrane pool of both wt p75NTR and mut p75NTR, while lower levels of membrane p75NTR were detected in untreated neurons (Fig. 7B and C). Inhibition of dynamin-dependent internalization with Dynasore (Fig. 7D), to maintain wt and mut S6-p75NTR–EGFP on the surface regardless of proNGF administration (Fig. 7E), was sufficient to drive the growth cone collapse, independently from ligands; indeed, proNGF had no further collapse-inducing effect, implying that p75NTR exposure is a downstream event to ligand binding (Fig. 7F and SI Appendix, Fig. S11A). Notably, Dynasore alone in untransfected neurons did not have such a prominent effect, although a trend could be observed upon drug treatment and proNGF administration (Fig. 7F); this is likely due to a fraction of hippocampal neurons expressing detectable levels of p75NTR, as previously shown (29). Inhibiting p75NTR internalization by expressing the K44A dominant negative form of dynamin had the same effect (SI Appendix, Fig. S11 D and E), confirming the results obtained with Dynasore. Furthermore, we found that the mechanism responsible for the removal of p75NTR from the plasma membrane in the absence of proNGF is dependent on clathrin, as blocking clathrin-dependent endocytosis by Pitstop2 was sufficient to accumulate surface p75NTR and trigger neuronal growth cone collapse (Fig. 7 G–I).

These data suggest that p75NTR has an intrinsic collapsing activity when retained on the growth cone membrane, and internalization inhibition is a sufficient driving force that does not
necessarily require NT-induced partitioning into raft domains. Indeed, both wt p75NTR and mut p75NTR constructs similarly regulate axonal complexity. Overexpression has been called into question in the evaluation of growth cone collapse (18). Indeed, p75NTR expression by a constitutively strong promoter results in a many-fold expression increase compared with that of p75NTR induced at 0.05 μg/mL doxycycline (SI Appendix, Fig. S12), which recapitulates the behavior of endogenous p75NTR (Fig. 5). We therefore evaluated the effect of proNGF on neurons infected with wt p75NTR or mut p75NTR and induced with 0.05 μg/mL or 1 μg/mL doxycycline (SI Appendix, Fig. S13A). We found that growth cone collapse could still be observed, although at lower levels than with overexpressed p75NTR. The growth cone area decreases with increasing surface p75NTR density: In particular, a threshold for growth cone collapse was found in the range of 1 to 2 receptors per square micrometer (SI Appendix, Fig. S13B and C). Although slightly higher than the receptor ranges explored in our advanced imaging (Fig. 3 and SI Appendix, Fig. S7) and apoptosis assays (Fig. 6A–F), these results are consistent with previous findings (18).
5), this value seems compatible with physiological levels observed at least in a subset of central neurons displaying sufficient p75NTR levels to drive cone retraction (29). At 0.05 μg/mL doxycycline, p75NTR explores a range of expression levels on the neuronal surface, with densities spanning from below to above the range of 1 to 2 receptors per square micrometer, and this explains why only a subpopulation of neurons undergoes growth cone collapse in this sample (SI Appendix, Fig. S13). Importantly, the growth cone area distribution for mut p75NTR neurons was not significantly different from that for wt p75NTR, and the 2 forms displayed similar area versus p75NTR level dependency (SI Appendix, Fig. S13C), thus ruling out overexpression as the cause for their identical behavior (Fig. 6). This demonstrates that growth cone collapse via p75NTR can occur at receptor levels close to or slightly higher than the natural average density in neurons, and that both p75NTR forms are equally capable of mediating it.

**Discussion**

To solve the oligomerization conundrum of p75NTR in a live cell context and to gain insight into its mechanisms of activation by NTs, we applied a single-molecule fluorescence approach that labeled antibodies or ligands and obviates the problem of Qdot aggregation with unknown proteins or lipids in close proximity. Indeed, both run length and intensity of higher weight bands in a gel critically depend on several technical parameters, such as lysis conditions, antibody, or composition of the gel (SI Appendix, Table S1 and compare, e.g., SI Appendix, Figs. S4 and S14). Heavier p75NTR-immunoreactive bands may be the result of p75NTR homo- or hetero-aggregation with unknown proteins or lipids in close proximity. Indeed, p75NTR oligomerization state in the cell membrane.

Evidence for oligomerization mostly came from the electrophoretic shift of the immunodetected receptor band of 2- or 3-fold the weight of the monomer, in nonreducing conditions or after chemical cross-linking. However, this constitutes an indirect way of investigating stoichiometry. Indeed, both run length and intensity of higher weight bands in a gel critically depend on several technical parameters, such as lysis conditions, antibody, or composition of the gel (SI Appendix, Table S1 and compare, e.g., SI Appendix, Figs. S4 and S14). Heavier p75NTR-immunoreactive bands may be the result of p75NTR homo- or hetero-aggregation with unknown proteins or lipids in close proximity. Indeed, p75NTR oligomerization state in the cell membrane.

Fig. 6. wt p75NTR and mut p75NTR mediate growth cone collapse and regulate axon complexity. (A) Hippocampal neurons from wt (Left) and p75NTR KO (Right) mice. Axons from EGFP- and TagRFP-actin-expressing neurons are drawn in black and superimposed to the EGFP channel (grayscale). (Scale bar, 100 μm.) (B) Magnification of axon terminals from images in A also showing immunofluorescence for the axonal marker NF-200. TagRFP-actin accumulates at growth cones. Branching points (arrow), terminal growth cones (filled arrowheads), and lateral growth cones (empty arrowheads) are indicated. (Scale bars, 5 μm.) (C) Quantification of axon length (C), number of branch points (D), and number of lateral growth cones per length unit (E) are illustrated in non-transfected (nt) wt hippocampal neurons (white columns), in nt p75NTR KO hippocampal neurons (black columns), or transfected with wt p75NTR or mut p75NTR constructs (wt/mut p75NTR, black columns). ***P < 0.001, Kruskal-Wallis test (Dunn’s multiple comparisons). Bars are mean ± SEM.
were quantified to be a 3/6-fold overestimation in similar ranges of receptor densities (54).

Our dynamics data question the possibility of a covalent TM p75NTR dimerization, challenging a previous model for the p75NTR mechanism of action, which postulates that NT binding to the putative preformed p75NTR covalent dimer induces a conformational change propagated via Cys256, leading to separation of receptor densities (55). We propose an alternative molecular mechanism of action, which postulates that NT binding by structural considerations on the flexibility of the JM and JH chopper domains (19). We propose an alternative molecular mechanism of action, which postulates that NT binding by structural considerations on the flexibility of the JM and JH chopper domains (19). We propose an alternative molecular mechanism of action, which postulates that NT binding by structural considerations on the flexibility of the JM and JH chopper domains (19).
partners like ephrin-A (57), eventually leading to Rac and RhoA activation (29, 58). It is also possible that p75NTR binds different proteins on the plasma membrane and intracellular stores, possibly regulating their availability. In any case, this activity is not dependent on the TM and JM residues mutated or missing in mut p75NTR, and therefore on the membrane partitioning necessary for proapoptotic signaling (Fig. 8). Accordingly, axonal extension and branching are enhanced in p75NTR KO hippocampal neurons (Fig. 6 A and B and SI Appendix, Fig. S10) and this enhancement is suppressed by either wt p75NTR or mut p75NTR expression (Fig. 6 C–E). These data corroborate the observation that sympathetic sprouting is enhanced in p75NTR–KO and p75NTR–KO KO mice (59). In addition, p75NTR–KO and p75NTR–KO KO neurons display increased dendritic complexity, and p75NTR-rich dendritic regions are particularly devoid of collateral branches (50).

In summary, our results demonstrate that p75NTR exists predominantly as a fast-diffusing monomer at the neuronal plasma membrane, and that its signaling capabilities depend on the membrane microdomains traversed and on the amount of surface-exposed receptor available in particular neuronal compartments. Interestingly, while this paper was in preparation, the existence of TrkB dimers was challenged by demonstrating that this receptor is mostly active as monomer on the plasma membrane: This posed severe doubts regarding a long-accepted view of TrkB dimerization as a key step in the transduction of BDNF signaling, partially supported by electrophoretic shifts after chemical cross-linking (60). A scenario emerges in which availability of NT receptor monomers is a fundamental step for signal transduction via Trk-NT-p75NTR constructs induced by the binding of clustered forms of NTs, as recently proposed based on structural data (61). It remains to be established, however, whether coexpression of Trks results in changes of the p75NTR oligomerization state in the cell plasma membrane. In any case, the multifaceted mechanisms of action of the p75NTR monomer suggested by our findings can successfully reconcile most of the apparently conflicting data reported in the literature for the structure and function of this pleiotropic receptor.

Material and Methods

Stoichiometry by Single-Molecule Step Photobleaching.

The wt p75NTR, mut p75NTR, and dim p75NTR constructs were transduced in 5K-N-B2E2 cells, labeled with Abberior 635P and then fixed for 90 min at room temperature with 4% paraformaldehyde, 5% sucrose, and 0.1% glutaraldehyde in phosphate-buffered saline (PBS); washed 5 times with PBS; and imaged in PBS on the TIRF microscope. Three thousand-frame movies were acquired in a 32.68 x 32.68-xm range of concentrations compatible with the endogenous levels (SI Appendix, Fig. S13). This is particularly plausible if the observed polarized distribution of surface p75NTR is taken into account (Fig. 1D). The mechanism is likely to support collapse in a cone-autonomous way. Indeed, collapsed cones show higher levels of surface p75NTR than extended ones even within the same neuron (Fig. 7). The p75NTR surface density is most probably controlled by removal from the plasma membrane and sequestration in intracellular stores. Acute proNGF administration increases both wt p75NTR and mut p75NTR surface pools at growth cones, and inhibition of p75NTR internalization mimics the proNT effect (Fig. 7 and SI Appendix, Fig. S11). Although we cannot tell whether proNGF prevents p75NTR internalization or increases p75NTR recycling onto the plasma membrane (Fig. 8B), we argue that a signaling cascade activated by surface-exposed p75NTR is responsible for growth cone retraction and axonal complexity. This mechanism could be activated by proNT binding, p75NTR local accumulation (28), or interactions with membrane

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measure of membrane versus total receptor pool; and 3) for all p75NTR constructs, the intensities in the QD channel as a measure of membrane abundance at the distribution levels.

More details on material and methods appear in SI Appendix. Readers will be able to access codes and materials by directly contacting the corresponding authors.

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