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RESEARCH ARTICLE

Disentangling the signaling complexity of nerve growth factor receptors by CRISPR/Cas9

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Abstract

The binding of nerve growth factor (NGF) to the tropomyosin–related kinase A (TrkA) and p75^{NTR} receptors activates a large variety of pathways regulating critical processes as diverse as proliferation, differentiation, membrane potential, synaptic plasticity, and pain. To ascertain the details of TrkA-p75^{NTR} interaction and cooperation, a plethora of experiments, mostly based on receptor overexpression or downregulation, have been performed. Among the heterogeneous cellular systems used for studying NGF signaling, the PC12 pheochromocytoma-derived cell line is a widely used model. By means of CRISPR/Cas9 genome editing, we created PC12 cells lacking *TrkA*, *p75^{NTR}*, or both. We found that TrkA-null cells become unresponsive to NGF. Conversely, the absence of p75^{NTR} enhances the phosphorylation of TrkA and its effectors. Using a patch-clamp, we demonstrated that the individual activation of TrkA and p75^{NTR} by NGF results in antagonizing effects on the membrane potential. These newly developed PC12 cell lines can be used to investigate the specific roles of TrkA and p75^{NTR} in a genetically defined cellular model, thus providing a useful platform for future studies and further gene editing.

KEYWORDS
gene editing, nerve growth factor, p75^{NTR}, PC12 cells, signaling, TrkA

1 | INTRODUCTION

NGF-mediated signaling regulates the development and physiology of a wide variety of cells,[1] such as sensory and sympathetic neurons,[2,3] cholinergic neurons,[4] glial,[5] and microglia cells,[6] as well as non-neuronal cells of epithelia, vessels, muscles, endocrine tissues,[7] and of the immune system.[8,9] In recent years, the role of NGF signaling has received increasing attention aiming at diverse clinical applications, including cancer,[10]

Abbreviations: Akt, Akt kinase, also known as protein kinase B; CD2AP, CD2-associated protein; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Grb2, growth factor receptor-bound protein 2; HRP, horseradish peroxidase; HSAN V, hereditary sensory and autonomic neuropathy type V; LINGO-1, leucine-rich repeat and immunoglobin-like domain-containing protein 1; NGF, nerve growth factor; NOGO, neurite outgrowth inhibitory protein; PC12, pheochromocytoma cell line 12; PI3K, phosphatidyl inositol 3-kinase; PLC-γ, phospholipase C-γ; REST, RE-1 silencer of transcription; SH2-B, SH2-B adaptor protein; Shc, Src homology and collagen containing transforming protein; Src, proto-oncogene tyrosine-protein kinase Src; TrkA, tropomyosin–related kinase A receptor.

Giovanna Testa and Marco Mainardi equally contributed.

pain-related disorders,[11-13] and neurodegenerative diseases.[14,15]

The biological action of NGF depends on the cellular context and is elicited through binding and activation of its receptors, namely TrkA (Tropomyosin-Related Kinase receptor type I)[16-19] and $p75^{NTR}$ (a member of the Tumor necrosis factor receptor superfamily).[20-23] TrkA has the highest affinity for NGF, and responds to ligand binding with dimerization and transphosphorylation, leading to the activation of key signaling pathways, such as extracellular signal-regulated kinase (ERK), phospholipase C-γ (PLC-γ) and phosphatidyl-inositol 3 kinase (PI3K).[21] These intracellular pathways promote NGF-mediated survival, differentiation, and synaptic plasticity.[24]

$p75^{NTR}$ is a single-pass transmembrane receptor with significant binding affinity for all neurotrophins and their immature forms (i.e., pro-neurotrophins[25]). The polarity of $p75^{NTR}$ effects depends on its interacting partners: with sortilin, $p75^{NTR}$ causes apoptosis mediated by pro-neurotrophins[26]; with LINGO-1 and Nogo-A, it participates in myelin-dependent inhibition of axonal growth[27]; with Trks, it promotes survival, axonal growth, and differentiation.[28] Regarding this latter cooperation, several works showed that interaction with $p75^{NTR}$ increases the NGF binding affinity of TrkA,[29] and potentiates signaling activation.[30] In addition, NGF regulates the ubiquitination of TrkA,[31,32] along with its increased endocytosis and retrograde transport.[33]

Thus, the physical or functional cooperation of $p75^{NTR}$ with TrkA is recognized to be a cellular process of paramount importance. On the contrary, the interaction mode and stoichiometry of TrkA and $p75^{NTR}$ in the absence or presence of NGF stimulation are still a matter of hot debate.[30,34,35,36]

Through the years, several studies on this cooperation were carried out using PC12 cells.[37-39] This cell line, which was established from a rat pheochromocytoma, is a gold standard model for assessing the biological activity of NGF in vitro,[40] owing to the expression of both NGF receptors. Indeed, PC12 cells are able to acquire the phenotype of sympathetic neurons (an NGF-dependent population) when exposed to NGF.[41]

To analyze the individual contribution of NGF receptors to downstream signaling and the consequent cellular responses, several PC12 clones have been generated. For instance, (i) the PC12nnr5 clone, selected by chemically mutagenized cultures and notably lacking a genomic characterization, does not express TrkA[42]; (ii) trk-PC12 cells stably overexpress TrkA, and have been used to investigate the role of TrkA in NGF-induced differentiation[43]; (iii) the PC12-27 clone has wild type-like levels of TrkA, while the expression of $p75^{NTR}$ is negligible, due to the repressive effect exerted by the REST (RE-1 silencer of transcription).[44] However, all these PC12 cell variants are genetically ill-defined.

Here, in order to dissect the contributions of TrkA and $p75^{NTR}$ in mediating NGF-dependent signaling effects in the context of a genetically controlled background, we exploited the CRISPR/Cas9 gene editing technology.[45] Using this approach, we generated and characterized three PC12 clones: (i) TrkA knockout; (ii) $p75^{NTR}$ knockout; (iii) TrkA-$p75^{NTR}$ double knockout. Upon comparison with wild type PC12 cells, these three gene-edited cellular models gave us the opportunity to assess the contribution and fine tuning of the individual and combined receptor effects to intracellular signaling, without any possible confounding source of variability caused by, e.g., the use of antisense oligonucleotides, chemical inhibitors, drugs, or protein overexpression.

2 | MATERIALS AND METHODS

2.1 | Molecular biology for gene editing

The human codon-optimized Cas9 and chimeric guide RNA expression plasmid (pX459) developed by the Zhang lab[46] were obtained from Addgene (Waterton, MA, USA). To generate gRNA plasmids, a pair of annealed oligonucleotides (20 base pairs) were cloned with the BbsI restriction enzyme into the single guide RNA scaffold of the pX459 plasmid.

The following gRNAs sequences were used to generate TrkA KO#1:

Forward1: 5′-caccGTTGGCATCGCCCGGCCGCG-3′;

Reverse1: 5′-aaacCGCGGCCGGGCGATGCCAAC-3′.

The following gRNAs sequences were used to generate $p75^{NTR}$-1 KO#2.

Forward2: 5′-caccCACGCCTTCGCCCAAGTTGC-3′;

Reverse2: 5′-aaacGCAACTTGGGCGAAGGCGTG −3′.

The following primers were used to genotype TrkA KO cells:

P1F: 5′-CAGCTGGGTTGGCATCGCCC-3′;

P1R 5′-CGCGGAGGGTATTCAGGGTCC-3′;

The following primers were used to genotype $p75^{NTR}$ KO cells:

P2F: 5′-TTGATCCCTTGGAAGACGCC-3′;

P2R: 5′-TAGTGGACTGGAGGAGAGGC-3′.

2.2 | Cell culture and transfection

Rat pheochromocytoma PC12 cells line were maintained at 37°C and 5% CO_2 in DMEM medium (Invitrogen, Monza, Italy) supplemented with 10% horse serum, 5% fetal bovine serum, 1% penicillin/streptomycin,

1% L-glutamine (Gibco-ThermoFisher, Monza, Italy), and grown as monolayer cultures according to ATCC standard protocols. Cells were plated at 80%–90% confluence in 3 cm diameter Petri dishes and transfected using Lipofectamine 2000 (Thermo-Fisher Scientific, Monza, Italy; 11 668-027) according to the manufacturer's instructions. Individual PC12 clones were obtained by treating the transfected PC12 cells with 6–9 μg/ml of puromycin for 24/36 h after transfection before replating transfected cells in 96-well plates at limiting dilution, in order to achieve single-cell seeding and subsequent monoclonal expansion. Then, the selected PC12 cell clones were routinely grown and frozen as described in ATCC protocols.

2.3 | Western blot

PC12 cells and mutant PC12 clones were cultured in 3 cm diameter petri dishes. PC12 cells were stimulated with wild type NGF (5 or 100 ng/ml) or maintained in basal conditions, then harvested at different post-stimulation times. Cells were lysed in RIPA buffer containing the following (in mM): NaCl (150), EDTA,[5] PMSF,[1] TRIS–HCl pH 7.5,[10] and Triton X-100 1%, Na-deoxycholate 1%, SDS 0.1%, 1× protease inhibitor cocktail (Sigma-Aldrich, Darmstadt, Germany). Complete mechanical dissociation was obtained by sonication. Then, cells were incubated for 30 min in ice and, finally, centrifuged at 20 000×*g* for 30 min at 4°C. The supernatant was recovered and the total protein concentration was measured using a BSA-based Bradford assay (Bio-Rad, Segrate, Italy). Protein extracts were run on 10% acrylamide gels and blotted on nitrocellulose membranes. Membranes were blocked for 1 h at RT using 5% milk and 0.5% Tween in TBS, then incubated O/N at 4°C under gentle rocking using primary antibodies solutions prepared in the same blocking solution. The primary antibodies used were: anti-TrkA (1:1000, Millipore, Darmstadt, Germany; 07-432), anti-p75^{NTR} (1:1000, Millipore 07-476), anti-phospho-Akt (1:1000, Cell Signaling Technology, Danvers, MA, USA; 9275), anti-Akt (1:1000, Cell Signaling Technology 9272), anti-phospho-p44/42 ERK (1:2000, Cell Signaling Technology 9101), anti-p44/42 ERK (1:1000, Cell Signaling Technology 9102), all produced in rabbit; mouse anti-GAPDH (1:10 000, Fitzgerald Industries International, Acton, MA, USA; 10R-G109a). Then, blots were rinsed 3 times for 10 min in TBS-Tween 0.1% and incubated with either goat anti-rabbit (1:5000, Santa Cruz Biotechnology, Dallas, TX, USA; SC-2004) or goat anti-mouse (1:10 000, Santa Cruz Biotechnology SC-2005) HRP-conjugated secondary antibodies. The signal was revealed with ECL reagents (Bio-Rad) and acquired using a ChemiDoc system (Bio-Rad). The optical density of bands was quantified using ImageJ (NIH, Bethesda, MD, USA).

2.4 | Cell differentiation

PC12 cells were maintained as described above. For differentiation assays, cells were plated into 12-well plates coated with 20 μg/ml Poly-L-Lysine (P4707 Sigma–Aldrich) at low density (1×10^4 cells/cm^2). Differentiation was induced by treatment with serum-free medium supplemented with wild type NGF at different concentrations: 5, 10, 20, 50, and 100 ng/ml. Exposure to serum-free medium alone was used as a control. Cells were imaged after 5 days of treatment using an AxioObserver microscope (Zeiss, Jena, Germany) at 40× magnification. Morphological analysis of differentiation was performed on imaged cells using ImageJ (NIH), and the average length of neurites of differentiated cells was measured, with the operator being blind to the genotype of cells.

2.5 | Patch-clamp recordings

Recordings were performed by adapting the procedure described in.[47] Briefly, cells were cultured on poly-L-lysine-coated glass coverslips, then transferred to a submerged recording chamber, continuously perfused with oxygenated Tyrode's solution containing (in mM): NaCl (150), KCl,[4] $MgCl_2$,[1] $CaCl_2$,[4] Glucose,[10] HEPES,[10] pH 7.4 with NaOH. Borosilicate glass pipettes (1B150F-4, WPI, Sarasota, FL, USA) were pulled with a P-97 puller (Sutter Instruments, Novato, CA, USA) to a resistance of 5–6 MΩ when filled with an internal solution containing (in mM): K-Gluconate (145), $MgCl_2$ 2, HEPES,[10] EGTA (0.1), Mg^{2+}-ATP (2.5), Na^+-GTP (0.25), phosphocreatine,[5] pH 7.35 with KOH. After achieving whole-cell configuration and allowing at least 3 min for complete equilibration between cytosol and internal solution, the membrane potential was recorded with the amplifier in the $I = 0$ configuration. NGF (100 ng/ml) was delivered via bath application. To analyze the dependency of NGF-induced variations in the membrane potential on K^+ and Na^+ currents, 5 mM tetraethylammonium (TEA) was added to the bath, or NaCl was substituted with 135 mM N-methyl-D-glucamine (NMDG), respectively.[48]

Recordings were performed at 32°C. Access resistance and membrane capacitance were monitored during each recording, which was discarded if series resistance varied more than 20% of the initial value. Data were acquired using a MultiClamp 700A amplifier, connected to a Digidata 1550A digitizer (Molecular Devices, San Jose,

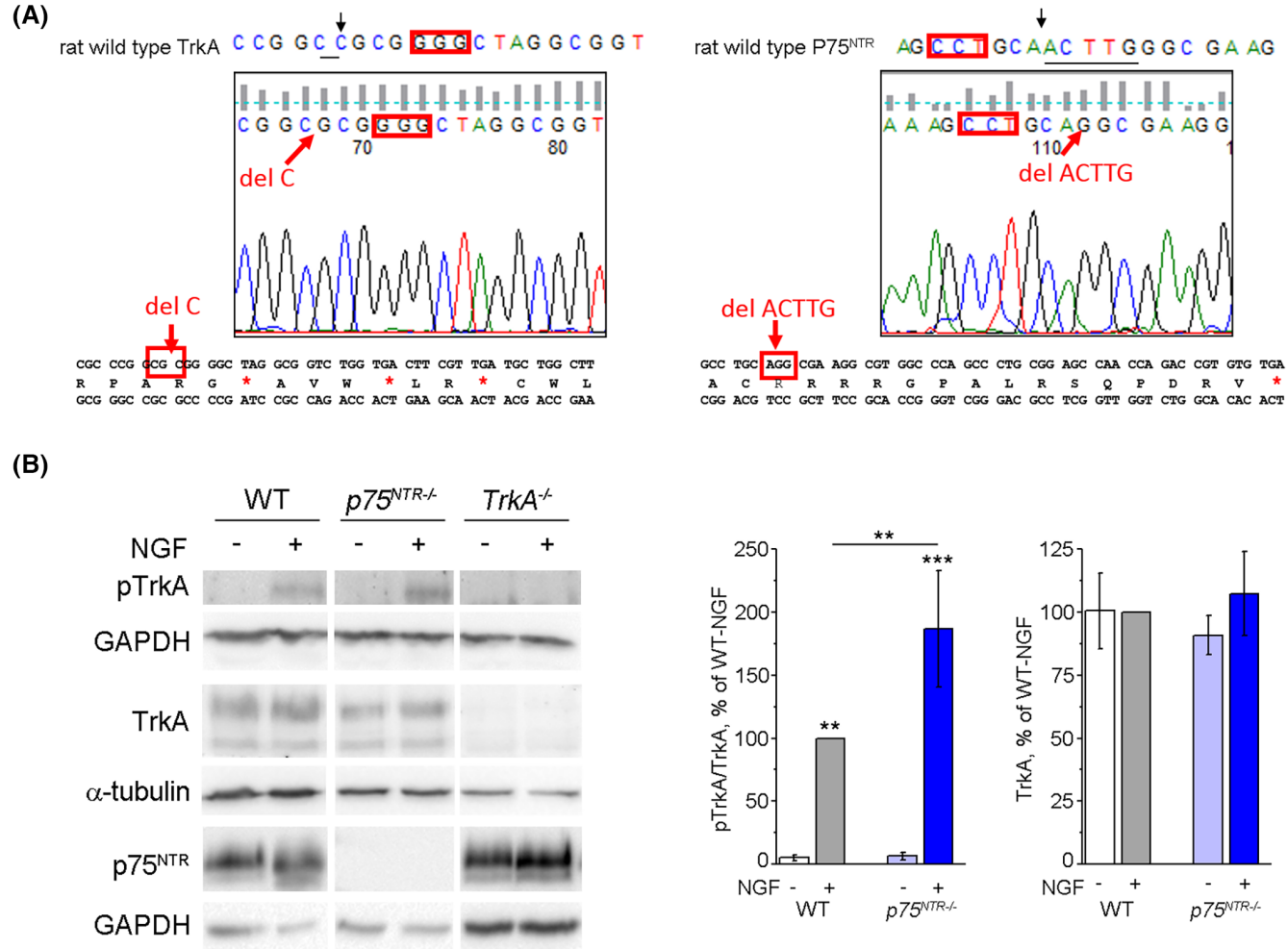


FIGURE 1 CRISPR-Cas9-assisted generation of PC12 cell lines lacking NGF receptors. (A) Sequencing spectropherograms for *TrkA* (left) and $p75^{NTR}$ (right) showing protospacer adjacent motifs (PAMs, bold red rectangles), mutation sites (black arrow and underlining), nucleotide deletions (del, red arrows) leading to the generation of premature stop codons (red asterisks). (B) Western blot analysis confirming the loss of $p75^{NTR}$ and TrkA expression in $p75^{NTR-/-}$ and $TrkA^{-/-}$ cells, respectively. Quantification of phospho-TrkA (pTrka) in wild type (WT) and $p75^{NTR-/-}$ cells shows increased levels in $p75^{NTR-/-}$ cells treated with NGF (ANOVA-2, p[genotype×treatment] = .033, followed by Student–Newman–Keuls post hoc test, WT + NGF vs. WT, $^{**}p = .002$; $p75^{NTR-/-}$ + NGF vs. $p75^{NTR-/-}$, $^{***}p < .001$; $p75^{NTR-/-}$ + NGF vs. WT + NGF, $^{**}p = .006$). No significant difference was observed in the total levels of TrkA (ANOVA-2, p(genotype) = .920, p(treatment) = .483, p(genotype×treatment) = .456). WT, $n = 5$; WT + NGF, $n = 5$; $p75^{NTR-/-}$, $n = 6$; $p75^{NTR-/-}$ + NGF, $n = 4$.

CA, USA), and sampled at 10 kHz. Analysis was done with Clampfit 11.1 (Molecular Devices).

3 | RESULTS

3.1 | Gene editing of PC12 cells to generate *TrkA* and $p75^{NTR}$ knock-outs and *TrkA*/$p75^{NTR}$ double knock-outs

To disentangle the specific contributions of TrkA and $p75^{NTR}$ to the effects of NGF, we used CRISPR/Cas9 gene editing to generate PC12 cell mutants lacking *TrkA*, $p75^{NTR}$, or both genes. Guide RNAs (gRNAs) were designed to avoid off-target editing (see Materials and Methods). To counteract possible phenotypic artifacts due to the selection of a single cell clone, we isolated and characterized at least 3 different independent PC12 clones for each genotype ($TrkA^{-/-}$, $p75^{NTR-/-}$, $TrkA^{-/-}$; $p75^{NTR-/-}$). The relevant genomic region from each PC12 clone was sequenced to verify the presence of a nonsense frameshift mutation near the protospacer adjacent motif (PAM) sequence (Figure 1A). Western blot analysis confirmed the lack of expression of the corresponding proteins, namely TrkA and $p75^{NTR}$, in gene-edited clones (Figure 1B and Supporting Information Figure S1A).

3.2 | Analyzing NGF-mediated signaling in *TrkA*$^{-/-}$ PC12 cells

After checking that *TrkA* knock-out results in the absence of the corresponding protein without affecting the levels of the other main NGF receptor, p75^{NTR}, we also wanted to verify that the corresponding signaling effectors were unresponsive to NGF.

Therefore, we stimulated *TrkA*$^{-/-}$ PC12 cells with 100 ng/ml NGF. In accordance with the absence of phosphorylated TrkA (Figure 1B), the phosphorylation of the main effectors of TrkA, i.e., ERK and Akt, was severely impaired in *TrkA*$^{-/-}$ clones (Figure 2A,B).

To demonstrate that the lack of responsiveness to NGF of *TrkA*$^{-/-}$ mutants do not affect the capability to respond to other stimuli, cells were treated with 100 ng/ml Fibroblast Growth Factor (FGF). Indeed, FGF binds to a different tyrosine kinase receptor (FGFR), but converges on the same intracellular pathways as NGF, including Akt and ERK.[49] Stimulation with FGF resulted in normal ERK and Akt phosphorylation, demonstrating the presence of active signaling mediated by FGFR in *TrkA*$^{-/-}$ PC12 cells (Supporting Information Figure S2A).

Finally, we wanted to prove whether the re-expression of TrkA in *TrkA*$^{-/-}$ null PC12 cells is sufficient to restore NGF-induced neural differentiation. To this end, we transfected *TrkA*$^{-/-}$ cells with a construct carrying wild type TrkA, and we observed a recovery of neurite outgrowth in response to NGF. On the contrary, transfection with a "dead" TrkA mutant[50] did not rescue responsiveness to NGF of *TrkA*$^{-/-}$ cells (Supporting Information Figure S2B).

These data demonstrate that (i) TrkA is required to activate the ERK and Akt pathways in response to NGF; (ii) the absence of TrkA does not disrupt the responsiveness of Akt and ERK to extracellular stimuli different from NGF; (iii) re-expression of wild type TrkA in PC12 *TrkA*$^{-/-}$ cells recover their responsiveness to NGF in terms of neurite outgrowth.

3.3 | Analyzing NGF-mediated signaling in *p75*$^{NTR-/-}$ PC12 cells

p75^{NTR} has been often referred to as a "co-receptor" collaborating with TrkA.[21,51,52,53] However, conflicting findings have been obtained from previous studies using antisense oligonucleotides, chemical inhibitors, or overexpression constructs.[54] Thus, we used our novel *p75*$^{NTR-/-}$ PC12 cell clone to address this point.

Western blot analysis confirmed the absence of the p75^{NTR} protein, while endogenous TrkA was normally expressed (Figure 1B). Mutated clones and wild type cells were then stimulated with 100 ng/ml NGF to evaluate the phosphorylation of TrkA, ERK, and Akt.

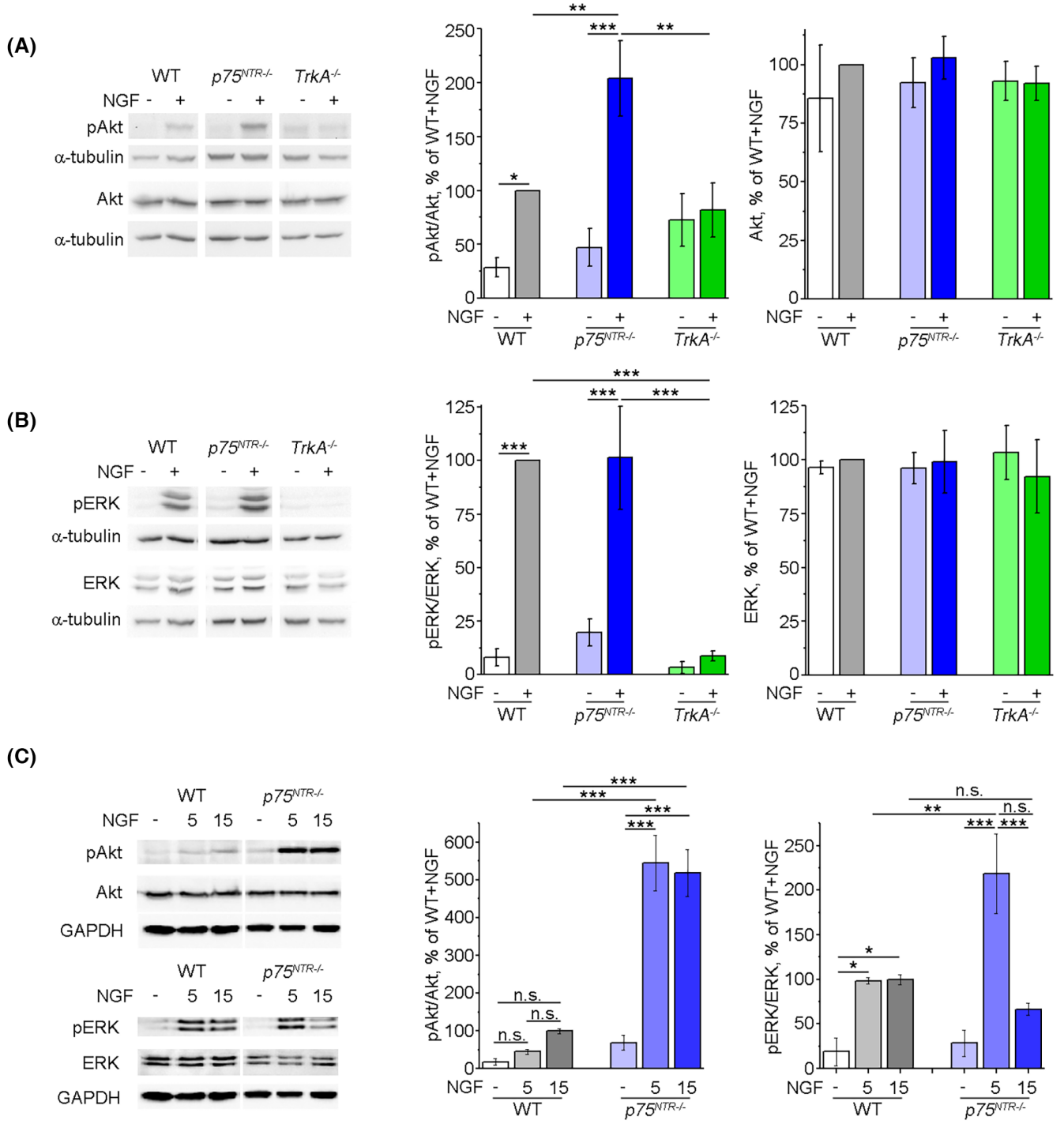
Interestingly, the absence of p75^{NTR} led to a significant increase in phospho-TrkA above the level displayed by control wild type PC12 cells subjected to the same treatment (Figure 1B). In keeping with this, at a high concentration (100 ng/ml) of NGF, the activation of Akt via phosphorylation in *p75*$^{NTR-/-}$ cells was also higher than in control cells (Figure 2A), whereas the phosphorylation of ERK was comparable between *p75*$^{NTR-/-}$ and control cells (Figure 2B). We also analyzed earlier time points, namely 5 and 15 min post-NGF application, and still detected a higher Akt phosphorylation in NGF-treated *p75*$^{NTR-/-}$ cells compared to WT cells (Figure 2C). Thus, the situation of 5- and 15-min NGF treatment matches that observed at 30 min, with a larger difference between *p75*$^{NTR-/-}$ and WT cells (5 min, 545.23 ± 73.53%, 15 min, 518.64 ± 62.46% of WT + NGF-15 min level; 30 min, 204.00 ± 35.04% of WT + NGF levels). In contrast, we found an early enhancement of ERK phosphorylation at 5 min post-NGF treatment in *p75*$^{NTR-/-}$ cells compared to WT cells, which disappeared at 15 min post-NGF treatment (Figure 2C).

In cells treated with 5 ng/ml NGF, the lack of p75^{NTR} did not result in increased phosphorylation of TrkA (Figure 3A) and Akt (Figure 3B), while ERK phosphorylation was significantly lower than in control cells (Figure 3C).

These data demonstrate that, at saturating concentrations of NGF (100 ng/ml), the absence of p75^{NTR} leads to increased NGF-TrkA signaling, suggesting that in normal PC12 cells, when TrkA and p75^{NTR} are coexpressed, the p75^{NTR} signaling stream sends an inhibitory signal to reduce the TrkA signaling stream. On the contrary, this mechanism of p75^{NTR} inhibition on TrkA signaling is not active at low concentrations of NGF (5 ng/ml). The level at which this inhibition is exerted remains to be ascertained.

3.4 | Exploring the contribution of p75^{NTR} and TrkA to PC12 cell differentiation

NGF plays a key role in promoting the survival of PC12 cells in the absence of serum[40] and is essential for their differentiation.[38,40] In addition to the downstream signaling mediated by TrkA, p75^{NTR} is also involved in neurotrophin-induced differentiation.[21] In order to elucidate the individual contribution of p75^{NTR} and TrkA to PC12 cell differentiation, we measured neurite length in response to treatment with a saturating concentration of NGF (100 ng/ml) and found that only wild type and



$p75^{NTR-/-}$ cells (i.e., only cells with functional TrkA) showed neurite outgrowth in response to NGF. Under these conditions, the response to NGF of $p75^{NTR-/-}$ cells was significantly higher than wild type cells. On the other hand, lack of TrkA completely abolished neurite elongation in response to NGF, an effect that was also observed in $TrkA^{-/-}$; $p75^{NTR-/-}$ double knock-out cells (Figure 4A).

Then, we tested different concentrations of NGF on wild type and $p75^{NTR-/-}$ cells and found that a low concentration of NGF (5 ng/ml) was enough to induce differentiation of wild type cells, resulting in neurite outgrowth, whereas no effect was observed in $p75^{NTR-/-}$ cells (Figure 4B). However, at higher concentrations of NGF (i.e., 10 ng/ml, 20 ng/ml, 50 ng/ml), the lack of $p75^{NTR}$ enhanced differentiation (Figure 4B), which is consistent with our findings on TrkA signaling (Figure 2).

We conclude that functional TrkA is required to induce PC12 cells differentiation and that $p75^{NTR}$ modulates this action, by showing differential effects at low or high NGF concentrations.

FIGURE 2 Analysis of signaling triggered by high-dose NGF. (A) NGF-treated $p75^{NTR-/-}$ cells show higher phosphorylation of Akt (pAkt) in comparison to both wild type (WT) and $TrkA^{-/-}$ cells. In addition, $TrkA^{-/-}$ cells lose their responsiveness to NGF stimulation. (ANOVA-2, p(genotype×treatment) = .019, followed by Student–Newman–Keuls post hoc test, WT + NGF vs. WT, $^{*}p = .044$; $p75^{NTR-/-}$ + NGF vs. $p75^{NTR-/-}$, $^{***}p < .001$; $p75^{NTR-/-}$ + NGF vs. WT + NGF, $^{**}p = .004$; $p75^{NTR-/-}$ + NGF vs. $TrkA^{-/-}$ + NGF, $^{**}p = .006$). No significant difference was observed in the total levels of Akt (ANOVA-2, p(genotype) = .889, p(treatment) = .449, p(genotype×treatment) = .839). WT, $n = 4$; WT + NGF, $n = 4$; $p75^{NTR-/-}$, $n = 5$; $p75^{NTR-/-}$ + NGF, $n = 5$; $TrkA^{-/-}$, $n = 3$; $TrkA^{-/-}$ + NGF, $n = 3$. (B) $TrkA^{-/-}$ cells do not display stimulation of ERK phosphorylation (pERK) upon NGF treatment; on the other hand, the response of $p75^{NTR-/-}$ cells is comparable to wild type (WT) cells (ANOVA-2, p(genotype×treatment) < .001, followed by Student–Newman–Keuls post hoc test, WT + NGF vs. WT, $^{***}p < .001$; $p75^{NTR-/-}$ + NGF vs. $p75^{NTR-/-}$, $^{***}p < .001$; WT + NGF vs. $TrkA^{-/-}$, $^{***}p < .001$, $p75^{NTR-/-}$ + NGF vs. $TrkA^{-/-}$ + NGF, $^{***}p < .001$). No significant difference was observed in the total levels of ERK (ANOVA-2, p(genotype) = .998, p(treatment) = .856, p(genotype×treatment) = .756). WT, $n = 6$; WT + NGF, $n = 4$; $p75^{NTR-/-}$, $n = 5$; $p75^{NTR-/-}$ + NGF, $n = 5$; $TrkA^{-/-}$, $n = 5$; $TrkA^{-/-}$ + NGF, $n = 5$. (C) *Left histogram*, NGF-treated $p75^{NTR-/-}$ cells show significantly elevated phosphorylation of Akt (pAkt) in comparison to wild type cells (WT) already after 5 and 15 min from NGF application (ANOVA-2, p(genotype×treatment) < .001, followed by Student–Newman–Keuls post hoc test, $p75^{NTR-/-}$ + NGF-5 min vs. $p75^{NTR-/-}$, $^{***}p < .001$; $p75^{NTR-/-}$ + NGF-15 min vs. $p75^{NTR-/-}$, $^{***}p < .001$; $p75^{NTR-/-}$ + NGF-5 min vs. WT + NGF-5 min, $^{***}p < .001$; $p75^{NTR-/-}$ + NGF-15 min vs. WT + NGF-15 min, $^{***}p < .001$). WT, $n = 4$; WT + NGF-5 min, n = 4; WT + NGF-15 min, $n = 4$; $p75^{NTR-/-}$, $n = 4$; $p75^{NTR-/-}$ + NGF-5 min, $n = 4$; $p75^{NTR-/-}$ + NGF-15 min, $n = 4$. *Right histogram*, $p75^{NTR-/-}$ cells show a transient increase in ERK phosphorylation 5 min after application of NGF (ANOVA-2, p(genotype×treatment) = .009, followed by Student–Newman–Keuls post hoc test, WT vs. WT + NGF-5 min, $^{*}p = .025$; WT vs. WT + NGF-15 min, $^{*}p = .038$; $p75^{NTR-/-}$ vs. $p75^{NTR-/-}$ + NGF-5 min, $^{***}p < .001$; $p75^{NTR-/-}$ + NGF-5 min vs. $p75^{NTR-/-}$ + NGF-15 min, $^{***}p < .001$; WT + NGF-5 min vs. $p75^{NTR-/-}$ + NGF-5 min, $^{**}p = .002$; n.s., not significant). WT, $n = 4$; WT + NGF-5 min, $n = 3$; WT + NGF-15 min, $n = 4$; $p75^{NTR-/-}$, $n = 4$; $p75^{NTR-/-}$ + NGF-5 min, $n = 4$; $p75^{NTR-/-}$ + NGF-15 min, $n = 4$. Numbers above blot images and below x-axes of histograms indicate the duration of NGF treatment before cell harvesting.

3.5 | The contribution of $p75^{NTR}$ and TrkA to PC12 cell membrane potential

After characterizing the contribution of TrkA and $p75^{NTR}$ to intracellular signaling and differentiation using classical, well-established assays, we decided to investigate a key aspect of neuronal function, namely the regulation of membrane potential by NGF.

To analyze the effect of TrkA and $p75^{NTR}$ on the membrane potential, we employed patch-clamp recordings on the three different gene-edited cell lines that we generated and compared their responses to non-engineered control cells. We first looked for possible effects of receptor knockout on the resting membrane potential of our cell lines in the absence of any manipulation and did not find any significant difference (Figure 5A). A strikingly different picture emerged when we treated cells with NGF (100 ng/ml) via bath perfusion. In line with previous literature,[48] control cells were quickly, but transiently, depolarized by NGF. This transient response was abolished by inactivation of the *TrkA* gene, which resulted in $TrkA^{-/-}$ cells being hyperpolarized by NGF. $p75^{NTR}$ inactivation ($p75^{NTR-/-}$ cells) had an opposite effect on the membrane potential, causing a strong membrane depolarization, which outlasted that observed in wild type cells. Finally, inactivation of both TrkA and $p75^{NTR}$ ($TrkA^{-/-}$; $p75^{NTR-/-}$ cells) abolished the response of PC12 cells to NGF, with only a mild and transient hyperpolarization being detected (Figure 5B).

Then, we sought to determine the possible currents mediating the effects of NGF on the membrane potential. Blockade of K^+ channels with tetraethylammonium (TEA) abolished NGF-induced hyperpolarization in $TrkA^{-/-}$ cells (Figure 5C). In contrast, replacing extracellular Na^+ with N-methyl-D-glucamine (NMDG) prevented NGF-induced depolarization in $p75^{NTR-/-}$ cells (Figure 5D).

This functional measure of the effect of NGF using single-cell electrophysiology agrees with our data on intracellular signaling and cell differentiation, further supporting an antagonistic role of TrkA and $p75^{NTR}$.

3.6 | Exploiting $TrkA^{-/-}$ and $p75^{NTR-/-}$ PC12 cells to the analysis of a pathologically relevant NGF mutant

Alterations in the NGF-TrkA-$p75^{NTR}$ axis are involved in a growing number of diseases.[14,15] Among these, mutations in NGF, including in the R100 residue, cause Hereditary Sensory and Autonomic Neuropathy type V.[12,55,56] Previous work has shown that the HSANV-related NGF^{R100E} mutant has an identical TrkA binding affinity as that of wild type NGF, and a 200-fold reduced affinity for $p75^{NTR}$,[14,57] and can be therefore described as a TrkA-biased agonist.

To investigate the effect of R100-mutated NGF on signaling pathways specific to TrkA or $p75^{NTR}$, we treated PC12 cells with either wild type NGF (NGF^{WT}), or NGF^{R100E}.[58] When treated with NGF^{R100E}, wild type PC12 cells showed reduced phosphorylation of TrkA in comparison to NGF^{WT} administration (Figure 6A). This effect was even more pronounced in $p75^{NTR-/-}$ cells subjected to the same treatments,

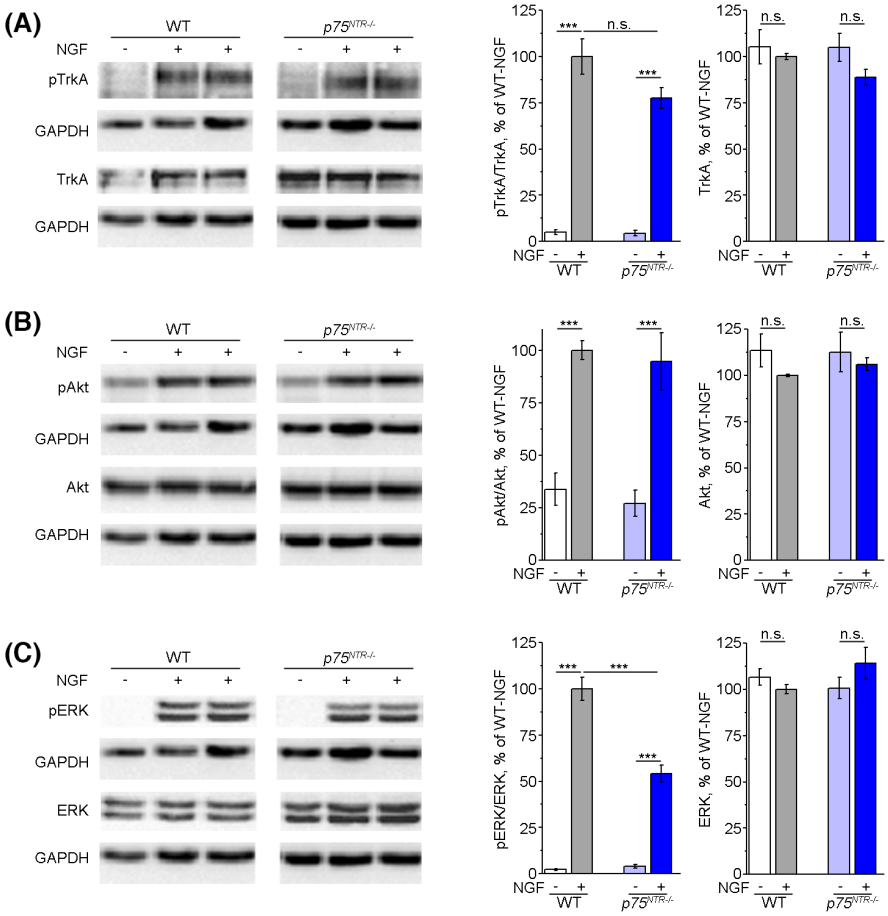


FIGURE 3 Analysis of signaling triggered by low-dose NGF. (A) $p75^{NTR-/-}$ retain normal NGF-induced TrkA phosphorylation (pTrkA) (ANOVA-2, p(treatment) < .001). No significant difference was observed in the total levels of TrkA (ANOVA-2, p(genotype) = .358, p(treatment) = .098, p(genotype×treatment) = .379). WT, $n = 5$; WT + NGF, $n = 6$; $p75^{NTR-/-}$, $n = 5$; $p75^{NTR-/-}$ + NGF, $n = 5$. (B) NGF-treated $p75^{NTR-/-}$ cells show normal NGF-induced phosphorylation of Akt (pAkt) in comparison to wild type (WT) cells. (ANOVA-2, p(treatment) < .001). No significant difference was observed in the total levels of Akt (ANOVA-2, p(genotype) = .702, p(treatment) = .157, p(genotype×treatment) = .618). WT, $n = 5$; WT + NGF, $n = 6$; $p75^{NTR-/-}$, $n = 5$; $p75^{NTR-/-}$ + NGF, $n = 5$. (C) $p75^{NTR-/-}$ cells display a significant reduction in ERK phosphorylation (pERK) upon NGF treatment; of note, NGF treatment of $p75^{NTR-/-}$ cells still causes a significant increase in pERK in comparison to non-treated cells (ANOVA-2, p(genotype×treatment) < .001, followed by Student–Newman–Keuls post hoc test, WT + NGF vs. WT, ***$p < .001$; $p75^{NTR-/-}$ + NGF vs. $p75^{NTR-/-}$, ***$p < .001$; WT + NGF vs. $p75^{NTR-/-}$ + NGF, ***$p < .001$). No significant difference was observed in the total levels of ERK (ANOVA-2, p(genotype) = .503, p(treatment) = .587, p(genotype×treatment) = .109). WT, $n = 5$; WT + NGF, $n = 6$; $p75^{NTR-/-}$, $n = 5$; $p75^{NTR-/-}$ + NGF, $n = 6$.

while, as expected, no signal could be detected in protein extracts from $TrkA^{-/-}$ cells (Figure 6A). Moreover, the absence of $p75^{NTR}$ was associated with a higher Akt phosphorylation than in wild type cells in response to NGF^{WT} (see above); but not to NGF^{R100E} (Figure 6B). The response of ERK to NGF^{R100E} was unaffected in wild type cells in comparison to NGF^{WT}, while a reduction was observed in the absence of $p75^{NTR}$ (Figure 6C).

In conclusion, these data demonstrate that by comparing the response of our gene-edited NGF receptor cells to ligands with a different receptor-engagement profile, a biochemical dissection of the contribution of TrkA and $p75^{NTR}$ to specific aspects of signaling can be easily carried out.

4 | DISCUSSION

Signaling by the "NGF system" is a complex process, with the TrkA receptor being more specific (but with some degree of promiscuity, such as NT3 binding) and $p75^{NTR}$ being common to all neurotrophins.[59] The pro-neurotrophin precursors add to the complexity. For instance, proNGF binds both TrkA[60] and $p75^{NTR}$,[61] in addition to the binding to sortilin, which mediates pro-apoptotic signals.[26] Over the years, NGF signaling has been investigated in a large number of different cellular systems. This heterogeneous set of results does not allow disentangling the cell-type specific aspects, from other more fundamental technical issues, such as, for instance, the fact that many studies

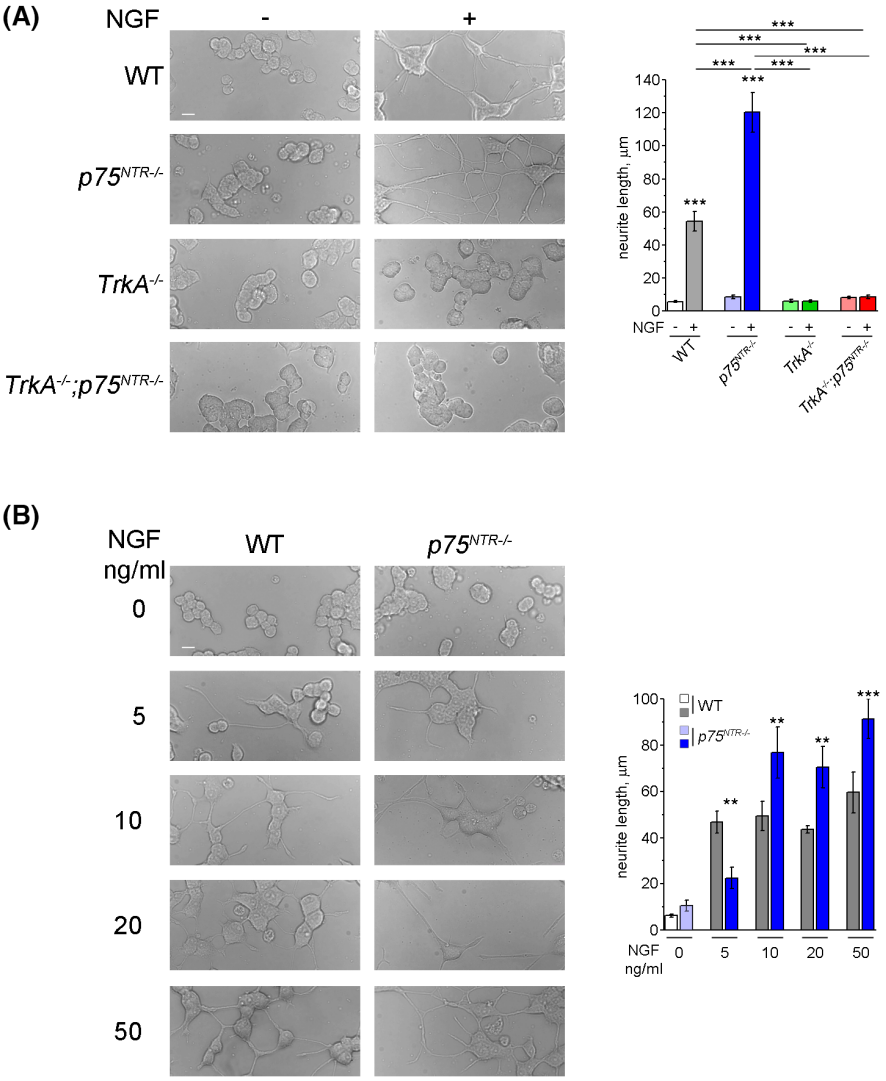


FIGURE 4 Analysis of NGF-induced differentiation in PC12 cells lacking one or both NGF receptors. (A) Wild type (WT) cells treated with NGF display the expected neurite outgrowth, and this response is significantly enhanced in $p75^{NTR-/-}$ cells, while it is absent in $TrkA^{-/-}$ and $TrkA^{-/-}$; $p75^{NTR-/-}$ cells (ANOVA-2, p(genotype×treatment) < .001, followed by Student–Newman–Keuls post hoc test, WT + NGF vs. WT, ***p < .001; WT + NGF vs. $TrkA^{-/-}$ + NGF, ***p < .001; WT + NGF vs. $TrkA^{-/-}$; $p75^{NTR-/-}$ + NGF, ***p < .001; $p75^{NTR-/-}$ + NGF vs. WT + NGF, ***p < .001; $p75^{NTR-/-}$ + NGF vs. $TrkA^{-/-}$ + NGF, ***p < .001; $p75^{NTR-/-}$ + NGF vs. $TrkA^{-/-}$; $p75^{NTR-/-}$ + NGF, ***p < .001). WT, n = 15; WT + NGF, n = 15; $p75^{NTR-/-}$, n = 17; $p75^{NTR-/-}$ + NGF, n = 14; $TrkA^{-/-}$, n = 6; $TrkA^{-/-}$ + NGF, n = 12; $TrkA^{-/-}$; $p75^{NTR-/-}$, n = 12; $TrkA^{-/-}$; $p75^{NTR-/-}$ + NGF, n = 12. (B) $p75^{NTR-/-}$ cells show concentration-specific differences in neurite outgrowth in comparison to wild type (WT) cells (ANOVA-2, p(genotype×treatment) < .001, followed by Student–Newman–Keuls post hoc test, 5 ng/ml, *p = .011; 10 ng/ml, **p = .008; 20 ng/ml, **p = .004; 50 ng/ml, ***p < .001). WT-0 ng/ml, n = 5; WT-5 ng/ml, n = 6; WT-10 ng/ml, n = 6; WT-20 ng/ml, n = 5; WT-50 ng/ml, n = 6; $p75^{NTR-/-}$-0 ng/ml, n = 9; $p75^{NTR-/-}$-5 ng/ml, n = 4; $p75^{NTR-/-}$-10 ng/ml, n = 6; $p75^{NTR-/-}$-20 ng/ml, n = 5; $p75^{NTR-/-}$-50 ng/ml, n = 5.

have been performed via receptor overexpression in heterologous systems.

In order to overcome these pitfalls, it would be very convenient to have a standardized cellular model system in which each signaling component can be genetically isolated and removed. In this respect, mouse models in which the receptors have been knocked out by homologous recombination could be helpful in investigating NGF signaling. Indeed, knockout mice for TrkA[62] and $p75^{NTR}$ have been created.[63,64] However, homozygous TrkA knockout mice show an early lethal phenotype[62] and cannot be bred as homozygotes with other knockout lines to yield double knockouts. As for $p75^{NTR}$ knockout mice, the two existing strains, carrying mutations in exon 3[63] or exon 4,[64] both display features that defy definitive conclusions. The $p75^{NTR\Delta Exon3}$ knockout mouse[63] still encodes an alternatively spliced isoform that might be (partially) functional. The $p75^{NTR\Delta Exon4}$ knockout mouse[64] still expresses an intracellular fragment of $p75^{NTR}$ that has pro-apoptotic properties.[65] To overcome these problems,

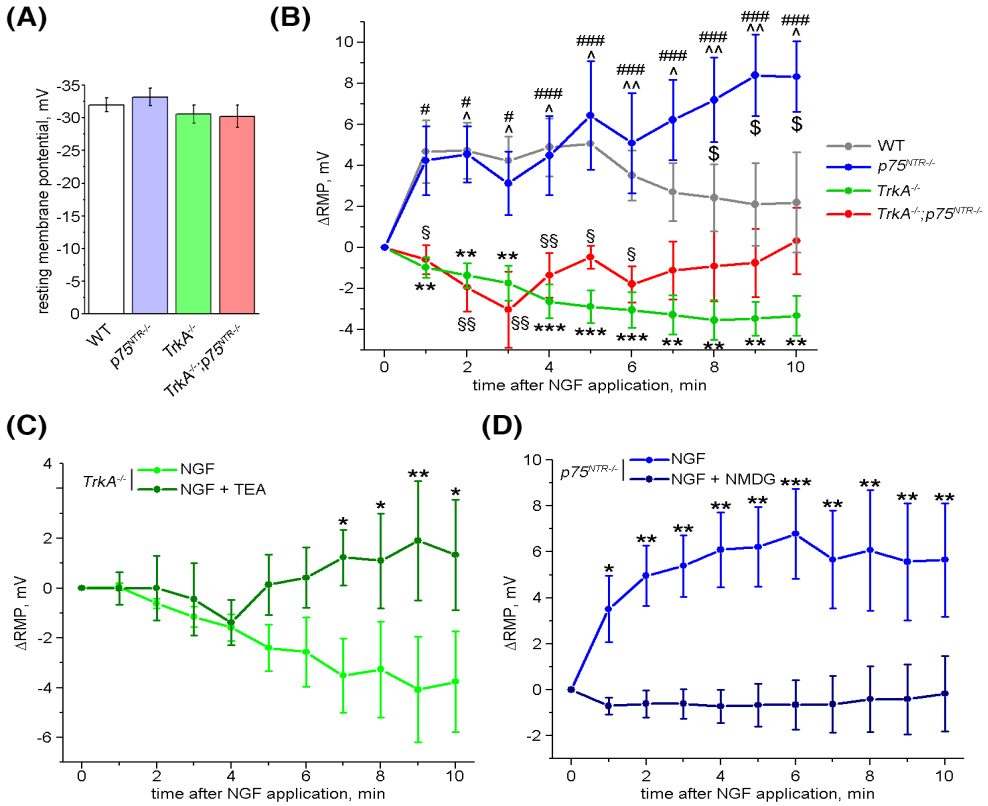


FIGURE 5 Electrophysiological recordings on PC12 cells lacking one or both NGF receptors. (A) No significant differences in resting membrane potential were observed among wild type (WT), $p75^{NTR-/-}$, $TrkA^{-/-}$, and $TrkA^{-/-}$; $p75^{NTR-/-}$ cells (ANOVA-1, p(genotype) = .410). WT, $n = 28$; $p75^{NTR-/-}$, n = 26; $TrkA^{-/-}$, n = 26; $TrkA^{-/-}$; $p75^{NTR-/-}$, $n = 16$. (B) Wild type (WT) cells respond to NGF with a transient depolarization, a response which turns into a prolonged one in $p75^{NTR-/-}$ cells, or into hyperpolarization in $TrkA^{-/-}$ cells; instead, $TrkA^{-/-}$; $p75^{NTR-/-}$ cells only display a transient hyperpolarization (ANOVA-2 for repeated measures, p(genotype×time) < .001; 1 min, WT vs. $TrkA^{-/-}$, $^{**}p = .006$; 1 min, WT vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\S}p = .042$; 1 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#}p = .029$; 2 min, WT vs. $TrkA^{-/-}$, $^{**}p = .002$; 2 min, WT vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\S\S}p = .007$; 2 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#}p = .013$; 2 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge}p = .017$; 3 min, WT vs. $TrkA^{-/-}$, $^{**}p = .003$; 3 min, WT vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\S\S}p = .003$; 3 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#}p = .034$; 3 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge}p = .023$; 4 min, WT vs. $TrkA^{-/-}$, $^{***}p < .001$; 4 min, WT vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\S}p = .011$; 4 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#\#\#}p = .001$; 4 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge}p = .026$; 5 min, WT vs. $TrkA^{-/-}$, $^{***}p < .001$; 5 min, WT vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\S}p = .022$; 5 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#\#\#}p < .001$; 4 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge}p = .011$; 6 min, WT vs. $TrkA^{-/-}$, $^{***}p = .001$; 6 min, WT vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\S}p = .030$; 6 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#\#\#}p < .001$; 6 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge\wedge}p = .008$; 7 min, WT vs. $TrkA^{-/-}$, $^{**}p = .003$; 7 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#\#\#}p < .001$; 7 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge}p = .011$; 8 min, WT vs. $TrkA^{-/-}$, $^{**}p = .003$; 8 min, WT vs. $p75^{NTR-/-}$, $^{\$}p = .029$; 8 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#\#\#}p < .001$; 8 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge\wedge}p = .004$; 9 min, WT vs. $TrkA^{-/-}$, $^{**}p = .004$; 9 min, WT vs. $p75^{NTR-/-}$, $^{\$}p = .025$; 9 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#\#\#}p < .001$; 9 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge\wedge}p = .004$; 10 min, WT vs. $TrkA^{-/-}$, $^{**}p = .006$; 10 min, WT vs. $p75^{NTR-/-}$, $^{\$}p = .047$; 10 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$, $^{\#\#\#}p < .001$; 8 min, $p75^{NTR-/-}$ vs. $TrkA^{-/-}$; $p75^{NTR-/-}$, $^{\wedge}p = .018$). WT, $n = 12$; $p75^{NTR-/-}$, $n = 8$; $TrkA^{-/-}$, $n = 13$; $TrkA^{-/-}$; $p75^{NTR-/-}$, $n = 6$. (C) Blocking K^+ channels with tetraethylammonium (TEA) abolishes NGF-induced hyperpolarization in $TrkA^{-/-}$ cells (ANOVA-2 for repeated measures, p(treatment×time) = .039; 7 min, $^{*}p = .022$; 8 min, $^{*}p = .035$; 9 min, $^{**}p = .005$; 10 min, $^{*}p = .015$). NGF, $n = 6$; NGF + TEA, $n = 8$. (D) Blocking Na^+ influx via substitution with N-methyl-D-glucamine (NMDG) abolishes NGF-induced depolarization in $p75^{NTR-/-}$ cells (ANOVA-2 for repeated measures, p(treatment×time) = .001; 1 min, $^{*}p = .041$; 2 min, $^{**}p = .009$; 3 min, $^{**}p = .005$; 4 min, $^{**}p = .002$; 5 min, $^{**}p = .002$; 6 min, $^{***}p < .001$; 7 min, $^{*}p = .004$; 8 min, $^{**}p = .010$; 9 min, $^{**}p = .006$; 10 min, $^{**}p = .007$). NGF, $n = 6$; NGF + NMDG, $n = 8$.

conditional knockout mice for TrkA[66] and p75^{NTR}[67] have been generated, but their crossbreeding to derive single or double knockout neurons, of the same overall genetic background, has not been reported.

In this regard, a simpler, standardized, and well-validated cellular system would facilitate investigations on signaling mechanisms controlled by the NGF system. The PC12 cell line, introduced by Greene and Tischler in 1976[38] has offered the gold standard system to the neurotrophin community to analyze NGF-induced signaling through its receptors TrkA and p75^{NTR}.[21,68]

In this report, we describe the generation and use of a set of new cell lines, based on the PC12 line, with genome editing inactivation of either *TrkA* or $p75^{NTR}$, or both.

Notably, a serendipitously isolated *TrkA* knockout variant (i.e., PC12nnr5) has been the main tool for cellular studies requiring the loss of function of this receptor.[42] However, the PC12nnr5 clone is genetically poorly defined.

These novel cellular tools allowed us to demonstrate, in a controlled manner, that: (i) TrkA is necessary and sufficient for NGF sensing; (ii) the absence of $p75^{NTR}$ enhances TrkA-mediated signaling at high concentration of NGF; (iii) TrkA and $p75^{NTR}$ collaborate to promote cell differentiation at low NGF concentrations; (iv) TrkA and $p75^{NTR}$ have opposing polarities on the regulation of the membrane potential by NGF.

4.1 | A genome editing approach to dissect the individual components of the NGF receptor system

Classical approaches (e.g., antisense oligonucleotides, gene overexpression, in vivo gene targeting) and a number of different heterogeneous experimental systems have been largely employed by different groups, over the years, to shape our current understanding of the cellular and functional mechanisms of NGF signaling.[2,29,54,62] However, the lack of a simple, robust, and standardized model susceptible to easy genetic manipulation left somewhat unresolved the analytical dissection of the early and late events of NGF signaling and the relationships between NGF, its co-receptors and the cellular context. In this regard, we chose the PC12 cell line, not only to disentangle the herein discussed roles of TrkA and $p75^{NTR}$, but also as a base for the future study of additional components of the NGF multireceptor system, such as sortilin, or pathologically relevant mutations of TrkA[69] and NGF (e.g.,[13,57,58,69]). Indeed, the random mutagenesis approaches so far used to select the currently used mutant PC12 clones, showing absent or reduced expression of TrkA or $p75^{NTR}$,[42,54] do not allow a precise control on the corresponding gene manipulation, nor are suitable for the specific manipulation of two genes in parallel, as we did in the present study.

4.2 | Modulation of NGF signaling by TrkA–$p75^{NTR}$ interplay

A critical and controversial question in NGF signaling is whether the TrkA and $p75^{NTR}$ receptors combine in heteromeric complexes[30,34,36] and cooperate or compete, resulting in new features compared to the simple sum of pathways activated by either TrkA and $p75^{NTR}$ receptor alone.

First, we confirmed that our CRISPR/Cas9-based approach abrogates NGF-induced phosphorylation of TrkA, along with abolishing the downstream phosphorylation of Akt and ERK. Then, we found that the absence of $p75^{NTR}$ enhances TrkA phosphorylation. These results demonstrate that, at a high concentration (100 ng/ml) of NGF, $p75^{NTR}$ antagonizes the effects of NGF-TrkA interaction, and that $p75^{NTR}$ alone is not able to transduce binding of NGF signaling into the activation of ERK and Akt. Our findings are consistent and support the previously postulated functional antagonism between TrkA and $p75^{NTR}$.[70]

Of note, TrkA-mediated activation of the two pathways depends on common (i.e., Shc and Grb2)[71] and specific interactors, such as SH2-B and CD2AP for Akt,[72,73] or Src for ERK.[74] Differential recruitment of these components of NGF receptor-associated signaling may contribute to explain the concentration-specific phosphorylation patterns of Akt and ERK, along with their different temporal kinetics.

Finally, we applied our cell platform to the study of the biochemical properties of NGF^{R100E}, a mutated isoform with important implications for pain insensitivity diseases.[12,58] The key feature of R100-mutated NGF is its reduced ability to elicit hyperalgesia while maintaining an unaltered neurotrophic activity.[12,13] The absence of this heavy side effect has led to testing NGF^{R100E} for the therapy of neurodegeneration.[14] Here, we show that NGF^{R100E} application results in lower phosphorylation of TrkA in wild type PC12 cells. However, when both receptors are present (i.e., in wild type cells), this has no effect on both Akt and ERK phosphorylation, which are equivalently stimulated by the two NGF ligands. Interestingly, in the absence of $p75^{NTR}$, the phosphorylation of Akt was significantly higher in response to $NGF^{WT,}$ but not NGF^{R100E} treatment, thus reproducing an analogous trend on TrkA phosphorylation. These data demonstrate that mutation-specific aspects of receptor engagement and signaling can be unmasked and dissected using our TrkA- and $p75^{NTR}$-null PC12 cells.

4.3 | Interplay between Trka and $p75^{NTR}$ in PC12 cell differentiation

Despite the controversy on the contribution of TrkA and $p75^{NTR}$ to downstream signaling, previous reports suggest that both ERK and Akt play a role in NGF-induced neurite outgrowth.[75,76] Our gene-edited PC12 clones allowed us to directly demonstrate that the absence of $p75^{NTR}$, in keeping with the increased phosphorylation of TrkA and Akt, results in stronger neurite-like processes outgrowth at high NGF concentrations.

Interestingly, p75^{NTR}-null cells expressing only TrkA are less sensitive to low-concentration (i.e., 5 ng/ml) NGF, showing less differentiation than wild type cells, accompanied by lower ERK phosphorylation. Despite being non-statistically significant, the decreased phosphorylation of TrkA observed in p75^{NTR}-null cells treated with 5 ng/ml NGF can contribute to the lower phospho-ERK levels and can be linked to the fact that p75^{NTR} also acts as a co-receptor for TrkA to increase its affinity for NGF.[51] Thus, it can be hypothesized that, in the absence of p75^{NTR}, the concentration of NGF must cross a critical threshold to fully exert its effects on cell differentiation. Thus, p75^{NTR} has a dual role in the initial steps of NGF signaling: (i) facilitating the presentation of NGF to TrkA and increasing its effectiveness, at low concentrations and (ii) negatively regulating the outcome of signaling, at higher concentrations. Moreover, our data extend previous findings of enhanced growth of primary cultures of sympathetic neurons from *p75$^{NTR-/-}$* mice.[70] Thus, our results fit very well with the general idea in the literature, but it is remarkable that a few simple experiments exploiting the newly generated PC12 cell lines show this very clearly.

Our data also show a direct correlation between the concentration of NGF and the level of differentiation; at the signaling level, this can relate to the idea that both ERK and Akt play a role to elicit NGF-induced neurite outgrowth in PC12 cells.[54,76]

4.4 | Interplay between TrkA and p75^{NTR} in regulating the membrane potential

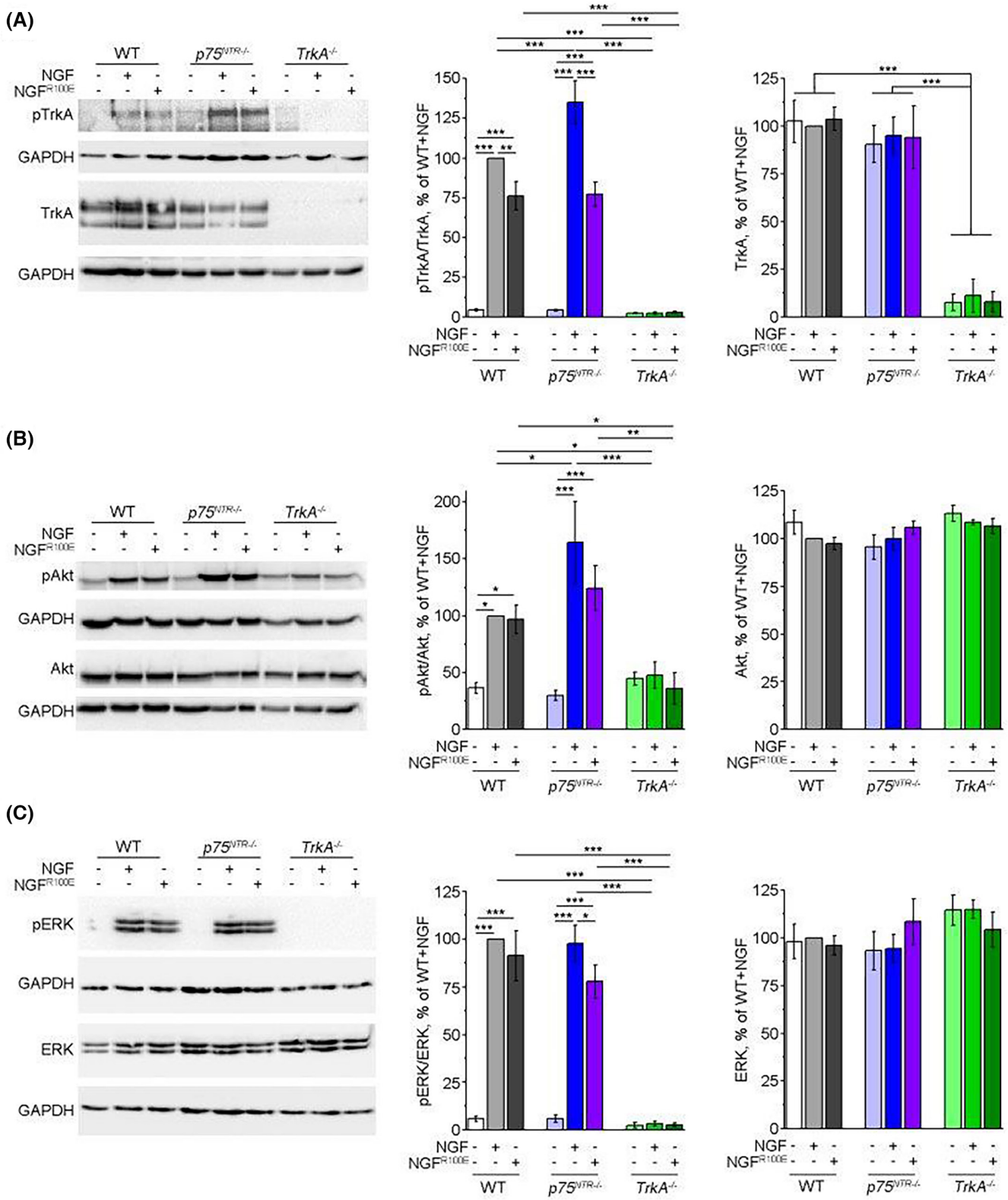
Our gene-editing approach also allowed a clean dissection of the role of the two receptors of NGF in regulating the membrane potential. The long-term development of electrical excitability in PC12 cells after 2 weeks of induction of differentiation with NGF was described in the early foundational study by Dichter et al.[77] Here, we investigated the receptor dependence of rapid effects of NGF on PC12 cell membrane potential.

First, in naïve PC12 cells, we found that NGF caused a transient depolarization, in agreement with Shimazu and colleagues.[48] Moreover, our findings showed that *TrkA* gene ablation abolishes the early depolarization and unmasks a late hyperpolarization. On the other hand, *p75^{NTR}* gene ablation had an opposite outcome and turned the early depolarization from transient to prolonged. Both effects were lost upon the knock out of both genes, thus pointing to opposite roles of TrkA and p75^{NTR} in controlling the membrane potential.

Of note, our system totally ablates the expression of either one or both receptors. This could explain why the presence of p75^{NTR} alone (i.e., in *TrkA$^{-/-}$* cells) resulted in NGF inducing hyperpolarization, whereas overexpression of this receptor in 3 T3 cells, which also express TrkA, resulted in depolarization.[48]

FIGURE 6 Using gene-edited, NGF receptor(s)-mutant PC12 cells to study the disease-related mutant NGF^{R100E}. (A) In wild type (WT) and *p75$^{NTR-/-}$* cells, NGF^{R100E} induces a lower TrkA phosphorylation (pTrkA) compared to NGF, while, as expected and regardless of the treatment, no pTrkA could be detected in *TrkA$^{-/-}$* cells (ANOVA-2, p(genotype×treatment) < .001, followed by Student–Newman–Keuls post hoc test, WT vs. WT-NGF, ***p < .001; WT vs. WT-NGF^{R100E}, ***p < .001; WT-NGF vs. WT-NGF^{R100E}, **p = .004; *p75$^{NTR-/-}$* vs. *p75$^{NTR-/-}$*-NGF, ***p < .001; *p75$^{NTR-/-}$* vs. *p75$^{NTR-/-}$*-NGF^{R100E}, ***p < .001; *p75$^{NTR-/-}$*-NGF vs. *p75$^{NTR-/-}$*-NGF^{R100E}, ***p < .001; WT-NGF vs. *TrkA$^{-/-}$*-NGF, ***p < .001; WT-NGF vs. *p75$^{NTR-/-}$*-NGF, ***p < .001; *p75$^{NTR-/-}$*-NGF vs. *TrkA$^{-/-}$*-NGF, ***p < .001; WT-NGF^{R100E} vs. *TrkA$^{-/-}$*-NGF^{R100E}, ***p < .001; *p75$^{NTR-/-}$*-NGF^{R100E} vs. *TrkA$^{-/-}$*-NGF^{R100E}, ***p < .001. No significant difference was observed in the total levels of TrkA between WT and *p75$^{NTR-/-}$* cells, while *TrkA$^{-/-}$* cells showed only background signal (ANOVA-2, p(genotype) < .001, followed by Student–Newman–Keuls post hoc test, WT vs. *TrkA$^{-/-}$*, ***p < .001; *p75$^{NTR-/-}$* vs. *TrkA$^{-/-}$*, ***p < .001). WT, n = 5; WT-NGF, n = 5; WT-NGF^{R100E}, n = 4; *p75$^{NTR-/-}$*, n = 5; *p75$^{NTR-/-}$*-NGF, n = 4; *p75$^{NTR-/-}$*-NGF^{R100E}, n = 4; *TrkA$^{-/-}$*, n = 4; *TrkA$^{-/-}$*-NGF, n = 4; *TrkA$^{-/-}$*-NGF^{R100E}, n = 4. (B) NGF^{R100E} has an unaltered capability to induce phosphorylation of Akt (pAkt) in wild type (WT) and *p75$^{NTR-/-}$* cells, while no response can be detected in *TrkA$^{-/-}$* cells (ANOVA-2, p(genotype×treatment) = .006, followed by Student–Newman–Keuls post hoc test, WT vs. WT-NGF, *p = .025; WT-NGF vs. WT-NGF^{R100E}, *p = .023; *p75$^{NTR-/-}$* vs. *p75$^{NTR-/-}$*-NGF, ***p < .001; *p75$^{NTR-/-}$* vs. *p75$^{NTR-/-}$*-NGF^{R100E}, ***p < .001; WT-NGF vs. *TrkA$^{-/-}$*-NGF, *p = .037; WT-NGF vs. *p75$^{NTR-/-}$*-NGF, *p < .015; *p75$^{NTR-/-}$*-NGF vs. *TrkA$^{-/-}$*-NGF, ***p < .001; WT-NGF^{R100E} vs. *TrkA$^{-/-}$*-NGF^{R100E}, *p = .032; *p75$^{NTR-/-}$*-NGF^{R100E} vs. *TrkA$^{-/-}$*-NGF^{R100E}, **p = .002). No significant difference was observed in the total levels of Akt (ANOVA-2, p(genotype) = .060, p(treatment) = .705, p(genotype×treatment) = .193). WT, n = 5; WT-NGF, n = 5; WT-NGF^{R100E}, n = 5; *p75$^{NTR-/-}$*, n = 5; *p75$^{NTR-/-}$*-NGF, n = 5; *p75$^{NTR-/-}$*-NGF^{R100E}, n = 5; *TrkA$^{-/-}$*, n = 4; *TrkA$^{-/-}$*-NGF, n = 4; *TrkA$^{-/-}$*-NGF^{R100E}, n = 4. (C) NGF^{R100E} treatment results in lower phospho-ERK (pERK) levels specifically in *p75$^{NTR-/-}$* cells (ANOVA-2, p(genotype×treatment) < .001, followed by Student–Newman–Keuls post hoc test, WT vs. WT-NGF, ***p < .001; WT-NGF vs. WT-NGF^{R100E}, ***p < .001; *p75$^{NTR-/-}$* vs. *p75$^{NTR-/-}$*-NGF, ***p < .001; *p75$^{NTR-/-}$* vs. *p75$^{NTR-/-}$*-NGF^{R100E}, ***p < .001; *p75$^{NTR-/-}$*-NGF vs. *p75$^{NTR-/-}$*-NGF^{R100E}, *p = .037; WT-NGF vs. *TrkA$^{-/-}$*-NGF, ***p < .001; *p75$^{NTR-/-}$*-NGF vs. *TrkA$^{-/-}$*-NGF, ***p < .001; WT-NGF^{R100E} vs. *TrkA$^{-/-}$*-NGF^{R100E}, ***p < .001; *p75$^{NTR-/-}$*-NGF^{R100E} vs. *TrkA$^{-/-}$*-NGF^{R100E}, ***p < .001). No significant difference was observed in the total levels of ERK (ANOVA-2, p(genotype) = .117, p(treatment) = .985, p(genotype×treatment) = .519). WT, n = 5; WT-NGF, n = 5; WT-NGF^{R100E}, n = 5; *p75$^{NTR-/-}$*, n = 5; *p75$^{NTR-/-}$*-NGF, n = 5; *p75$^{NTR-/-}$*-NGF^{R100E}, n = 5; *TrkA$^{-/-}$*, n = 4; *TrkA$^{-/-}$*-NGF, n = 4; *TrkA$^{-/-}$*-NGF^{R100E}, n = 4.

As a step toward the identification of the channels and conductances responsible for the effects of TrkA and $p75^{NTR}$ activation on the membrane potential, we found that K^+ channel blockade and replacement of extracellular Na^+ prevented NGF from causing hyperpolarization in $TrkA^{-/-}$ cells and depolarization in $p75^{NTR-/-}$ cells, respectively. These findings are in line with Shimazu et al.,[48] who demonstrated that a Na^+-free extracellular solution abolishes NGF-induced membrane depolarization, while blockade of K^+ channels with TEA eliminated NGF-induced membrane hyperpolarization in wild type PC12 cells. By performing TEA



experiments on *TrkA*$^{-/-}$ cells, we did not need to combine TEA administration with Na^+-free extracellular solution, as knockout of this receptor abolished NGF-induced depolarization per se.

These electrophysiological results provide a quantitative and robust experimental read-out for the early effects of NGF signaling and might form the basis for an experimental assay for the activity of small molecule NGF agonists or antagonists and a comparison of proNGF with NGF.

5 | CONCLUSIONS

In conclusion, we employed CRISPR/Cas9-based gene editing to generate new PC12 cell lines that can be used to disentangle the complexity of the NGF/TrkA/p75^{NTR} system. Our data on intracellular signaling, cell differentiation, and membrane electrical potential point to an antagonistic role of TrkA and p75^{NTR} in transducing the binding of NGF at the cell surface. As a further expression of a widely used physiological building motif, this interaction creates a push-pull system, which expands the dynamic range of NGF-associated cellular responses. Identifying the molecular effectors supporting this system will be the focus of our next experiments.

The newly generated PC12 mutants will be very useful for genetic reconstitution experiments. For instance, the many TrkA mutants described as being responsible for congenital insensitivity to pain diseases, such as Hereditary Sensory and Autonomic Neuropathy type IV (HSAN IV; 77) can now be expressed on a genetically clean background. Similarly, the expression of a single NGF receptor, or the absence of both, can be exploited to detect differences in signaling elicited by, e.g., NGF mutants responsible for HSAN type V,[13,78,79] proNGF,[35,60] neurotrophin NT3, or by synthetic ligands or antagonists.[80,81]

Finally, our three new PC12 clones, *TrkA*$^{-/-}$, *p75*$^{NTR-/-}$, *TrkA*$^{-/-}$/*p75*$^{NTR-/-}$ will be available as a cellular platform for further gene-editing operations to dissect the downstream key components mediating the wide array of NGF effects on cell pathophysiology.

AUTHOR CONTRIBUTIONS

Mario Costa, Antonino Cattaneo, Marco Mainardi, and Giovanna Testa designed research. Giovanna Testa, Marco Mainardi, Laura Pancrazi, Eleonora Vannini, and Mario Costa performed research. Giovanna Testa, Marco Mainardi, Eleonora Vannini, and Mario Costa analyzed data. Giovanna Testa, Marco Mainardi, Mario Costa, and Antonino Cattaneo wrote the paper.

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DISCLOSURES

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All the data produced during the experiments of the present manuscript (western blots, microscope images, electrophysiological recordings) are available upon request to the corresponding authors.

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