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Modifications in the C-terminal tail of TrkC significantly alter neurotrophin-3-promoted outgrowth of neurite-like processes from PC12 cells

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ABSTRACT

TrkB and TrkC are quite common neurotrophin receptors found on the same cells in CNS. In the C-terminal tail, TrkB and TrkC differ only in two amino acid residues at positions immediately preceding the tyrosine residue, which, upon phosphorylation, becomes the docking site for phospholipase Cγ1 (PLCγ1). The question arose whether such a difference near the PLCγ1 docking site might contribute to differential response to neurotrophin. PC12 clones with the following receptors were obtained: wild-type TrkC, TrkC-Y820F with a defective PLCγ1 binding site, TrkC-T817S–I819V with two amino acid residues replaced with those in the TrkB tail. The outgrowth of neurite-like processes from TrkC-Y820F-containing cells appeared to be impaired, while the TrkC-T817S–I819V variant appeared more effective than wild-type TrkC in promoting the outgrowth of neurite-like processes after neurotrophin stimulation, at least in the compared PC12 cell clones. Taken together, both the tyrosine residue at the PLCγ1 docking site and the amino acid residues immediately preceding it appear important for TrkC-supported outgrowth of neurite-like processes.

1. Introduction

TrkA, TrkB and TrkC are transmembrane neurotrophin receptors with the enzymatic activity of a tyrosine protein kinase, belonging to the tropomyosin-related kinase (Trk) family: TrkA binds nerve growth factor (NGF), TrkB binds brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4), while TrkC is specific for neurotrophin 3 (NT-3). According to the model of Schlessinger [1], in the absence of ligand, inactive receptor tyrosine kinase dimers may be in equilibrium with dimers adopting an active conformation capable of *trans*--autophosphorylation. In turn, ligand binding to the extracellular domains of Trk stabilizes the active dimer conformation and thus activates Trk, resulting in cross-phosphorylation of tyrosine residues within the activation loops of the intracellular tyrosine kinase catalytic domains, as well as outside the catalytic domains, both in the juxtamembrane regions and in the C-terminal tails of Trk proteins [1]. The activated Trk then, *via* its phosphorylated tyrosine residues, recruits various signaling proteins causing them to be activated by phosphorylation on their own tyrosine residues. This leads to the activation of the extracellular signal-regulated kinases 1 and 2 (Erk-1/2), PI3K/Akt and phospholipase Cγ1 (PLCγ1) signaling pathways, which transmit neurotrophic signals intracellularly [2]. Secondary messengers produced by PLCγ1 also appear to directly influence the processes of neurite outgrowth and axon guidance [3]. Although the cytoplasmic domains of Trks share a high degree of sequence homology and appear to utilize the same signaling pathways, it has been suggested that subtle differences in the dynamics of activation of signaling pathways may lead to distinct cellular responses to activation of different Trks [4].

In the peripheral nervous system, neurotrophins are indispensable factors for the survival of nerve cells equipped with a receptor appropriate for a given neurotrophin, and developing axons compete for access to limited amounts of neurotrophins secreted at the innervation target sites [5]. In the central nervous system (CNS), although neurotrophins do not appear to be key determinants of the emerging neural

Abbreviations: **PLCγ1**, phospholipase Cγ1; **Trk**, tropomyosin-related kinase; **NT-3**, neurotrophin 3; **Dox**, doxycycline; **Y820F**, substitution of a tyrosine residue with a phenylalanine residue at position 820 of TrkC; **T817S–I819V**, substitutions of threonine and isoleucine residues with serine and valine residues at positions 817 and 819 of TrkC; **Erk-1/2**, extracellular signal-regulated kinases 1 and 2 (p44/42 MAPK); **p-Erk-1/2**, phosphorylated Erk-1/2 (phospho-p44/42 MAPK).

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network, it can be assumed that during CNS development, neurotrophins may cooperate with other signals to regulate the migration of neuronal precursors [6], the survival or programmed death of neurons [7], growth and branching of dendritic trees and axons [8]. Neurotrophins may also regulate synaptic plasticity of the developing and adult CNS [9], while alterations in synaptic plasticity have been reported as an early event in the progression of Alzheimer's disease [10,11]. It should also be noted that impaired transport of neurotrophins to sites of interaction with receptors and unbalanced distribution of Trk ligands occur in severe neurodegenerative diseases such as Alzheimer's disease [12]. A recent report demonstrated that PTX-BD10-2, a small molecule agonist of TrkB/TrkC receptors, may have neuroprotective effects in advanced Alzheimer's disease [13]. It is also worth noting that TrkB appears to be a direct target of antidepressants interacting with the transmembrane portion of TrkB [14].

Understanding subtle differences in the activation of signaling pathways by different neurotrophin receptors may also be important for more effective treatment of patients with tumors characterized by abnormal Trk expression [15]. For example, a fusion protein containing a constitutively active TrkC catalytic domain has been shown to be oncogenic in breast cancer [16]. A recent report also indicated for the first time the involvement of TrkC in the pathogenesis of Ewing's sarcoma [17].

All this justifies the search for differences in the functioning of neurotrophin receptors. Finally, it should be noted that the presence of TrkB and TrkC on the same nerve cells [18,19] is quite common in the CNS, especially in the neurons of the cerebral cortex, the hippocampus, and the basal forebrain [20]. Interestingly, it has also been shown that neurotrophins acting through TrkB or TrkC located on the same neural stem cells can trigger different developmental programs [21]. In other studies, a significantly weaker increase in calcium levels in the cytosol of cultured rat cerebral cortical neurons was observed following treatment with NT-3 (TrkC ligand) compared to treatment with BDNF (TrkB ligand) [22].

TrkB and TrkC share approximately 40 % identical amino acid residues in the extracellular domains, while the intracellular tyrosine kinase catalytic domains share more than 80 % identical amino acid residues. Differences in the functioning of Trk receptors may result from their different location inside or outside lipid rafts in the cell membrane [7], as well as from differences in the affinity of activated receptors for adaptor proteins and enzymes involved in intracellular signal transduction pathways. Given the high degree of sequence homology of TrkB and TrkC, two general reasons for the differences in the effects of their activation can be considered: (a) TrkB and TrkC activations lead to the activation of the same signaling pathways, and the observed differences in biological effects may be due to differences in the localization of these receptors in the cell; (b) despite similar cellular localization but slight differences in the receptor affinities for adaptor proteins and enzymes, activation of TrkB and TrkC receptors can lead to at least quantitatively different biological effects.

In the C-terminal tail, TrkB and TrkC differ in two amino acid residues at positions −3 (S/T) and −1 (V/I) relative to the last tyrosine residue, i.e. immediately before the PLCγ1 binding site to the phosphorylated tyrosine residue in the sequence consensus pY-(L/V/I)-X-(P/L/V/I) [23] (see Fig. 1A). Therefore, a more specific question was asked whether even such a subtle difference between Trk proteins in their cytosolic regions is sufficient to induce a differential biological effect in response to stimulation with an appropriate neurotrophic ligand. PC12 cells contain TrkA but not TrkB and TrkC and are not stimulated by BDNF and NT-3 [22,24,25]. This is why PC12 cells were chosen to express exogenous TrkC and its variants, and also because much earlier studies have shown that PC12 cells containing exogenous wild-type TrkC can produce processes upon NT-3 stimulation in a neurite outgrowth assay [24,25]. Therefore, to answer the above question, PC12-Tet-On clones were derived with doxycycline (Dox)-inducible expression of the following proteins: (a) wild-type TrkC; (b) TrkC variant with weakened PLCγ1 recruitment site (the tyrosine residue was replaced by a phenylalanine residue); (c) TrkC variant with two amino acid residues in the C-terminal tail changed to residues like those in the TrkB tail. Our results show that even such a subtle difference between Trk proteins in the C-terminal tail beyond the PLCγ1 binding site was already sufficient to induce differences in neurotrophin-stimulated outgrowth of neurite-like processes from PC12-Tet-On cells. In turn, the defect in the phosphorylation of the tyrosine residue in the PLCγ1 binding site significantly reduced the outgrowth of neurite-like processes in response to NT-3 stimulation.

2. Materials and Methods

2.1. Plasmids

The pTRE-tight-trkC plasmid contains the wild-type rat *trkC* complementary DNA (cDNA) under the transcriptional control of the tight tetracycline-responsive promoter (Clontech, Heidelberg, Germany) and has been previously described [26]. The pTRE-tight-trkC-Y820F plasmid contains the cDNA encoding the TrkC variant with the substitution of a tyrosine residue with a phenylalanine residue at position 820 of TrkC (Y820F), and its construction has already been described [26]. The pTRE-tight-trkC-T817S–I819V plasmid contains the cDNA encoding the TrkC variant with two substitutions of threonine and isoleucine residues with serine and valine residues at positions 817 (T817S) and 819 (I819V) of TrkC. The substitutions were made using the Transformer Site-Directed Mutagenesis Kit (Clontech), similar to the Y820F substitution. The pTRE-tight-trkC plasmids enable Dox-induced expression of the *trkC* cDNAs in cells containing the reverse tetracycline-controlled transactivator (rtTA). In turn, Erk-1/2 activity was measured indirectly using a luciferase reporter system consisting of pFR-Luc and pFA2-Elk1 plasmids of the PathDetect Elk1 trans-Reporting System (Stratagene, La Jolla, CA, USA) and the Dual-Luciferase Reporter assay (Promega, Madison, WI, USA) according to the manufacturers' protocols and using a GloMax-20/20 single-tube luminometer (Promega). Plasmid phRG-B (Promega) encoding *Renilla* luciferase was used as an internal control in transient transfection experiments performed using Lipofectamine 2000 (Life Technologies, Poland). Erk-1/2 activity measured by the luciferase reporter system is denoted in the figures as 'Activation of GAL4-Elk1'.

2.2. Cells

The PC12-Tet-On cell line [27] containing rtTA was purchased from Clontech (Heidelberg, Germany). Cells were grown in DMEM/GlutaMAX medium (Life Technologies, Carlsbad, NM, USA) with high glucose content (4.5 g/L), supplemented with 10 % donor horse serum (HS, Sigma, St. Louis, MO, USA) and 5 % fetal calf serum (FCS, Thermo Scientific, HyClone, Cramlington, UK) and with antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.1 mg/mL G418, purchased from Sigma and 0.075 mg/mL hygromycin B purchased from Life Technologies) at 37 °C in 5 % CO_2. The derivation of stably transfected clones with Dox-induced expression of wild-type *trkC* (clone 4.1) and *trkC-Y820F* variant (clones 36.1, 55.1, 65.1) was previously described [26]. Similarly, a clone 2.1 of PC12-Tet-On cells with Dox-induced expression of the *trkC-T817S–I819V* variant was obtained (see Fig. 1).

2.3. Quantitative RT-PCR (RT-qPCR)

Cells suspended in the culture medium were seeded into the wells of poly-L-lysine coated 12-well culture plates (600,000 cells per well). Cells were then treated with various concentrations of Dox (Sigma) for 24 h to induce expression of the *trkC* cDNA introduced into the genome of PC12-Tet-On cells under the transcriptional control of the tetracycline promoter, or cells were left untreated in controls. After removal of the culture medium, cell lysis and RNA isolation were performed using TRI

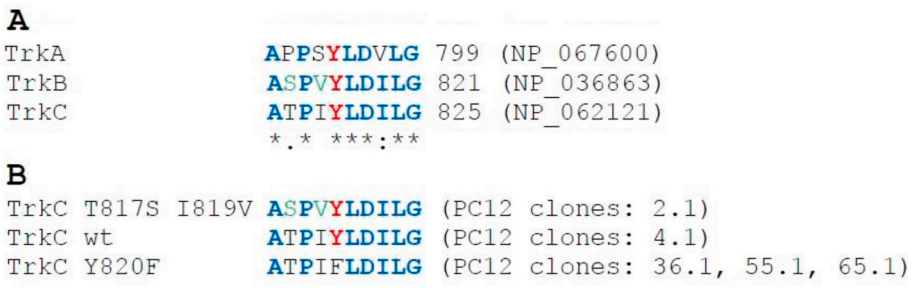

Fig. 1. C-terminal protein sequences of Trk receptors and generated TrkC variants used in this work. A. C-terminal sequence comparison of rat Trk receptors: TrkA (NGF receptor), TrkB (BDNF and NT-4 receptor), TrkC (NT-3 receptor). The accession numbers are provided in brackets. B. Sequence comparison of the C-terminal TrkC variants transfected into PC12-Tet-On cells: TrkC wt (wild-type), TrkC with the C-terminal sequence as in TrkB (variant with T817S–I819V substitutions), TrkC with an inactivated phosphorylation site on the C-terminal tail of the protein, presumably unable to recruit PLCγ1 (variant with the Y820F substitution). The names of the derived PC12-Tet-On clones with doxycycline (Dox)-induced expression of the given TrkC variant are given in brackets.

Reagent RT and BAN BN 191 (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's protocol. Real-time PCRs were performed in the Eco Real-Time PCR System (Illumina, San Diego, CA, USA) with the SYBR Green qPCR EURx Master Mix (EURx, Poland) with specific primers:

trkC forward 5′-TCTTCAACAAGCCCACCCACTACA,

trkC reverse 5′-AATCTGTGCTCTCTGGAAAGGGCT (PCR product 126 bp),

hprt1 forward 5′- GCTTGCTGGTGAAAAGGACC,

hprt1 reverse 5′- CCCTGAAGTACTCATTATAGT (PCR product 117 bp).

The results of *trkC* mRNA quantification were normalized to *hprt1* mRNA quantified in the same samples and calculated by the ΔΔCt method [28]. Briefly, ΔCT (of a given sample) = CT (*trkC* in a given sample) - CT (*hprt1* in a given sample) and ΔΔCT = ΔCT (of a given sample) - ΔCT (of the calibrator), where ΔCT (of the calibrator) = the average ΔCT of all untreated clones (Dox = 0). Finally, normalized relative quantities (NRQ) = 2^-(ΔΔ CT). The calculated NRQ are presented in the figures as the relative level of *trkC* mRNA, while the statistical analysis was performed on the data after logarithmic transformation: Log [2^-(ΔΔCt)].

2.4. Western Blot

Cells were seeded in poly-L-lysine-coated 6-well cell culture plates (million cells per well) and then starved in DMEM/GlutaMAX medium containing 0.5 % FCS and 0.5 % HS for 24 h. Cells were then treated for 24 h with Dox (1.0 μg/mL in most, but also 0.01 and 0.10 μg/mL in some experiments) to induce expression of wild-type or variant TrkC. Cells were then stimulated with 100.0 ng/mL (10.0 ng/mL in some experiments) NT-3 (PeproTech, Cranbury, NJ, USA) for 5, 15 or 300 min or left unstimulated in control samples. After stimulation, the cell monolayer plates were quickly placed on ice, the culture medium was removed, and the cells were washed twice with phosphate-buffered saline and lysed in a buffer containing 50 mM Tris-HCl pH 7.5, 5 M NaCl, 0.5 % (v/v) Igepal, protease inhibitors, phosphatase inhibitors (50 mM NaF, 0.1 mM Na_3VO_4), 0.5 M dithiothreitol, 0.05 % (v/v) β-mercaptoethanol and 4 × Laemmli sample buffer. Whole cell lysates were then incubated at 99 °C for 10 min and resolved by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membranes (Amersham, UK) and Western blot analysis was performed. For this purpose, the membranes were blocked by incubating for 2 h in a blocking solution containing 1 % casein in TBS buffer (50 mM Tris-HCl pH 8.0, 0.15 M NaCl) with 0.05 % Tween 20, after which the membranes were washed twice in TBS and once in TBS with 0.05 % Tween 20. The membranes were then incubated overnight at 4 °C with the appropriate primary antibodies: anti-TrkC (goat polyclonal IgG, Upstate Biotechnology, code 07–226, 1:1000), monoclonal anti-c-Myc (clone 9E10, Sigma), monoclonal anti-β-actin (Sigma, 1:1000), phospho-p44/42 MAPK (p-Erk-1/2, XP rabbit antibodies, Cell Signaling Technology, Danvers, MA, USA, code 4370, 1:2000), p44/42 MAPK (Erk-1/2, rabbit antibodies, Cell Signaling Technology, code 4695, 1:1000), phospho-p38 MAPK (Thr180/Tyr182) (p-38, rabbit antibodies, Cell Signaling Technology, 1:1000), p38 MAPK (rabbit antibodies, Cell Signaling Technology, 1:1000), phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK, rabbit antibodies, Cell Signaling Technology, 1:1000), phospho-Akt (Ser473) (p-Akt, rabbit antibodies, Cell Signaling Technology, 1:1000), Phospho-Tyrosine (p-Tyr, rabbit antibodies, Cell Signaling Technology). The membranes were then washed four times in TBS with 0.05 % Tween 20 and then incubated for 1 h at room temperature with fluorochrome-conjugated secondary antibodies (all from LI-COR Biosciences, Lincoln, NE, USA): IRDye 800CW donkey anti-goat IgG (after anti-TrkC), IRDye 680RD donkey anti-mouse IgG (after anti-β-actin, c-Myc), and IRDye 800CW goat anti-rabbit IgG (after anti-Erk-1/2, p38, JNK, Akt, p-Tyr). Immunoreactivities were detected using the ODYSSEY Infrared Imaging System (LI-COR Biosciences). Finally, Western Blot band optical densities were determined for some results using ImageJ software (US National Institutes of Health, Bethesda, USA) and expressed in relative densitometric units. Band density ratios of phosphorylated Erk-1 (phospho-p44 MAPK) and Erk-2 (phospho-p42 MAPK) proteins to the corresponding total Erk-1 and Erk-2 proteins (i.e., phosphorylated and unphosphorylated) were then calculated for a given sample.

2.5. Flow cytometric analysis of the TrkC receptor stained by immunofluorescence on the surface of living cells

Cells were seeded in a 6-well plate (CytoOne, Ocala, FL, USA) at a density of 1.5 million cells per well in medium supplemented with 0.1 mg/mL G418 and 0.075 mg/mL hygromycin B. The next day, cells were treated with 1.0 μg/mL Dox for 24 h. The cells were then suspended in 1 mL of Cell Dissociation Solution Non-enzymatic (Sigma) and samples were washed twice with phosphate-buffered saline (PBS) containing 2 % FCS. Then, after brief centrifugation, 5 μL of PBS with 1 % HS and 100 μL of anti-TrkC antibody (Upstate Biotechnology, Charlottesville, VA, USA, 1:500) were added to the cell pellet and the cell suspension was incubated for 20 min on ice. The cells were then washed twice with PBS supplemented with 2 % FCS, then 5 μL of PBS with 1 % HS and 100 μL of the antibody-biotin conjugate (BD Biosciences, Franklin Lakes, NJ, USA, 1:500) were added to the cell pellet, followed by incubation in suspension for 20 min on ice. The cells were washed again, then 100 μL PE-Cy7 Streptavidin (BD Biosciences, 1:1000) was added to the cell pellet and the cell suspension was incubated for 20 min on ice. Cells were washed twice with PBS supplemented with 2 % FCS and finally suspended in 500 μL PBS supplemented with 2 % FCS. Addition of the anti-TrkC antibody was omitted from the immunostaining controls. Readings were taken on a BD Biosciences flow cytometer and results analyzed

using WinMDI software.

2.6. Immunocytochemistry

Cells seeded at a density of 20,000 cells per well on a poly-L-lysine-coated 8-well Chamber Slide (Nunc-LabTek, Thermo Fisher Scientific) were treated with 1.0 μg/mL Dox for 24 h to induce TrkC expression. Then, the cells were fixed with 70 % ethanol for 10 min, washed with PBS supplemented with 0.01 % Tween 20 (PBS-T) and permeabilized for 30 min with PBS buffer containing 0.3 % Triton X-100. Next, cells were incubated for 30 min with PBS supplemented with 10 % HS and subsequently washed with PBS-T. Then, cells were incubated for 1 h with goat IgG antibodies directed against the extracellular domain of the rat TrkC receptor (Upstate Biotechnology) diluted at 1:700 in PBS-T. After washing with PBS-T, cells were incubated for 30 min with Cy3-conjugated donkey anti-goat IgG antibodies (Millipore, Burlington, MA, USA, code AP180C) diluted at 1:1200 in PBS-T, and for 10 min with a solution of YO-PRO-1 iodide (Molecular Probes, Eugenem OR, USA). Fluorescence imaging was performed using an Axio Imager A1 microscope (Carl Zeiss Microscopy, Jena, Germany). TrkC stained with primary antibody and Cy3-conjugate appeared red, while cell nuclei stained with YO-PRO-1 were green.

2.7. Analysis of the outgrowth of neurite-like processes

Cells were seeded (4,000 cells per well) into the wells of poly-L-lysine-coated 96-well cell culture plates (CytoOne) and starved for 48 h in DMEM/GlutaMAX medium containing 0.5 % FCS and 0.5 % HS to synchronize the cell cycle. Cells were then treated for 24 h with 1.0 μg/mL Dox to induce TrkC expression (wild-type or variant) or left untreated in controls, and then cells were stimulated with 0.1, 10.0, or 50.0 ng/mL NT-3 or left unstimulated in controls (Dox alone). In positive controls, cells were treated with 50 ng/mL NGF (Sigma). Dox and NT-3 were added every other day during medium replacement. Seven days after the first addition of neurotrophin, cells were assessed under a phase-contrast light microscope, cells bearing neurite-like processes were counted, and the length of processes was measured using Zeiss-ZEN software. Cells bearing neurite-like processes were defined as those whose processes were longer than the diameter of the cell passing through its geometric center.

2.8. Statistics

NRQ of *trkC* mRNA, calculated from RT-qPCR results, were statistically evaluated after logarithmic transformation. Differences in NRQ of *trkC* mRNA, encoding wild-type or variant TrkC, in different clones were analyzed by one-way analysis of variance (ANOVA) using online calculator ([29]: https://www.statskingdom.com). In turn, for a given clone, differences between the NRQ of *trkC* mRNA induced by Dox and untreated cells were assessed using the Welch's T-test for unknown and unequal variance, using online calculator [29]. A value of $p<0.05$ was regarded statistically significant.

The results of the analysis of neurite-like outgrowth from cells of the compared clones treated with a given concentration of NT-3 were tabulated in 2 x 2 contingency tables (cells with "neurites" vs. cells without "neurites" for the two compared clones) and analyzed using Fisher's exact test, and where possible, Pearson chi-square test of independence [30]. The null hypothesis tested was that the proportions of cells with neurite-like processes were the same for cells with wild-type TrkC and cells with mutant TrkC (i.e., no association was found between the tested TrkC variant and the proportion of cells with neurite-like processes for the two compared clones), and the alternative hypothesis was accepted if $p<0.05$.

3. Results

3.1. PC12-Tet-On cells carrying wild-type (wt) or variant TrkC

To test the importance of phosphorylation of the C-terminal tyrosine residue for the process of neurite outgrowth following TrkC stimulation by NT-3, PC12-Tet-On cell clones 36.1, 55.1 and 65.1 carrying TrkC-Y820F were obtained (Fig. 1B). In the TrkC-Y820F variant, the tyrosine (Y) residue at position 820 was replaced with a phenylalanine (F) residue because the Y -> F substitution prevents the recruitment of downstream molecular targets that require phosphorylation of a tyrosine residue, but not those whose recruitment does not depend on tyrosine phosphorylation. In turn, the TrkC-T817S–I819V variant had substitutions of two amino acid residues T817S and I819V upstream of the PLCγ1 binding site. This TrkC-T817S–I819V variant had a C-terminal sequence of 11 amino acid residues identical to that of the TrkB protein, and its activation still depended on stimulation by NT-3 rather than BDNF, so its effects could be directly compared to TrkC wt. Clone 2.1 of PC12-Tet-On cells carrying the TrkC-T817S–I819V variant was obtained to test whether a difference of just two amino acid residues between the C-terminal tails of TrkB and TrkC might already be sufficient to induce different neurite outgrowth.

The Dox-induced gene expression system (Fig. S1) was chosen to express TrkC because this system allows cell proliferation without undesirable overexpression of the neurotrophin receptor and, to some extent, also allows the study of cells carrying different TrkC variants at a similar levels. In all PC12-Tet-On cell clones tested, treatment with Dox resulted in a concentration-dependent increase in the expression level of *trkC* mRNA (Fig. S2). No significant differences were found in the expression of *trkC* wt and its variants at a Dox concentration of 1.0 μg/mL (Fig. 2), and this Dox concentration was used in further studies to stimulate the outgrowth of neurite-like processes from cells containing TrkC wt or its variants. TrkC protein levels also depended on the Dox concentration used (Fig. S3). TrkC protein was detected on the surface of cells treated with 1 μg/mL Dox for 24 h (Fig. 3 and Fig. S4). In turn, Erk-1/2 activation after TrkC stimulation by NT-3 was measured using luciferase reporter assays (Fig. S5). Measurements of Erk-1/2 activation indirectly indicate that after 12 h of induction of *trkC* expression by Dox, the maximum level of TrkC on the cell surface was reached (Figure S6A). TrkC transport to the cell surface and/or TrkC-dependent activation of

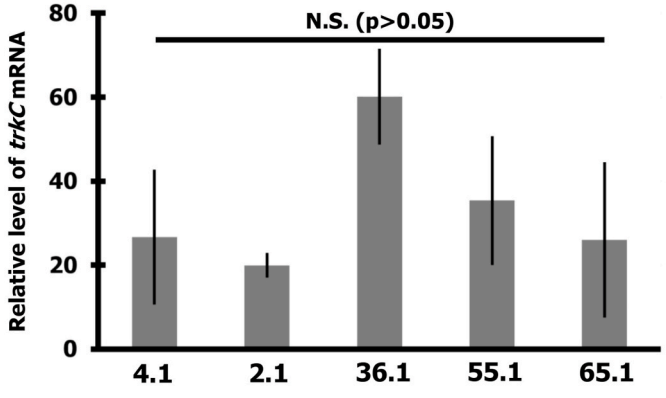

Fig. 2. Expression of NT-3 receptors in PC12-Tet-On cells stably transfected with TrkC. Data shown are for: clone 4.1 with Dox-induced *trkC* wt; clone 2.1 with Dox-induced *trkC*-T817S–I819V; clones 36.1, 55.1 and 65.1 with Dox-induced *trkC*-Y820F. Expression levels of *trkC* mRNA were determined after 24 h treatment with 1.0 μg/ml Dox and evaluated by RT-qPCR. The data presented are averaged from two (for clones 2.1, 36.1), three (for clone 55.1) or four (for clones 4.1, 65.1) independently performed experiments. As a result of one-way ANOVA analysis, it was found that the differences between the mean values of relative *trkC* mRNA levels in the tested clones were not statistically significant ($p>0.05$).

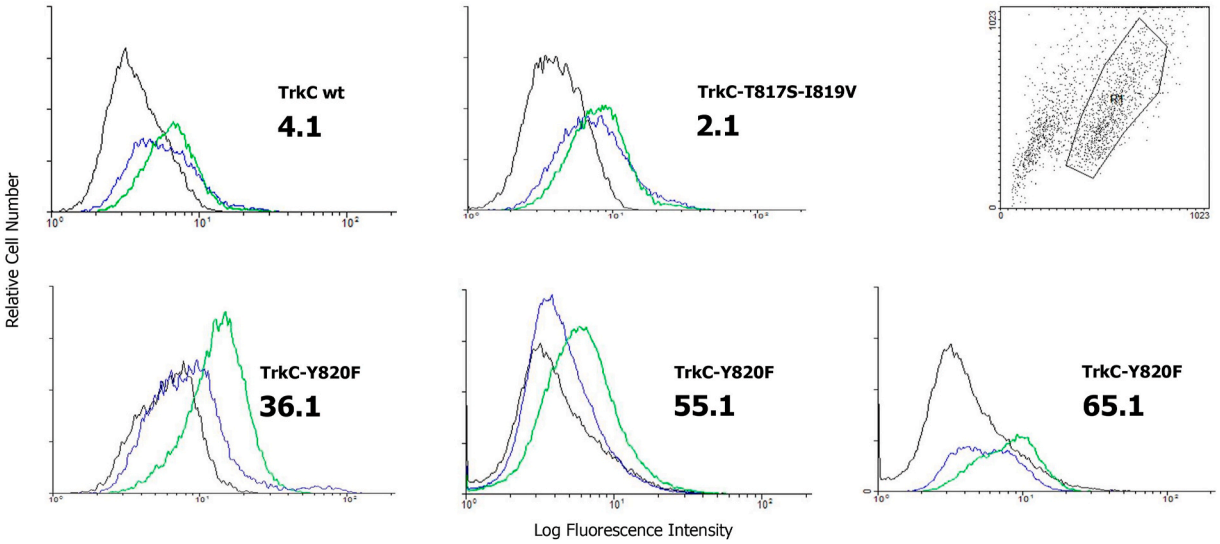

Fig. 3. Presence of TrkC proteins on the surface of viable cells after treatment with 1 μg/ml Dox for 24 h. TrkC proteins were labeled using goat IgG antibodies directed against the extracellular domain of the rat TrkC receptor (Upstate Biotechnology) followed by an antibody conjugate with biotin and PE-Cy7 Streptavidin (BD Biosciences). Staining details are described in the Materials and Methods section. Three flow cytometer fluorescence histograms are shown for each clone: for cells not labeled with anti-TrkC antibodies (black line), for cells not treated with Dox but then labeled with anti-TrkC antibodies (blue line), for cells treated with Dox and then labeled with anti-TrkC antibodies (green line). Cell suspensions were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). Cellular debris was excluded from analysis by electronic gating on the FSC/SSC dot plot. Histograms show the results of three independent experiments with a similar trend. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Erk-1/2 were partially inhibited by brefeldin A (Figure S6.B).

3.2. Phosphorylation of selected protein kinases by TrkC wt and its variants

TrkC proteins induced in cells of clones stably transfected with *trkC* genes after 24 h of treatment with 1 μg/mL Dox were found to be already phosphorylated on tyrosine residues, and it was not possible to convincingly detect an increase in the level of phosphorylation following stimulation of the TrkC receptor with the NT-3 ligand (Fig. S7). However, the increase in Erk-1/2 activity after NT-3 treatment was most likely dependent on the tyrosine kinase activity of the catalytic domain of the TrkC protein, as the selective K252a inhibitor of Trk family receptor tyrosine kinases significantly reduced Erk-1/2 activation as measured by luciferase reporter assays (Fig. 4).

Next, the effect of NT-3 stimulation on the phosphorylation of Erk-1/2 protein kinases in PC12-Tet-On cells carrying TrkC wt or its variants was investigated. The time course of Erk-1/2 phosphorylation was found to be quite similar in cells containing different TrkC receptor variants in response to NT-3 ligand stimulation (Fig. S8), although quantitatively such Erk-1/2 phosphorylation was variable from experiment to experiment. However, a noticeable trend towards stronger Erk-1/2 phosphorylation was observed in clone 2.1 cells after stimulation of the TrkC-T817S–I819V variant of the NT-3 receptor (Figure S8 and Figure S9), although this was not supported by robust statistical analysis. No apparent differences were observed between TrkC variants in their ability to phosphorylate the protein kinases Akt, JNK, and p38 MAPK (Fig. S10). On the other hand, inhibition of Erk-1/2 phosphorylation by U73122, a phospholipase C inhibitor, in the clones tested except clone 36.1, suggests that PLCγ1 may have been involved to some extent in the

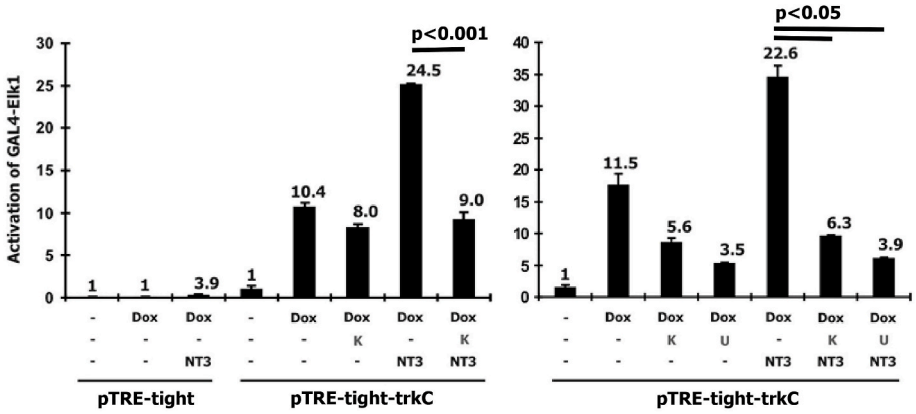

Fig. 4. The increase in Erk-1/2 activity triggered by NT-3 depended on the activity of the receptor tyrosine kinase of the Trk family. PC12-Tet-On cells were transiently transfected in a 24-well plate with pTRE-tight-trkC or pTRE-tight (empty vector) at 25 ng/well and phRG-B (Promega, encodes *Renilla* luciferase) and plasmids (both from Stratagene): pFR-Luc (GAL4 luciferase reporter) and pFA2-Elk1 (encodes the GAL4-Elk1 fusion protein). Then, the cells were treated for 24 h with 1 μg/ml Dox to induce TrkC expression and stimulated for 5 h with 100 ng/ml NT-3 without or with inhibitors: 1 μM K252a (K, inhibits the activity of the receptor tyrosine kinase of the trk family, $IC_{50} = 3$ nM, purchased from Biosource, Camarillo, USA), 25 μM U0126 (U, selective MEK-1/2 inhibitor, $IC_{50} = 58–72$ nM, purchased from Calbiochem). The results of two independent experiments are shown. Inhibition was statistically significant as assessed by two-sample *t*-test (Welch): $p<0.05$.

signaling of even the TrkC variant with a defective PLCγ1 docking site, as suggested by the results for two of three tested clones containing TrkC-Y820F (Fig. S11).

3.3. NT-3-stimulated differential outgrowth of neurite-like processes from PC12-Tet-On cells carrying TrkC variants that differ in amino acid residues in the C-terminal tail of the protein

To study the outgrowth of neurite-like processes supported by TrkC wt and its variants, PC12-Tet-On cells with Dox-induced *trkC* expression were stimulated for seven days with NT-3 (Fig. 5 and Fig. S12), and then the proportions of cells bearing neurite-like processes were assessed, taking these values as a quantitative measure of the neurite outgrowth process. First, it was checked whether the TrkC variant with a putative impairment of the PLCγ1 recruitment site had an altered ability to support the outgrowth of neurite-like processes from cells compared to TrkC wt. After treatment with 0.1 ng/ml NT-3, no more than 1 % of cells containing TrkC-Y820F (clones 36.1, 55.1, 65.1) had protrusions compared to a significantly higher proportion of 5.2 % of clone 4.1 cells with TrkC wt (Fig. 6 and Table S1). In turn, after stimulation with 10 ng/ml NT-3, in two of the three PC12-Tet-On clones examined, the TrkC-Y820F variant supported NT-3-promoted outgrowth of neurite-like processes from statistically significantly fewer cells (less than 2 % in clones 36.1 and 55.1) compared with 10.2 % of clone 4.1 cells harboring TrkC wt. However, stimulation of clone 65.1 (TrkC-Y820F) with 10 ng/ml NT-3 caused the outgrowth of protrusions from 3.6 % of cells, which was still statistically significantly lower than in the case of clone 4.1 (TrkC wt). Extension outgrowth from cells of clones 36.1 and 55.1 (TrkC-Y820F) was still statistically significantly weaker than that of clone 4.1 with TrkC wt after treatment with a higher concentration of 50 ng/ml NT-3 (Fig. 6 and Table S1). Only in clone 65.1 was the outgrowth of "neurites" noticeable after treatment with a rather high concentration of 50 ng/mL NT-3 (Figs. 5 and 6).

Finally, the TrkC-T817S–I819V variant was found to be significantly more effective than TrkC wt in supporting the outgrowth of neurite-like processes from cells, as evidenced by almost twice the percentage of cells bearing "neurites" (19.3 % *v.* 10.2 %, and 13.5 % *v.* 7.7 %) after stimulation with 10.0 ng/ml and 50 ng/ml NT-3 (see Supplementary Table S1 and Fig. 6).

4. Discussion

The C-terminus of receptor tyrosine kinases of the Trk family is the site of recruitment of PLCγ1 and potentially other proteins that may be involved in processes leading to neurite outgrowth. Two questions arise in this context. Is phosphorylation of the C-terminal tyrosine residue essential for TrkC-stimulated neurite outgrowth? Could the difference in two amino acid residues at the C-terminus of TrkB and TrkC upstream of the PLCγ1 recruitment site (see Fig. 1A) have any significance in the neurite outgrowth processes stimulated by these receptors? To test the importance of the C-terminal tyrosine residue and its immediate vicinity for the outgrowth of neurite-like processes from neurotrophin-treated PC12 cells, used as a neuronal cell model, clones of PC12-Tet-On cells carrying either wild-type (wt) TrkC or a TrkC variant were obtained (Fig. 1B).

The Tet-On system [31] and the second-generation tetracycline-responsive promoter P_{tight} were selected for *trkC* expression, which in PC12-Tet-On cells allows for at least a tenfold increase in the expression level of the regulated gene following Dox treatment compared to the background in untreated cells, as shown in our much earlier publication [32]. In this expression system in PC12-Tet-On cells, the maximum expression level of the regulated gene is achieved after 12 h of treatment with 1 μg/mL Dox, as shown for TrkC (Fig. S6) and other regulated genes [27]. The tested TrkC variants in selected clones of stably transfected PC12-Tet-On cells were induced by treatment with Dox at a concentration of 1 μg/mL at approximately similar levels (Fig. 2), and the presence of TrkC protein was detected by immunofluorescence on the surface of live cells (Fig. 3). TrkC variants in the tested clones were also found to be functional, as a robust increase in Erk-1/2 proteins phosphorylation was observed after NT-3 treatment (Figure S8 and Figure S9). The increase in Erk-1/2 activity induced by NT-3 in PC12-Tet-On cells transfected with the TrkC-encoding gene was rather dependent on the activity of TrkC as a receptor tyrosine kinase, as it appeared to be inhibited by the K252a inhibitor (Fig. 4).

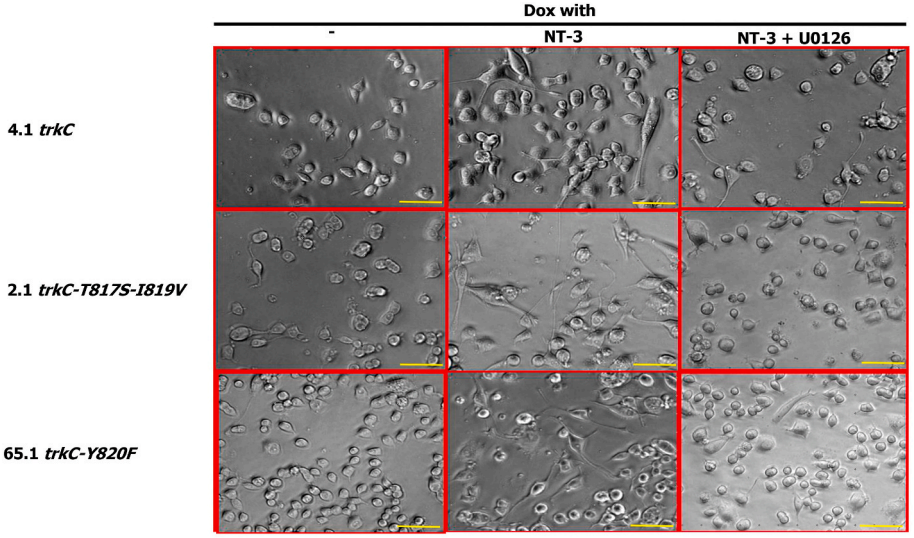

Fig. 5. NT-3-promoted outgrowth of neurite-like processes from PC12-Tet-On cells with Dox-induced expression of wild-type TrkC or the TrkC-T817S–I819V variant. One of the three clones containing the TrkC-Y820F variant is also shown, in which cell extensions were also observed after NT-3 stimulation. In contrast, no processes were observed growing from the cells of the other two clones containing the TrkC-Y820F variant (see Supplementary Fig. S13). Cells were treated with 1.0 μg/mL Dox to induce *trkC* expression and then stimulated with 50 ng/mL NT-3 for 7 days or left unstimulated. In some samples, cells were additionally treated with 10 μM U0126 to check the dependence of the growth of elongated cell processes on Erk-1/2 activation. Cells images were taken using a phase contrast light microscope. Shown are enlargements of the red-boxed images in Supplementary Fig. S12. Scale bar = 50 μm. Note the spindle shape of the cells and the formation of elongated neurite-like processes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

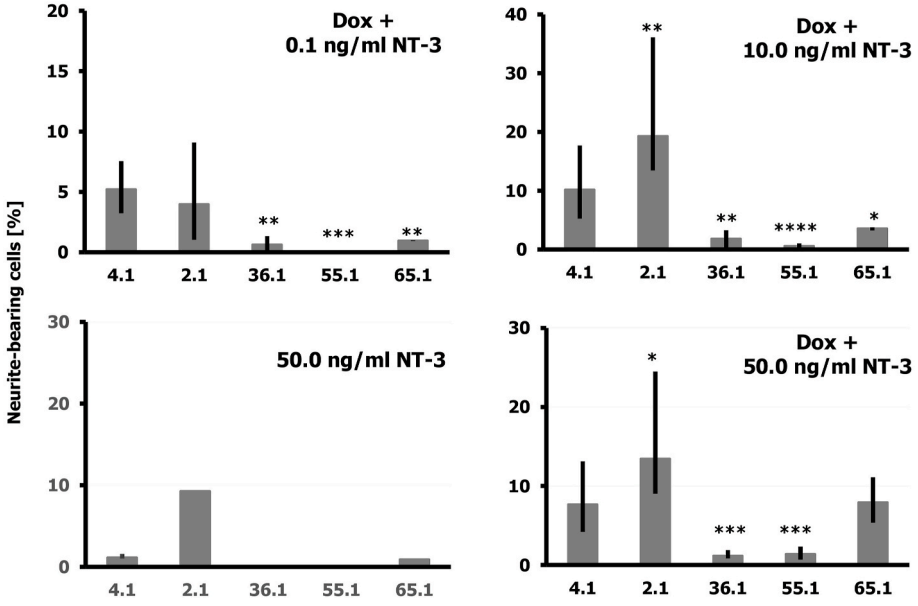

Fig. 6. Differential outgrowth of neurite-like processes from PC12-Tet-On cells carrying TrkC variants differing in amino acid residue substitutions in the C-terminal tail: wild-type in clone 4.1; T817S–I819V in clone 2.1; Y820F in clones 36.1, 55.1 and 65.1. Cells were pretreated with Dox (1.0 μg/mL) for 24 h to induce TrkC expression and then treated with Dox and NT-3 (0.1, 10.0, and 50.0 ng/mL) for seven days. In the control, cells were treated for seven days with only NT-3 (50.0 ng/mL) without Dox (no induction of TrkC expression and trace TrkC expression is presumably the result of "leaky" expression in the Tet-On system). Cells with long processes were counted as described in the Materials and Methods section. The proportion of "neurite"-bearing cells was calculated from the number of cells with long processes to the total number of cells. The bars indicate the proportions calculated from the cumulative counts from all experiments (two for clones 2.1, 36.1, 65.1 and three for clones 4.1, 55.1), while the whiskers indicate the minimum and maximum proportions in individual experiments (see Supplementary Table S1 for details). Clone 4.1 cells expressing wild-type TrkC were compared with cells expressing mutant TrkC variant for their ability to support neurite-like outgrowth from the cells. Statistical analyses were performed using Fisher's exact test. The null hypothesis was that the proportions of neurite-bearing cells were the same for 4.1 cells (TrkC wt) and for cells expressing mutant TrkC. The null hypothesis was rejected at a significance level of 5 % (*), 1 % (**), 0.1 % (***).

Very early work showed that NGF-stimulated TrkA activation then stimulated signal transduction pathways that depend on the recruitment of adaptor proteins to a phosphotyrosine residue in the membrane-adjacent region of TrkA (the SHC/FRS2 docking site) and the recruitment of PLCγ1 to a phosphotyrosine residue in the C-terminal tail of TrkA, which redundantly transmitted signals leading to Erk-1 phosphorylation [33]. In turn, another study found that the mutation of only the PLCγ1 docking site (Y816 in TrkB) did not appear to be critical for the level of Erk-1/2 phosphorylation in response to BDNF stimulation [6]. The Y820F substitution in the C-terminal tail of TrkC prevents phosphorylation of the tyrosine residue at the PLCγ1 binding site and therefore this TrkC variant should not recruit PLCγ1 (or hypothetically another protein) upon receptor stimulation with the NT-3 ligand, as has been shown much earlier for TrkA [33]. Overall, Erk-1/2 phosphorylation after TrkC stimulation by NT-3 was found to be quite similar in cells of clones 36.1, 55.1, 65.1 containing TrkC-Y820F compared to clone 4.1 containing TrkC wt, which is generally consistent with the cited results of previous studies [6,33], despite some variability from experiment to experiment. There were also no significant differences in the phosphorylations of Akt, JNK and p38 MAPK in cells equipped with TrkC-Y820F compared to TrkC wt (Fig. S10). In contrast, in some experiments, noticeable higher levels of Erk-1/2 phosphorylation were noted in clone 2.1 (TrkC-T817S–I819V) compared to clone 4.1 (TrkC wt) after 5 and 15 min of NT-3 stimulation (Figure S8 and Figure S9), although this was not supported by demonstration of statistical significance. On the other hand, the result of the experiment using the PLCγ1 inhibitor suggests that PLCγ1 may, to some extent, participate in signaling from TrkC to phosphorylate Erk-1/2, which we could not confirm based on the results of the analysis of the interaction of PLCγ1 with TrkC due to unresolved technical problems with the antibody directed against PLCγ1 and the calcium wave measurement. Furthermore, no significant differences were observed between TrkC variants in their ability to phosphorylate protein kinases such as Akt, JNK, and p38 MAPK. Finally, it cannot be excluded that proteins other than PLCγ1 may interact with the C-terminal region of Trk family proteins and influence signal transduction pathways, although such a suggestion was not supported by the results presented here.

Previous studies have shown that the human TrkA-Y785F variant with a phenylalanine residue substituted for a tyrosine residue at position 785 (Tyr-794 in rat TrkA) and therefore defective in PLCγ1 recruitment to TrkA was nevertheless able to support NGF-promoted outgrowth of neurite-like processes from PC12 cells to an extent comparable to the wild-type TrkA [34]. In contrast to those studies on TrkA, we present here our results for TrkC indicating that the TrkC-Y820F variant supported the outgrowth of neurite-like processes from statistically significantly fewer cells in all clones tested after treatment with NT-3 at lower concentrations of 0.1 ng/ml or 10.0 ng/ml. Only after applying a higher concentration of 50 ng/ml NT-3, in two of the three tested clones containing TrkC-Y820F, a statistically significantly lower number of only a few cells with extensions was recorded, while in the third clone 65.1 (TrkC-Y820F) the proportion of cells with extensions was close to the proportions for clone 4.1 (TrkC wt) (Figs. 5 and 6). Nevertheless, this result also indicates some variability between cell clones containing the same TrkC variant.

Comparing the results regarding the support for the outgrowth of neurite-like processes by TrkC wt and its Y820F variant and the phosphorylation of selected protein kinases, especially Erk-1/2, it seems that the signal from neurotrophin receptors to the final biological effect, such as neurite outgrowth, is transmitted in parallel *via* several signaling pathways, and despite the lack of significant differences in the phosphorylation of the tested protein kinases after activation of various TrkC variants, only the analysis of the final biological effect allowed for the detection of significant differences between cells carrying these trkC variants.

Going further and considering the involvement in neuronal differentiation of PLCγ1 [35,36] and possibly also other, as yet unrevealed

proteins recruited to the C-terminal tails of TrkB and TrkC receptors, we also examined whether the difference of only two amino acid residues between TrkB and TrkC near the PLCγ1 binding site may already be sufficient for differential outgrowth of neurite-like processes from PC12-Tet-On cells. Because the TrkC-T817S–I819V variant is stimulated by NT-3 and not BDNF, this highly simplified experimental system allowed for direct comparison of the effects of the receptor tyrosine kinase with the C-terminal tail of TrkB *vs.* TrkC in the context of the same remaining part of the protein molecule and under essentially the same experimental conditions (except, of course, for inserting the *trkC* genes into different positions in the genome). After stimulation with 10 or 50 ng/ml NT-3, TrkC-T817S–I819V was found to support the outgrowth of neurite-like processes from a statistically significant and almost twice the percentage of clone 2.1 cells compared to clone 4.1 containing TrkC wt (Fig. 6). This suggests that even small differences between Trk family receptors, located on these proteins outside sites directly involved in recruiting proteins involved in signal transduction (in this case PLCγ1), may contribute to differences in biological effects either by affecting the interaction between phosphorylated tyrosine residues and proteins recruited from the cytosol, or through interaction with yet unknown components of signal transduction pathways. In the absence of appropriate experimental results conforming or excluding different affinities of PLCγ1 for the C-terminus of TrkC wt and TrkC-T817S–I819V, this remains an unresolved issue. As mentioned above, it cannot be excluded that proteins other than PLCγ1 may also interact with the C-terminal region of TrkC and influence the outgrowth of neurite-like processes from PC12-Tet-On cells, differentiating the biological effect supported by TrkC variants with different C-terminal tails. It is worth adding, however, that in order to develop an interpretation of the observations, it would be worth making an effort to obtain cells that would allow the measurement of biological effects also after activation of the TrkB variant with a TrkC tail compared to the wild-type TrkB. It should be strongly emphasized here that the influence of inter-clone variability on the observed difference between clones with TrkC wt compared to TrkC-T817S–I819V cannot be ruled out. However, of several clones of PC12-Tet-On cells stably transfected with *trkC-T817S–I819V*, only one clone expressed *trkC* at a sufficiently high level, so in this case it was not possible to examine inter-clone variability. The result presented here was obtained in the PC12-Tet-On cell model system, and future studies may reveal whether differences in the C-terminal sequence of TrkB and TrkC are indeed associated with the generation of diversity in response to stimulation of these receptors in CNS cells.

5. Conclusions

First, the tyrosine residue at position 820 in the C-terminal tail of TrkC, the PLCγ1 binding site, appears critical for TrkC-supported outgrowth of neurite-like processes from PC12-Tet-On cells. Next, the interesting result of the analyzes performed with the TrkC variants presented here is that which suggests that the TrkC-T817S–I819V variant is more effective than wild-type TrkC in supporting the outgrowth of neurite-like processes from cells, at least in the context of PC12 cell-specific intracellular mechanisms, although due to potential variability between clones, this will still require future verification.

CRediT authorship contribution statement

Pawel Krawczyk: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Dagmara Klopotowska:** Writing – review & editing, Resources, Methodology, Conceptualization. **Janusz Matuszyk:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Data availability

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101853.

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