Journal of Cell Biology and Histology
Volume 5 | Issue 1
ISSN: 2638-082X

Research Article Open Access

7,8-Dihydroxyflavone Affects Cellular Behavior of PC12 Cells

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Citation: Lipinski, Stuppardt, Stoycheva, Oezcan and Wiese (2023) 7,8-Dihydroxyflavone Affects Cellular Behavior of PC12 Cells. J Cell Biol Histol 5(1): 101

Received Date: September 12, 2023 **Accepted Date:** October 12, 2023 **Published Date:** October 16, 2023

Abstract

The BDNF/TrkB signaling is a robust regulatory feature of neuronal cells that promotes survival, differentiation, and proliferation in a variety of cell types and animal models for neurodegenerative diseases. The activation of TrkB by BDNF in clinical trials is accompanied by remarkable side effects and appears to be challenging due to BDNF's adverse molecular profile. Herein, a human TrkB-overexpressing PC12 cell line ($PC12^{TrkB}$) was generated, and the effects of BDNF and the TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) on cell survival, apoptosis, and differentiation were compared to investigate alternative activation modes of TrkB in more detail. It was observed that 7,8-DHF could reduce the apoptotic activity in a dose- dependent manner via activating the PI3K/AKT pathway using western blot analyses and immunocytochemical stainings. Moreover, a decrease in proliferating cells was detected in a concentration-dependent way. Neurite growth was also enhanced after 1 and 5 days of differentiation, however, 7,8-DHF showed a more potent effect after 1 day than BDNF. Furthermore, a TrkB-independent activation of PI3K/AKT and Ras/Raf/MAPK and an impact on cellular response could be observed in native $PC12^{wt}$ cells. This outcome could imply the TrkB-independently action of 7,8-DHF in cells lacking the TrkB receptor through an unknown mechanism.

Keywords: TrkB agonist; differentiation; transfected cells; TrkB signaling

List of Abbreviations: 7,8-DHF: 7,8-dihydroxyflavone; AKT: Protein kinase B; BDNF: Brain derived neurotrophic factor; BrdU: Bromdesoxyuridin; div: Days in vitro; DAB: Diaminobenzidine tetrahydrochloride; DMEM: Dulbecco's Modified Eagle Medium; EDTA: Ethylenediaminetetraacetic acid; G418: Geneticin disulfate 418; MEM: Minimum Essential Medium; MAPK: Mitogen-activated protein kinase; NGF: Nerve growth factor; NT-3: Neurotrophin-3; NT-4/5: Neurotrophin-4/5; PMSF: Phenylmethylsulfonyl fluoride; PBS/A: Phosphate buffered saline with 0.1% BSA; PBS: Phosphate buffered saline; PBT1: Phosphate buffered saline with 0.1% TritonX-100; PI3K: Phosphoinositide-3-kinase; PVDF: Polyvinylidene difluoride; PIC: Protease inhibitor cocktail; Raf: Rapidly accelerated fibrosarcoma; Ras: Rat sarcoma; rpm: Revolutions per minute; RT: Room temperature; TBS: Tris-buffered saline; TBST: Tris-buffered saline with 0.1% Tween; Trk: Tropomyosin

receptor kinase; TrkA: Tropomyosin receptor kinase A; TrkB: Tropomyosin receptor kinase B; TrkC: Tropomyosin receptor kinase C

Introduction

Neurotrophic factors are essential regulators of neuronal development in vertebrates [1, 2]. They participate in a variety of processes such as neurogenesis, the release of neurotransmitters, and plasticity [3, 4]. In addition, they have survival-promoting properties on motoneurons [6] and play a crucial role in nervous system regeneration after injuries [7, 8]. Neurotrophins are known as a type of neurotrophic factor family, including nerve growth factor (NGF) [9], neurotrophin-3 (NT-3) [10], neurotrophin-4/5 (NT-4/5) [11], and brain-derived neurotrophic factor (BDNF) [12]. These growth factors are synthesized by nervous system cells and tissues [13, 14] and exert a wide range of influential properties [2] through the activation of specific tropomyosin receptor kinase (Trk) receptors. Trk receptors belong to the receptor tyrosine kinase family and are primarily expressed by neuronal cells [15]. TrkA, TrkB, and TrkC all have a cytoplasmatic domain in common, but their extracellular domains differ [16], resulting in their specificity. NGF activates TrkA [17], whereas TrkC is stimulated by NT-3 [18]. Moreover, BDNF and NT-4/5 enable TrkB [19, 20]. The binding causes autophosphorylation of the catalytic intrinsic tyrosine kinase domain in the cytoplasm [21], which is followed by the recruitment of adapter and effector proteins. This results in activating signaling pathways such as PI3K/AKT, Ras/Raf/MAPK, and PLCγ, influencing cellular behavior [22,14].

The neurotrophin BDNF, discovered in 1982 [12], influences survival [23], proliferation [24], synaptogenesis [25], and plasticity [26]. According to the strong regulatory role of BDNF, changes or variations in its expression and its signaling through TrkB are linked to various psychiatric and neurodegenerative diseases [27]. The use of BDNF in clinical trials appears to be accompanied by some severe side effects and poses a research challenge due to its poor molecular profile [28]. The administration of BDNF intrathecally to patients with amyotrophic lateral sclerosis could not improve the survival and function of the motoric system [29] or the autonomic nervous system [30]. It is assumed that the difficulties are attributed to BDNF's unstable molecular structure, short half-life period, and inability to cross the blood-brain barrier [28, 29]. These problems are addressed in current research by using innovative approaches like for example the use of nanoparticles to increase the permeability of the blood-brain barrier and enhance the delivery of BDNF [31]. Because of the beneficial effects of TrkB signaling, the investigation of alternative TrkB activation has received wide attention by researchers, which could be a promising approach for future treatment.

7,8-dihydroxyflavone (7,8-DHF) is a naturally occurring flavonoid [32] that was characterized as a TrkB agonist in hippocampal neurons for the first time [33, 34]. The protective function on endangered neurons, improvement of neuronal functions, and stimulation of neuronal regeneration have been represented for flavonoids in the literature [35, 36]. Neuroprotective effects, as well as improved phenotypes have been observed for 7,8-DHF in animal models for different central nervous system diseases, including Alzheimer's disease [37], amyotrophic lateral sclerosis [38], Parkinson's disease [39], and Huntington's disease [40]. Additionally, 7,8-DHF can promote the survival of dopaminergic hippocampal neurons [33] and retinal ganglia cells [41], protects neurons against degeneration, and improves axon regeneration in a TrkB-dependent manner [42, 43]. Previous research conducted by our group could also reveal a protective and differentiation-promoting effect on motoneurons [5]. Moreover, the ability of 7,8-DHF to cross the blood-brain barrier [33] makes the small TrkB agonist a promising candidate for therapeutic treatments. Meanwhile, it is unclear how the TrkB receptor is activated in a BDNF-independent manner and what cellular responses this alternative activation method causes, as motoneurons and hippocampal neurons have opposing effects [5].

The majority of studies on alternative TrkB activation and its effects on cellular behavior have been carried out using primary cells like for example retinal ganglia cells, motoneurons or spiral ganglion cells [41, 5, 42]. Selected cell lines with neuronal characteristics can overcome the labor-intensive cultivation attached to primary cell isolation from animals. Furthermore, stem cell differentiation to neuronal cells is associated with a low number of differentiated neurons [44].

PC12 cells, derived from a rat pheochromocytoma, are a commonly used model system for analyzing neuronal differentiation and neurotrophin-induced signaling transduction. They proliferate rapidly and differentiate into neuron-like cells after serum removal and NGF addition [45, 46]. In the present evaluation, a stable transfected human TrkB overexpressing PC12 cell line ($PC12^{TrkB}$) has been generated to enable TrkB- dependent analyses because they lack TrkB [47].

This study aims to characterize a new $PC12^{TrkB}$ cell line in order to establish it for future analyses concerning TrkB activation and induced cellular responses. Moreover, the effect of TrkB activation by BDNF versus stimulation via the TrkB agonist 7,8-DHF on survival, proliferation, and differentiation was investigated. Furthermore, to determine whether TrkB mediates the effect, the same experiments were repeated on native PC12 cells ($PC12^{wt}$) and after inhibiting TrkB with the Trk inhibitor K252a on $PC12^{TrkB}$ cells. Successful TrkB activation in the PC12TrkB cell line could be obtained by BDNF and 7,8-DHF, leading to different cellular responses in a dose-dependent manner. It turned out that 7,8-DHF could inhibit the apoptotic activity of $PC12^{TrkB}$ cells. Additionally, it was represented that 7,8-DHF had no impact on $PC12^{TrkB}$ cells proliferation, similar to BDNF, or had a lower effect. Compared to BDNF, 7,8-DHF significantly enhanced neurite growth stronger after 1 day than after 5 days. Interestingly, a TrkB-independent activation of the signaling pathways PI3K/AKT and Ras/Raf/MAPK, as well as an effect on the cellular response, could be observed in $PC12^{wt}$ cells. The observed trends raise questions about the exact effect mechanism of 7,8-DHF. Hence, it appears that the TrkB agonist may also act in a TrkB-independent way, which should be investigated more intensively in the future.

Materials and Methods

Materials

The $PC12^{wt}$ cells were provided by the German Collection of Microorganisms and Cell Cultures. Medium ingredients or cell culture solutions were purchased from Thermo Scientific (horse serum, fetal calf serum, DMEM, and MEM) and Sigma-Aldrich (Geneticindisulfate 418 (G418), BDNF, 7,8-DHF, poly-D-lysine, penicillin/streptomycin, NGF, phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail (PIC), diaminobenzidine tetrahydrochloride (DAB), trypan blue solution, and K252a). In addition, primary antibodies were obtained from Sigma- Aldrich (caspase-3, βIII-tubulin, α-Tubulin, and pTrkB), Cell Signaling (AKT, pAKT, pERK, and TrkA), and Santa Cruz Biotechnology (ERK and TrkB). Moreover, secondary antibodies were purchased from Dianova (anti-rab-Cy3, anti-m-HRP, anti-m-Cy3, and anti-rab-HRP), and the BrdU detection kit was provided by Roche.

Cultivation of $PC12^{trkB}$ and $PC12^{wt}$ cells

All experiments were performed using native $PC12^{wt}$ cells as well as TrkB overexpressing PC12 cells ($PC12^{TrkB}$) generated previously via stable transfection. Regarding cultivation, cells were incubated on 0.001% poly-D-lysine coated culture dishes (10 cm Ø) at 5% CO2 and 37°C in a high-serum medium containing DMEM, 10% (v/v) horse serum, and 5% (v/v) fetal calf serum. To ensure an absolute stable transfected PC12TrkB cell line, 1 mg/ml G418 was added to $PC12^{TrkB}$ cells as a selection factor. After reaching a 70-80% confluence, cells were split, and the medium was changed every 2-3 days. To singularize the cells for passaging or seeding, they were washed twice with PBS (A. bidest, 1.5 mM KH_2PO_4, 3 mM KCl, 137 mM NaCl, and 6.5 mM Na_2HPO_4 x $2H_2O$) and treated for 1-3 min with 500 µl trypsin/EDTA. Afterward, cells were mechanically detached from the culture dish via pipetting. After centrifugation for 5 min at 800 rpm at room temperature (RT), the pellet was gently resolved in 1 ml MEM. The cell suspension was then ready for further cultivation or subsequent experiments.

Differentiation, apoptosis, and proliferation assays

For differentiation and apoptosis assays, 2.500 cells per well were seeded on poly-D- lysine coated 4-well culture dishes in low-serum medium containing DMEM and 1% (v/v) horse serum (1 mg/ml G418 for $PC12^{TrkB}$ cells). Regarding the proliferation assay, 5.000 cells were seeded in MEM. Before starting the assays, cells were synchronized for 24 h, and the medium was changed.

To investigate, if the effect of 7,8-DHF is mediated by TrkB, the TrkB antagonist K252a was added to $PC12^{TrkB}$ cells before stimulation. By adding the antagonist (1 ng/ml K252a) to inhibit TrkB, and the agonists (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM 7,8-DHF; 1 ng/ml BDNF; 20 ng/ml NGF) to stimulate TrkB (or TrkA in case of NGF), assays were started. The differentiation assay was run for 5 days, during which time the medium was changed, and factors were added again after 2 days. Proliferation and apoptosis assays were carried out for 2 days. Cells used in proliferation assays were labeled with 10 μM BrdU for 1 h at 37°C before being fixed for 10 min in Ethanol-Fixative at -20°C. Notably, cells provided for other assays were fixed for 15 min in 4% (v/v) paraformaldehyde.

Immunocytochemical Staining

Fixed cells were first permeabilized with PBT1 (PBS containing 1% (w/v) BSA and 0.1% Triton-X) and then incubated with the primary antibody (βIII-tubulin, caspase-3; 1:300 in PBT1) for 1 h at RT. BrdU-labelled cells, however, were incubated directly with the primary antibody (BrdU; 1:50 in incubation buffer from BrdU detection kit). Following that, cells were washed three times with PBS and incubated with secondary antibodies (anti-m-HRP, anti-rab-HRP, anti-m-Cy3; 1:300 in PBS/A (PBS containing 0.1% (w/v) BSA) and Hoechst-33258 (1:50) for 1 h light-protected at RT. Subsequently, cells were washed three times with PBS. Fluorescent stainings were mounted with a PBS-Glycerin (1:1) mixture, whereas cells of the differentiation assay were incubated with DAB for 3-30 min at 37°C, light-protected. Finally, the cells were washed three times with PBS before mounting with Mowiol.

Microscopy, Evaluation, and Neurite Measurements

Standard fluorescence microscopy was performed using a Zeiss Axioplan 2 Axiophot microscope, and images were captured with a Zeiss AxioCam HRc digital camera. The percentage of proliferating cells was estimated by counting the total cell number and the number of positive cells in three independent pictures per well and three wells per condition using the ImageJ program. Standard light microscopy was performed using the Leica DM2500 microscope, and images were captured with a Leica EC3 digital camera. The total cell number and amount of caspase-3 positive cells were counted directly at the light microscope of three independent pictures per well and three wells per condition to investigate the apoptosis rate. ImageJ software was applied to measure the longest neurite and the number of neurites in 200 cells per experimental setup (n=3) to analyze neurite outgrowth. Meanwhile, neurites shorter than 15 μm were included and counted as "zero."

Viability assay with trypan blue staining

To investigate the viability 2.500 cells per well were seeded in a low-serum medium containing DMEM and 1% (v/v) horse serum (1 mg/ml G418 for $PC12^{TrkB}$ cells) on poly-D-lysine coated 4-well culture dishes. Cells were stimulated for 1 day with 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM), BDNF (1 ng/ml), or NGF (20 ng/ml), then stained for 5 min with trypan blue and washed twice with PBS. Using the Leica DM2500 light microscope, total cells and positive stained cells (dead cells) were counted directly in four independent visual fields per well and four wells per condition.

Western blot analysis

Western blot analyses were used to determine the activation of the signaling pathways Ras/Raf/MAPK and PI3K/AKT, as well as the expression and activation of TrkB. Cell lysates were generated by seeding 1 million cells on poly-D-lysine coated 35 cm culture dishes in MEM. After 24 h of synchronization, MEM was replaced and inhibitor and factors were added for 3 min (10 ng/ml BDNF; 0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM 7,8-DHF; 20 ng/ml NGF) or 90 min (20 ng/ml NGF). Cells were lysed after 1 day to detect receptor expression. Cells were washed once with PBS and lysed for 20 min on ice in membrane lysis buffer (A. bidest, 50 mM Tris buffer, 50 mM sodium acetate, 60 mM N-Octyl-β-D-Glycopyranoide, 10 ng/ml PMSF, 20 ng/ml PIC). The supernatant was stored at -20°C for further use after centrifuging for 10 min at 16.400 rpm at 4°C.

In order to run gel electrophoresis, 15 µl of lysates were mixed with 5 µl of 4x sample buffer (A. bidest, 0.25% (w/v) bromphenol blue, 40% (v/v) glycerol, 20% (v/v) β-Mercaptoethanol, 9.2% (v/v) SDS, 250 nM Tris/HCl (pH 6.8)), heated for 5 min at 95°C, and briefly centrifuged. Protein lysates were separated on 10% (v/v) acrylamide gels by electrophoresis (BioRad) and transferred to a polyvinylidene fluoride (PVDF) membrane (Roth) by semi-dry western blotting. After blocking for 1 h with a blocking solution containing 5% (w/v) milk powder in TBST (10 mM Tris buffer (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween20) at RT, the PVDF membrane was incubated overnight at 4°C with primary antibodies diluted in blocking solution against pAKT, pERK, AKT, ERK, TrkA, TrkB, pTrkB (1:1000), or α-Tubulin (1:5000). The blot was washed three times with TBST and incubated for 2 h at RT with secondary antibody anti-rabbit-HRP (1:5000) or anti-mouse-HRP (1:1000). Afterward, the membranes were washed three times with TBST and once with TBS (A. bidest, 10 mM Tris buffer (pH 7.4), 150 mM NaCl) before proteins were detected using ECL chemiluminescence substrate (BioRad) and the chemiluminescence system MicroChemi 4.0 (Biostep, Germany).

Statistical analysis

The statistical analysis of the data sets and the graphical representation were carried out using the Graph Pad Prism 6 software. The data in the graphs were displayed by the mean with standard error (± SEM). The one-way ANOVA with Tukey's Multiple Comparison Test was used to detect significant deviations. The significance levels were considered as follows: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Results

TrkB is expressed and activated by 7,8-DHF in PC12^{TrkB} cells

Western blot analyses were applied to detect TrkA and TrkB in both cell lines (Figure 1a, b). As expected, a strong expression of TrkB in PC12^{TrkB} cells (Figure 1a) and almost no expression in PC12^{wt} cells (Figure 1b) could be observed. While TrkA was expressed in PC12^{wt} cells (Figure 1b), it appeared to be absent in PC12^{TrkB} cells (Figure 1a). We aimed to verify if the planned concentrations of 7,8-DHF could activate TrkB in the generated PC12^{TrkB} cell line (Figure 1c). Therefore, a signal for phosphorylated TrkB (pTrkB) could be noted after stimulation with BDNF as well as with different concentrations of 7,8-DHF employing western blot analyses, whereas no TrkB activation could be observed in PC12^{wt} cells (Figure 1d).

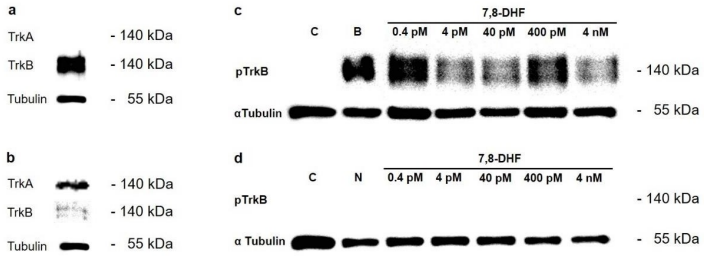

Figure 1: TrkA and TrkB receptor expression and TrkB activation

(a-b) Expression of TrkA and TrkB after 1 day without factor stimulation in PC12TrkB cells (a) and PC12wt cells (b).(c-d) To investigate the activation of TrkB, PC12^{TrkB} cells (c) and PC12^{wt} cells (d) were stimulated for 1 day with BDNF (10 ng/ml), NGF (20 ng/ml), and 7,8-DHF in various concentrations (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM). C: control, B: BDNF, N: NGF.

7,8-DHF Does Not Promote Cell Viability in a TrkB-dependent Manner

The viability of both cell lines was investigated using a trypan blue staining after 1 day of stimulation. Trypan blue-stained cells indicated dead cells, with the amount conversely representing viability. The analysis of the PC12^{TrkB} cell line (Figure 2a) revealed that treatment with 7,8-DHF resulting in nearly the same proportion of trypan blue-positive cells, depending on the concentration (0.4 pM: 26.6 ± 2.28%; 4 pM: 26.8 ± 1.8%; 40 pM: 33.0 ± 2.4%; 400 pM: 29.6 ± 1.82%; and 4 nM: 28.9 ± 3.33%), when compared to the

control (26.1 ± 2.57%) and BDNF (35.0 ± 2.73%). In comparison, the analysis of PC12wt cells appeared to show a higher proportion of trypan blue-positive cells, as displayed in figure 2b. Stimulation of $PC12^{wt}$ cells with 400 pM 7,8-DHF (46.38 ± 2.61%) led to a significantly increased proportion of dead cells.

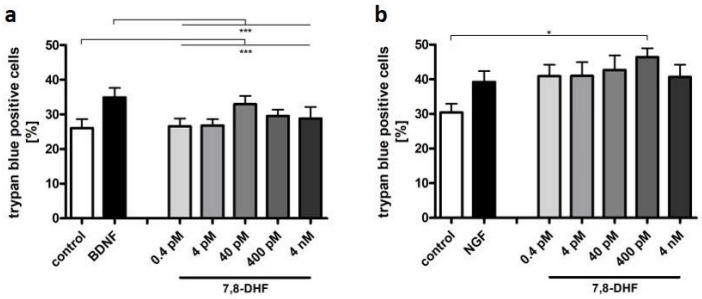

Figure 2: The viability of $PC12^{TrkB}$ and $PC12^{wt}$ cells after 1 day *in vitro*

(a) Viability of PC12TrkB cells is shown in the form of a percentage of trypan blue-stained cells after stimulation with BDNF (10 ng/ml) or various concentrations of 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 1 day, n=3. (b) Viability of PC12wt cells after stimulation with NGF (20 ng/ml) or various concentrations of 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 1 day, n=3.

The Apoptotic Activity can be Reduced by 7,8-DHF

Cells were immunocytochemically stained after stimulation for 2 days against caspase- 3 with DAB to investigate if 7,8-DHF influences apoptosis in $PC12^{TrkB}$ and $PC12^{wt}$ cells. According to figure 3a, the statistical evaluation showed that BDNF stimulation significantly reduced the number of apoptotic $PC12^{TrkB}$ cells by more than half to 7.99 ± 1.19% compared to the control (22.53 ± 2.07%). In addition, 7,8-DHF significantly reduced the apoptosis rate in a dose-dependent manner (4 pM: 5.37 ± 0.56%; 40 pM: 5.87 ± 0.45%; and 400 pM: 6.17 ± 0.55%) than BDNF. As depicted in figure 3b, the statistical analysis of $PC12^{wt}$ cells showed no significant differences in the proportion of apoptotic cells. To analyze whether the antiapoptotic effect of 7,8-DHF is mediated by TrkB and therefore to inhibit TrkB, PC12TrkB cells were treated additionally with the TrkB antagonist K252a (Figure 3e). We could observe that a previous inhibition of TrkB by K252a could significantly enhance the number of apoptotic cells (0.4 pM + K252a: 36.81 ± 1.83%; 4 pM + K252a: 44.14 ± 2.47%; 40 pM + K252a: 39.43 ± 2.12%; 400 pM + K252a: 45.82 ± 2.18%; and 4 nM + K252a: 48.36 ± 1.58%).

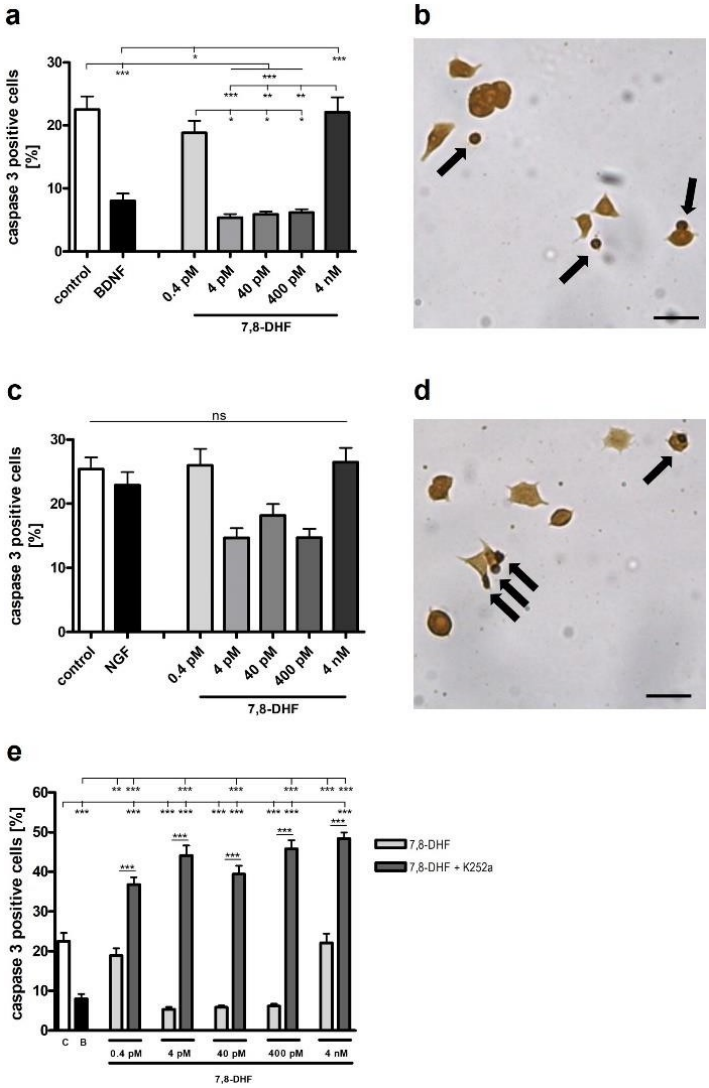

Figure 3: Analysis of apoptotic activity after 2 days

(a) Percentage of caspase-3 positive PC12TrkB cells after stimulation with BDNF (1 ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 2 days.

(b) Caspase-3 positive PC12TrkB cells (marked by black arrows) as shown by immunostaining. Scale bar, 50 µm.

(C) Percentage of caspase-3 positive PC12wt cells after stimulation with NGF (20 ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 2 days.

(d) Caspase-3 positive PC12wt cells (marked by black arrows) as shown by immunostaining. Scale bar, 50 µm.

(e) Percentage of caspase-3 positive PC12TrkB cells after stimulation with BDNF (1 ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) and additionally K252a (1 ng/ml) for 2 days. n = 3-7.

7,8-DHF reduces the Number of DNA-replicating PC12^{TrkB} and PC12^{wt} cells

The proliferation behavior influenced by 7,8-DHF was determined through labeling the cells with the marker BrdU (Figure 4), which identifies DNA-replicating cells in the S-phase of the cell cycle [48]. The analysis of PC12^{TrkB} cells (Figure 4a, b) revealed that 22.21 ± 0.97% of non-stimulated cells were BrdU-positive, and the addition of 7,8-DHF resulted in a significant reduction at a concentration of 0.4 pM (14.25 ± 0.79%) and 400 pM (13.61 ± 0.69%). Similar outcomes could be determined using PC12^{wt} cells (Figure 4c, d). 7,8-DHF significantly reduced the number of BrdU-positive cells in a concentration-dependent manner (0.4 pM: 8.93 ± 0.5% and 400 pM: 10.25 ± 0.46%).

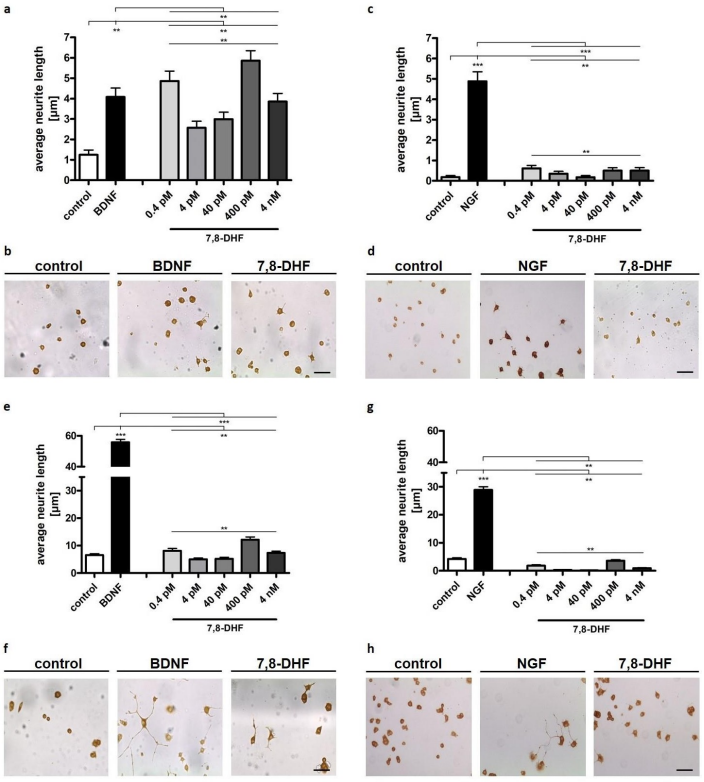

Figure 5: Neurite growth of PC12^{TrkB} and PC12^{wt} cells after 1 and 5 days

(a,c) Average neurite length of PC12TrkB cells (a) and PC12wt cells (c) after stimulation with BDNF (1 ng/ml), NGF (20ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 1 day.

(b,d) DAB stained PC12TrkB cells (b) and PC12wt cells (d) against βIII-tubulin as shown by immunostaining after 1 day of stimulation. Scale bar, 50 µm.

(e,g) Average neurite length of PC12TrkB cells (e) and PC12wt cells (g) after stimulation with BDNF (1 ng/ml), NGF (20ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 5 days.

(f,h) DAB stained PC12TrkB cells (f) and PC12wt cells (h) against βIII-tubulin as shown by immunostaining after 5 days of stimulation. Scale bar, 50 µm. n = 3.

7,8-DHF Enhances Neurite Growth After 1 div More Than BDNF

To investigate the impact of 7,8-DHF stimulation on neurite growth, βIII-tubulin stainings were performed after 1 and 5 days in vitro, and the longest neurites were measured. When neurite growth of PC12TrkB cells was examined after 1 day (Figure 5a, b), a significant increase in growth could be observed not only after the BDNF addition (4.08 ± 0.44 µm) but also after stimulation with 7,8-DHF, which had a stronger effect than BDNF stimulation in a dose-dependent manner. Cells with 0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM 7,8-DHF showed average lengths of 4.86 ± 0.49 µm, 2.57 ± 0.32 µm, 2.3 ± 0.34 µm, 5.86 ± 0.49 µm, and 3.85 ± 0.4 µm, respectively. The analysis of neurite growth of PC12wt cells after 1 day (Figure 5.c, d) displayed basal neurite growth (0.19 ± 0.08 µm), which was significantly increased to 4.88 ± 0.47 µm after addition of NGF, while stimulation with 7,8-DHF enhanced the average neurite length to 0.61 ± 0.15 µm (0.4 pM), 0.35 ± 0.12 µm (4 pM), 0.18 ± 0.07 µm (40 pM), 0.51 ± 0.14 µm (400 pM), and 0.51 ± 0.14 µm (4 nM).

After differentiation for 5 days, the statistical analysis of PC12TrkB cells (Figure 5e) revealed a significant increase in neurite growth due to BDNF (55.79 ± 1.84 µm) compared to the control (6.56 ± 0.49 µm). Stimulation with 7,8-DHF could significantly increase the average neurite length at a concentration of 0.4 pM (8.15 ± 0.86 µm), 400 pM (12.15 ± 0.99 µm), and 4nM (7.37 ± 0.62 µm). When examining the neurite length of PC12wt cells (Figure 5g, h), a generally shorter average neurite length was observed after NGF treatment (28.88 ± 1.13 µm).

Stimulation with 7,8-DHF significantly reduced neurite growth compared to control (0.4 pM: 1.87 ± 0.29 µm; 4 pM: 0.33 ± 0.12 µm; 40 pM: 0.19 ± 0.08 µm; 400 pM: 3.57 ± 0.43 µm; 4 nM: 0.96 ± 0.20 µm, and control: 4.23 ± 0.41 µm).

To investigate, if the neurite growth promoting effect of 7,8-DHF was mediated by TrkB, PC12TrkB cells were additionally treated with the TrkB antagonist K252a. Interestingly, the effect of 7,8-DHF after 1 day was affected differently after TrkB inhibition by K252a in PC12TrkB cells (Figure 6a). While 0.4 pM + K252a (3.98 ± 0.43 µm) and 4 nM + K252a (3.66 ± 0.40 µm) significantly reduced average neurite length and 400 pM + K252a had no effect on neurite growth (5.83 ± 0.51 µm), stimulation with 4 pM + K252a (4.67 ± 0.49 µm) and 40 pM + K252a (4.43 ± 0.46 µm) showed a significant increase in neurite length. In contrast, K252a significantly reduced the effect of 7,8-DHF after 5 days (Figure 6b; 0.4 pM: 1.77 ± 0.29 µm; 4 pM: 1.83 ± 0.35 µm; 40 pM: 3.39 ± 0.48 µm; 400 pM: 3.61 ± 0.49 µm; and 4 nM: 5.55 ± 0.62 µm).

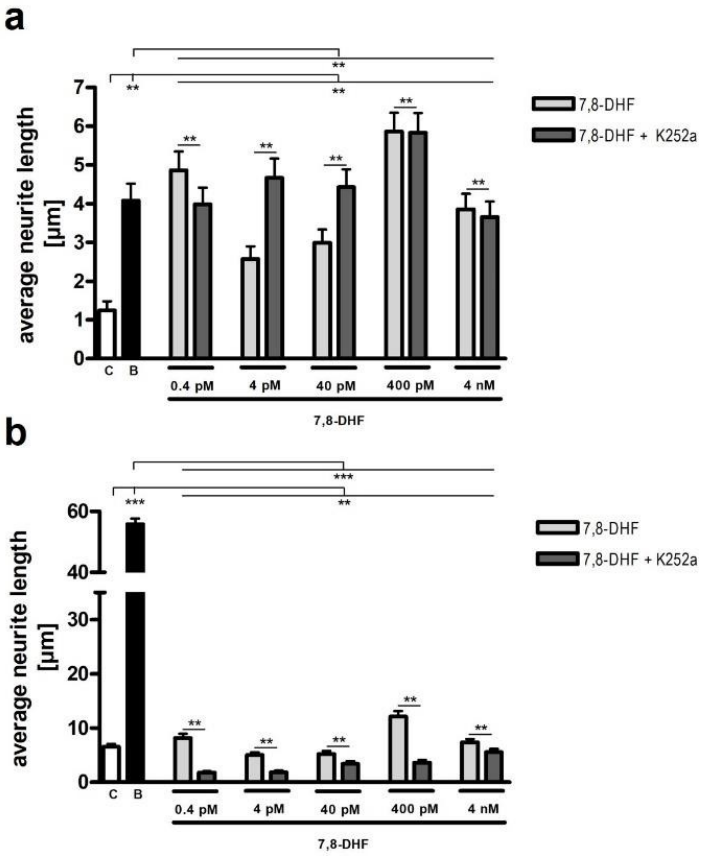

Figure 6: Neurite growth in PC12^{TrkB} cells after 1 and 5 days of stimulation after TrkB inhibition

(a) Average neurite length of PC12TrkB cells after stimulation with BDNF (1 ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM), and K252a (1 ng/ml) for 1 day.

(b) Average neurite length of PC12TrkB cells after stimulation with BDNF (1 ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM), and K252a (1 ng/ml) for 5 days. n = 3.

Activation of PI3K/AKT and Ras/Raf/MAPK Pathways in PC12^{TrkB} and PC12^{wt} Cells by 7,8- DHF

The activation of signaling pathways was then characterized following 7,8-DHF stimulation. Western blot analyses were performed 3 min after stimulation with 7,8-DHF, BDNF, and NGF, and 90 min after stimulation with NGF. PC12^{TrkB} cells (Figure 7a, b) showed a significant activation after BDNF application, and 7,8-DHF stimulation resulted in AKT activation. After 90 min of NGF stimulation, AKT activation could be observed in PC12^{wt} cells (Figure 7c, d, e, f). In comparison, the 7,8-DHF treatment caused a slight AKT activation after 3 min of stimulation, but with substantial concentration-related variations.

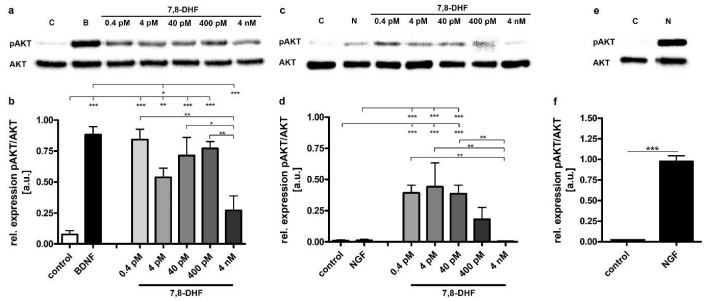

Figure 7: Activation of PI3K/AKT signaling pathway

(a,c) Westernblot analysis of pAKT and AKT in PC12TrkB cells (a) and PC12wt cells (c) after stimulation with BDNF (10 ng/ml), NGF (20ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 3 min.

(b,d) Relative expression of pAKT/AKT in PC12TrkB cells (b) and PC12wt cells (d) after stimulation with BDNF (10 ng/ml), NGF (20ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 3 min.

(e) Westernblot analysis of pAKT and AKT in PC12wt cells after stimulation with NGF (20ng/ml) for 90 min.

(f) Relative expression of pAKT/AKT in PC12wt cells after stimulation with NGF (20ng/ml) for 90 min. n = 3-8.

In terms of the Ras/Raf/MAPK pathway, ERK activation could be detected in PC12TrkB cells (Figure 8a, b) after BDNF stimulation. Treatment with 7,8-DHF resulted in tendential activation, with only 4 pM 7,8-DHF having significant activation strength comparable to BDNF. In PC12wt cells (Figure 8c-f), there was a significant NGF-mediated activation of ERK after 90 min of stimulation, as well as a dose-dependent activation after 3 min of 7,8-DHF stimulation.

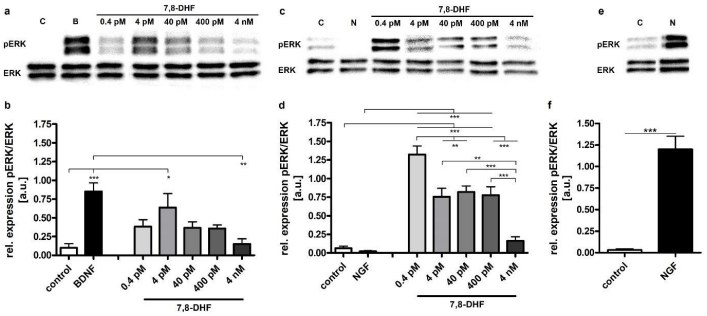

Figure 8: Activation of Ras/Raf/MAPK signaling pathway

(a,c) Westernblot analysis of pERK and ERK in PC12TrkB cells (a) and PC12wt cells (c) after stimulation with BDNF (10 ng/ml), NGF (20ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 3 min.

(b,d) Relative expression of pERK/ERK in PC12TrkB cells (b) and PC12wt cells (d) after stimulation with BDNF (10 ng/ml), NGF (20ng/ml), or 7,8-DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM) for 3 min.

(e) Westernblot analysis of pERK and ERK in PC12wt cells after stimulation with NGF (20ng/ml) for 90 min.

(f) Relative expression of pERK/ERK in PC12wt cells after stimulation with NGF (20ng/ml) for 90 min. n = 3-7.

Furthermore, we investigated how TrkB inhibition affects signaling pathway activation by 7,8-DHF in PC12TrkB cells (Figure 9). Our data revealed that cells treated with the TrkB inhibitor K252a previously suppressed pathway activation significantly.

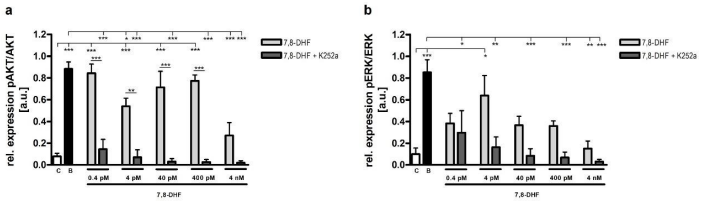

Figure 9: Activation of the PI3K/AKT and Ras/Raf/MAPK signaling pathways in PC12TrkB cells after 3 min stimulation and TrkB inhibition

(a) Relative expression of pAKT/AKT in PC12TrkB cells after stimulation with BDNF (10 ng/ml), or 7,8- DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM), and K252a (1ng/ml) for 3 min.

(b) Relative expression of pERK/ERK in PC12TrkB cells after stimulation with BDNF (10 ng/ml), or 7,8- DHF (0.4 pM, 4 pM, 40 pM, 400 pM, and 4 nM), and K252a (1ng/ml) for 3 min. n = 3-8.

Discussion

This study aimed to characterize the new PC12^{TrkB} cell line to use it in future analyses on TrkB activation and induced cellular responses. According to the literature and our findings, native PC12^{wt} cells did not express TrkB [47]. Trk detection issues mentioned in some studies [49] could explain the weak Western blot signal in the obtained results and possibly assume the detection of a TrkB isoform, as also declared in a previous study [50]. As expected, and in line with previous studies [5, 33, 41], the generated TrkB overexpressing PC12TrkB cell line displayed a high level of TrkB expression, which could be successfully activated by the TrkB agonist 7,8-DHF.

Then, the effect of the TrkB activation by BDNF was compared to that via 7,8-DHF. Based on the findings, 7,8-DHF did not affect viability and could reduce apoptotic activity even stronger than BDNF in a dose-dependent manner. There was no significant influence, but an apparent impairment could be observed in the viability of PC12^{TrkB} cells after the common activation of TrkB by BDNF. Since BDNF promotes survival in different types of neurons through a TrkB-mediated manner [12, 13], an improvement in viability would be expected. Because such an improvement in TrkB-expressing neurons was observed after seven days [51], it is possible that the promoting influence would occur with delay. In a TrkB-expressing PC12 cell line generated in 1996 that was transfected with murine cDNA of TrkB, treatment with BDNF improved viability after two days and seemed to worsen it beforehand [52]. In addition, a concentration dependence could be responsible for the lack of promotion, as other studies declared different results in a dose-dependent manner [53, 54]. It should also be noted that the PC12TrkB cells were in a stressful situation due to the low serum content, so the outcome may differ [55]. Cultivation in low serum media and the absence of growth factors has also been illustrated to reduce PC12wt cell survival [52, 56]. Studies showed that BDNF treatment of stressed TrkB-expressing neuroblastoma cells resulted in viability decrement [57]. As a result, it could be assumed that factors such as stress, concentration, and a possible delay in action influenced the effect of BDNF on the viability of PC12^{TrkB} cells. TrkB activation by 7,8-DHF displayed little impact on PC12^{TrkB} cell viability. The absence of the expected promoting effect could be explained by a similar delayed action, as relevant studies revealed unchanged viability after 24 h [41, 58]. The influence of 7,8-DHF on viability after several days has not yet been investigated therefore, no comparison could be made. Moreover, together with other studies, no clear concentration-dependent effect was determined. Concentrations of 20 µM led to a reduction in viability [59], whereas concentrations up to 200 µM resulted in an improvement, though this is often in conjunction with induced stress [41, 60]. In contrast, the viability of PC12^{wt} cells appeared to be slightly reduced by 7,8-DHF and was significantly declined by almost 16%, especially through applying 400 pM 7,8-DHF. The obtained results are consistent with the fact that PC12wt cells do not express TrkB [47] and 7,8-DHF [33], thus cannot cause a survival-promoting effect.

The apoptotic activity of PC12TrkB cells was not only significantly reduced by 7,8-DHF, but it also showed a stronger effect than BDNF in a dose-dependent manner. This result appears conclusive because 7,8-DHF has been widely reported as a TrkB agonist with survival-promoting properties [33, 34, 61]. The stronger effect could be attributed to the lack of ubiquitination and TrkB

degradation following internalization by 7,8-DHF [62, 38]. The anti- apoptotic impact was mediated by the PI3K/AKT signaling pathway, as it was activated after 7,8-DHF stimulation, which is consistent with the literature [41, 63]. Moreover, the obtained data showed that the survival-promoting effect of 7,8-DHF was TrkB-mediated after TrkB inhibition with K252a. K252a has been reported as a well-established Trk inhibitor [64], which was already used in studies regarding 7,8-DHF [33]. Blocking the ATP binding site disrupts intracellular phosphorylation of TrkB and thus prevents signal transduction [65]. The use of K252a enabled the investigation, if the obtained effects of 7,8-DHF were mediated by TrkB activation. Additionally, a concentration-dependent effect could be predicted since 0.4 pM and 4 nM 7,8-DHF did not appear to reduce the apoptosis rate and had a significantly weaker effect than with BDNF. While initial studies with 7,8-DHF described a stronger effect with increasing concentration [33, 34], subsequent research supported the theory of different concentration- dependent effects for 7,8-DHF [5, 59], BDNF [53] and flavonoids [66]. Too low concentrations may result in insufficient activation of the survival-promoting PI3K/AKT signaling pathway, whereas too high concentrations could cause a protective down-regulation of TrkB [28]. In fact, 4 nM 7,8-DHF led to a weaker activation of AKT, suggesting that the receptor's possible downregulation requires to be investigated further. The Western blot results could exclude a too weak activation by 0.4 pM 7,8-DHF, allowing for modified regulation of AKT downstream effector proteins [67] or the expression of pro- and anti-apoptotic proteins [68]. Although no significant differences were observed in PC12wt cells, the unsignificant reduction of apoptotic activity stayed remarkable. In studies with oxidatively stressed $PC12^{wt}$ cells, 7,8-DHF was found to have an anti-apoptotic effect, which was probably mediated via PI3K/AKT [58, 69]. Similar coherences have been figured out for NT-3 [70], indicating that in $PC12^{wt}$ cells, TrkA- unspecific substances such as 7,8-DHF could activate PI3K/AKT signaling.

The activation of TrkB by 7,8-DHF led to the reduction of DNA-replicating cells in a dose-dependent manner. Similar results were reported by Park et al. [71]. It is worth noting that at the lowest concentration of 7,8-DHF (0.4 pM), impaired proliferation and an increased rate of apoptosis were observed. Such a dual effect could be beneficial in developing cancer treatments [72, 73]. In comparison, the opposite effect appeared to occur at the highest concentration (4 nM). As it is apparent, with a higher overall number of apoptotic cells, the surviving cells were not influenced in their proliferation. Therefore, a connection between apoptotic and proliferating activity could not be excluded and has already been observed in other coherences [74, 75]. So far, there have been few studies to investigate the influence of 7,8-DHF on proliferation. Analyzes on murine brain slices showed that a two-week treatment with 7,8-DHF had no effect on the number of DNA-replicating cells [76, 77], while a slight increase was observed after three weeks [34]. This seems to evidence a time-dependent effect of 7,8-DHF, which could be decisive for therapeutic applications in cancer diseases. Although 7,8-DHF stimulation causes significant changes in the proliferation rate of $PC12^{wt}$ cells, a TrkB- mediated influence on $PC12^{TrkB}$ cells could be concluded because TrkB inhibition by K252a significantly reversed the effect. Meanwhile, if the suggesting trend of a decrease is taken into account, a TrkB-independent mechanism could be assumed in the same way as described for the apoptotic activity above.

BDNF and TrkB activation have represented a positive effect on differentiation in many cell types [6, 12, 13, 22, 78]. In the present research, the neurite growth of the $PC12^{TrkB}$ cell line was significantly increased after stimulation with BDNF, which is in line with previous studies [79]. The enormous increase after five days was remarkable, and it could be explained by TrkB receptor overexpression. The enhanced differentiation of $PC12^{TrkB}$ cells could probably be mediated by Ras/Raf/MAPK, which has already been demonstrated to promote differentiation [22]. The alternative activation of TrkB via 7,8-DHF significantly increased neurite growth of $PC12^{TrkB}$ cells after 1 and 5 days. The differentiation-promoting characteristic of 7,8-DHF was also observed in other cell types [5, 40, 41, 43, 80] and confirms the results obtained. It is also clear that, depending on the cell type, 7,8-DHF could enhance differentiation to different degrees in a dose-dependent manner and that excessively high concentrations might cause negative feedback mechanisms, resulting in a reduced differentiation [5]. A similar effect was observed with 4 nM of 7,8-DHF, which had a lower promoting effect than 400 pM 7,8-DHF. A clear dose-effect curve, however, could not be identified. Notably, 0.4 and 400 pM 7,8-DHF had the strongest differentiation-promoting and proliferation-inhibiting effects. A connection between differentiation and proliferation has been established in the NGF-induced differentiation of PC12wt cells, whose addition terminates mitotic division and initiates the formation of neurites [45]. While proliferation was not completely stopped by 7,8-DHF after 1 day, the strength of the

effect on proliferation seemed to play a crucial role in the development of differentiation. Another intriguing finding is that 7,8-DHF could cause stronger neurite growth than BDNF after 1 day, but this superiority disappeared at 5 days. The delayed effect of BDNF could be explained by postponed (NGF-induced) neurite outgrowth in $PC12^{wt}$ cells [81, 82], which began significantly from day three or four and plateaued after seven days [82]. Since different ligands of a receptor can produce various cellular responses [16, 83, 84], such differences between two TrkB ligands would be conceivable. Accordingly, when compared to BDNF, 7,8-DHF initiated neurite growth in PC12TrkB cells equally early, but not with a comparable intensity in the longer term. The differentiation-promoting impact of 7,8-DHF on $PC12^{TrkB}$ cells appeared to be mediated by PI3K/AKT, as 7,8-DHF mainly activated AKT. This assumption is supported by similar findings, which show AKT-mediated differentiation of primary neurons [36] as well as motor neurons [5]. Further studies revealed that AKT activation is involved in neurite growth in specific contexts such as axon survival, elongation, and branching [85, 86].

$PC12^{wt}$ cells were also stimulated with 7,8-DHF to assess whether the effect was TrkB- mediated in PC12TrkB cells. Comparing these two cell lines represented no promotion of neurite growth in PC12wt cells, implying a TrkB-mediated effect of 7,8-DHF in PC12TrkB cells. Additionally, inhibition of TrkB by K252a showed a suppressed impact of 7,8-DHF on neurite growth of $PC12^{TrkB}$ cells on 5 div. Interestingly, various effects could be observed after TrkB- inhibition on 1 day in vitro, for reasons that are still unclear. An insufficient inhibition of TrkB could be thereby excluded as no activation of the PI3K/AKT and Ras/Raf/MAPK pathways was observed after K252a treatment. Surprisingly, other researchers reported differentiation- promotion of K252a in combination with EGF in $PC12^{wt}$ cells [87], despite the fact that EGF is known to promote proliferation rather than differentiation in $PC12^{wt}$ cells [88]. Notably, compared to non-stimulated cells, 7,8-DHF had a minimal effect after 1 day in PC12wt cell analysis. These results could be attributed to TrkB-independent signaling pathway activation since a slight activation of AKT along with stronger ERK activation were detected in $PC12^{wt}$ cells. Considering that activation of PI3K/AKT by 7,8-DHF resulted in cellular response of $PC12^{wt}$ cells in another study [58], a similar mechanism with a minor effect on differentiation cannot be excluded. PI3K/AKT and Ras/Raf/MAPK are known to be interconnected, and individual effector proteins can exhibit a variety of reactions [22]. Accordingly, results such as those of $PC12^{wt}$ cells imply an essential evaluation of such relationships.

The stimulation of PC12TrkB cells with 7,8-DHF resulted in a significant activation of AKT, while only 4 pM 7,8-DHF could significantly activate ERK. The obtained results are consistent with previous research that used 7,8-DHF to activate AKT or ERK in TrkB- expressing cells such as hippocampal neurons [5, 33] cortical neurons [34, 36, 62], motor neurons [5], and retinal ganglion cells [41]. Since TrkB inhibition by K252a significantly suppressed AKT and ERK activation, a TrkB-mediated activation could be assumed. A partially weaker activation compared to BDNF could be linked with differences in receptor kinetics [62]. Due to differences in activation patterns and cultivation conditions, it is clear that activation of both signaling pathways does not only depend on concentration but also on the activation timeframe, which varies based on cell type [5, 33, 34, 41]. Although Liu et al. [62] declared an enhancing activation response with an increasing concentration of 7,8-DHF, no clear dose- effect curve could be observed within $PC12^{TrkB}$ cells. Meanwhile, it should be noted that in the above-mentioned study, higher concentrations of 7,8-DHF were used and that receptor activation might result in different cellular responses depending on cell type [89]. Interestingly, stimulation of $PC12^{wt}$ cells with 7,8-DHF could also lead to a brief activation of ERK and AKT. Since these cells lack TrkB [47], a TrkB-independent mechanism must be responsible for the cellular response, which was postulated in a previous study [69].

Conclusion

Overall, this research could demonstrate that neurotrophin-mediated activation of TrkB via BDNF, as well as alternative activation by the TrkB agonist 7,8-DHF, result in a cellular response in terms of proliferation, apoptotic activity, differentiation, and signaling activation in the new generated TrkB-overexpressing $PC12^{TrkB}$ cells. $PC12^{TrkB}$ cells have a similar morphology and basic behavior in a non-stimulated condition, and their cellular behavior can be investigated using various methods. In comparison to native $PC12^{wt}$ cells, $PC12^{TrkB}$ cells appear to have a fundamentally higher proliferation rate, which could be attributed to TrkB receptor overexpression and could thus be a beneficial tool for experimental performance. Furthermore, according to the findings, 7,8-

DHF seems to induce a cellular response in native PC12^{wt} cells, which is assumed to be TrkB-independent since these cells lack TrkB. The unknown activation mechanism of 7,8-DHF must be investigated in more detail, so that this ligand can be determined further for future therapeutic approaches.

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