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M.Sc. Thesis

Evaluation of novel microneurotrophin compounds on activating neurotrophin receptors.

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Abstract

Neuronal cell survival and differentiation is determined by multiple events, one of which is the binding of neurotrophic factors to their respective high affinity receptors, leading to a cascade of molecular interactions. Neurotrophins and their receptors have been reported as deregulated in many neurodegenerative diseases, however due to their polypeptidic nature and their restricted penetration to the blood-brain barrier (BBB), their therapeutic application is compromised. Studies in our laboratory have proven that the neurosteroid dehydroepiandrosterone (DHEA) interacts with the nerve growth factor (NGF) receptors, namely TrkA and $p75^{NTR}$, efficiently activating downstream signaling pathways and preventing neuronal apoptosis. Nevertheless, DHEA as a steroid hormone is metabolized *in vivo* to sex hormones; thus its pharmacological potential for neurodegenerative conditions is limited. Overcoming these limitations, small DHEA-like molecules deprived of androgenic-estrogenic actions and effectively mimicking the effects of neurotrophins have been recently synthesized and characterized. We have shown that BNN27, a synthetic DHEA derivative, interacts with TrkA and $p75^{NTR}$ receptors and activates downstream signaling pathways aiming to prevent neuronal apoptosis and promote neuroprotection *in vitro* and *in vivo*. The present study focuses on the characterization of newly synthesized, neurotrophin-like small molecules named as *microneurotrophins*. By utilizing receptor's phosphorylation assays, we assessed the efficacy of each analog to activate TrkA receptor and by employing cell survival assays we investigated their anti-apoptotic properties. Our results indicate that compounds BNN397 and BNN403 efficiently activate the receptor in a 20-minute trial; BNN218, BNN219, BNN396 and BNN398 activate the receptor after 30 minutes while BNN218 and BNN398 have a greater impact on rescuing cells from apoptosis. The microneurotrophin analogs which stand out from both experiments are BNN218 and BNN398 and we propose that future work shall validate our results and further investigate their functional properties *in vitro* and *in vivo*. We hope that these new microneurotrophin compounds will exhibit good pharmacological properties and will qualify as therapeutic agents towards the treatment of neurodegenerative diseases.

Abbreviations

CHO: Chinese Hamster Ovary; SF: serum free; N,N,N',N'-tetramethylethylene-diamine :TEMED; Ammonium Persulfate: APS; Sodium dodecyl sulfate polyacrylamide gel electrophoresis: SDS-PAGE; TBS: Tris-buffered saline; BSA: Bovine Serum Albumine; RT: Room Temperature; NGF: Nerve Growth Factor; BDNF: Brain Derived Neurotrophic Factor; AD: Alzheimer's disease; PD: Parkinson's disease; ALS: Amyotrophic Lateral Sclerosis; BBB: Blood-Brain Barrier; PLC-γ: Phosphoinositide phospholipase C-γ ; PKC: protein kinase C; SH_2: Src Homology 2; JNK: Jun N-terminal kinase; p/s: penicillin/streptavidin; IB: immunoblotting; IP: immunoprecipitation

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Introduction

1. Neurotrophins

The neurotrophic factors (neurotrophins) are a family of secreted target-derived proteins that are essential for the development of the vertebrate central and peripheral nervous system, promoting cell survival and differentiation [1], [2]. Discovered in the early 1950s, Nerve Growth Factor (NGF) was the first neurotrophic factor identified [3], [4]. Later, isolated and characterized by Barde et al., Brain-Derived Neurotrophic Factor (BDNF) [5] paved the way for the 'neurotrophin family' concept. Additional members of this family became neurotrophin-3 (NT-3) [6]–[9], neurotrophin-4/5 (NT-4/5) [9], neurotrophin-6 (NT-6) [11] and neurotrophin-7(NT-7) [12].

Mature neurotrophins exist as stably, non-covalently associated dimers of ~13,500 Da monomers [13], [14] with high resolution structures been determined for each of them [15]–[17]. Premature forms of neurotrophins (proneurotrophins) can be produced in either neuronal or non-neuronal cells which are packaged in secretory vesicles, processed into mature neurotrophins and secreted [2]. In some cases, proneurotrophins can be as well secreted leading to different activation of signaling pathways [18].

Neurotrophins are produced in a well defined spatiotemporal manner, with different patterns of expression for each of them [19] and levels varying throughout development, in order to control the number of surviving neurons at each time point. Neurotrophin availability is required during adulthood, where they control synaptic function and plasticity and sustain neuronal cell survival, morphology and differentiation [20]. Mice that completely lack the expression of any of the neurotrophins, die during the first few emryonic weeks or following birth, and mice with reduced neurotrophin levels are viable but show other deficits (in memory, behavior etc.) [1].

In addition to their classic effects on neuronal cell survival, neurotrophins can also regulate axonal and dendritic growth and guidance, synaptic structure and connections, neurotransmitter release, long-term potentiation (LTP) and synaptic plasticity [21]. Also, there is considerable evidence that NGF and BDNF contribute to pain homeostasis, with NGF being up-regulated in a wide variety of inflammatory conditions and BDNF acting as central modulator of pain [22].

2. Neurotrophin receptors and downstream signaling pathways

Neurotrophins interact with two major receptor classes: Trks (Tropomyosin related kinases) and the pan-neurotrophin receptor $p75^{NTR}$, which is a member of tumor necrosis factor receptor (TNFR) family. Each neurotrophin shows preferential binding specificity for Trk receptors: NGF preferentially binds to TrkA, BDNF and NT-4 to TrkB and NT-3 to TrkC [23]–[26] (**Figure 1.1**). These interactions are considered of high-affinity, which is promoted by receptor dimerization, structural modifications or association with the p75 receptor [27]. Also, it has been reported that NGF, BDNF and NT-3 could also bind to the receptor α9β1 integrin leading to activation of pro-survival signaling pathways like the mitogen-activated protein kinase (MAPK) pathway [28].

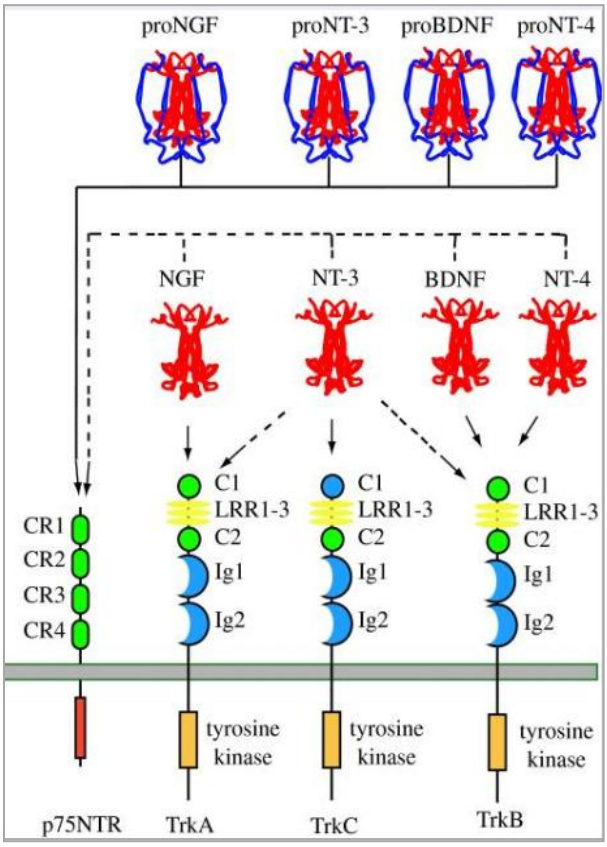


Figure 1.1: Structural characteristics of neurotrophin receptors. CR: cystein rich domains; LRR: leucine-rich repeats; Ig: Ig-like domains; C1 or C2: cysteine rich cluster. Neurotrophins bind preferentially in specific receptors as indicated, the dotted line represents binding of lower affinity and the normal line represents binding of higher affinity (*Reichardt L. F., 2006*).

The Trk receptors consist of an extracellular domain with a cysteine-rich cluster (CR) followed by three leucine-rich repeats (LRR), another cysteine-rich cluster (CR) and two Ig-like domains [29] (**Figure 1.1**) . All receptors have a single transmembrane region terminating in a cytoplasmic tyrosine kinase domain containing several tyrosine residues which serve as phosphorylation sites for cytoplasmic adaptors and enzymes. Ligand-induced receptor oligomerization and activation via trans-phosphorylation by the cytoplasmic kinases is an established mechanism for receptor-tyrosine kinase activation. Neurotrophin signals can be propagated retrogradely along the axon to the cell body and are now known to be required for proper neuronal survival, axonal growth, gene expression, neuronal subtype specification and synapse formation [30].

Following phosphorylation at specific tyrosine residues, adaptor proteins containing phosphotyrosine-binding or Src homology 2 (Sh2) domains, are recruited in specific membrane compartments and mediate the signal transduction inside the cell [31]–[33]. Briefly,

phosphorylation of Trks at the most C-terminal tyrosine *Y785*, leads to recruitment and activation of Phosphoinositide phospholipase C-γ (PLC-γ), which initiates an intracellular signalling cascade, resulting in the release of inositol phosphates and activation of protein kinase C (PKC). Phosphorylation of Trk at the tyrosine residue closest to the trans-membrane domain, *Y490* in TrkA or *Y515* in TrkB, creates a Sh2 binding site where Grb2 and Gab1 are recruited, so PI3K-Akt is activated leading to axonal growth and neuronal survival, by inhibiting the expression of pro-apoptotic genes. Additional event following *Y490* phosphorylation is the activation of Ras and MEK/Erk cascade leading to local axonal growth and initiation of CREB-mediated transcriptional events (**Figure 1.2**).

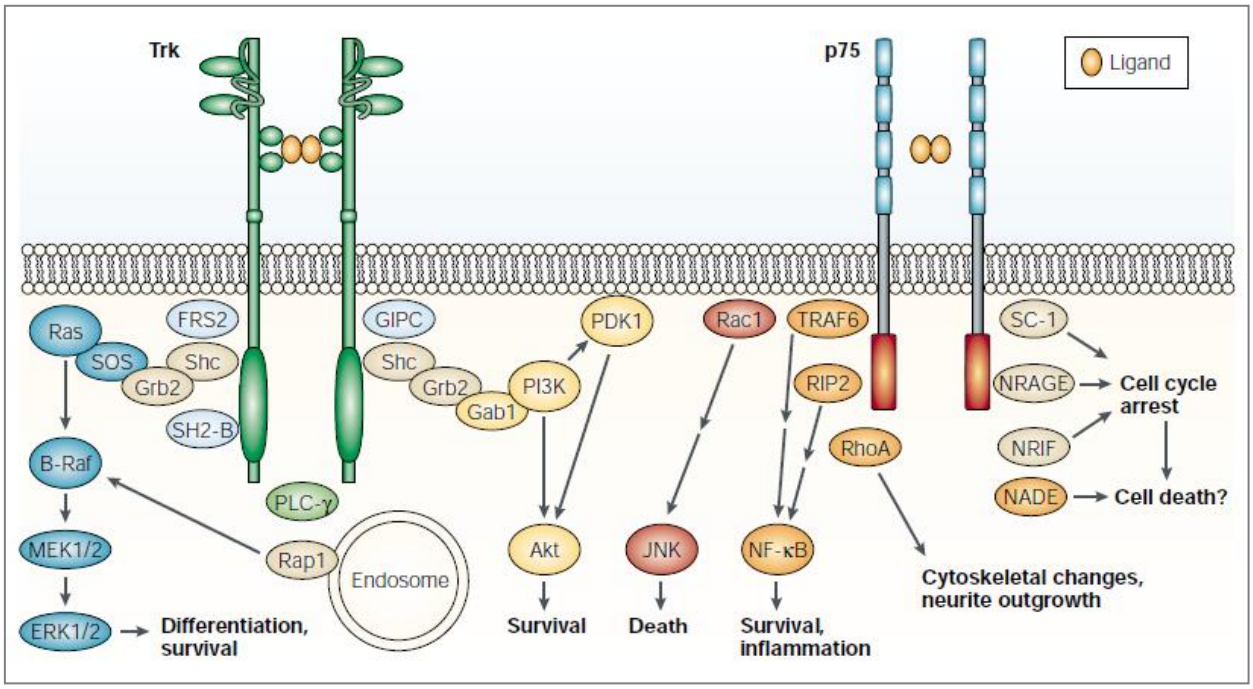


Figure 1.2: Neurotrophin activated signaling pathways. Neurotrophin binding to Trk receptors results in cell differentiation and survival through activation of multiple signaling cascades. p75NTR mediated neurotrophin signaling through a different set of adaptor proteins, leads to multiple events like cell cycle arrest, death or survival, cytoskeletal changes, neurite outgrowth etc. (*Chao M.V., 2003*)

Through a different set of adaptor proteins, $p75^{NTR}$ promotes activation of Jun N-terminal kinase (JNK) , NF-κB and other signaling pathways [34], [35] (**Figure 1.2**). Pro-neurotrophins are more selective ligands for the p75 receptor than mature forms, being more effective at inducing p75-dependent apoptosis, after its dimerization with the Sortilin receptor. [36]. The $p75^{NTR}$ and Trk receptors can function independently, but in neurons that express both, the receptors interact physically and functionally, in ways that may enhance or alter the signaling properties of each (**Figure 1.3**, Left). It has been reported that the aminoacid residue Cys^{257} in

the trans-membrane domain of $p75^{NTR}$ is essential for transmitting conformational changes from the extracellular to the intracellular domains upon ligand-binding, inducing the separation of the dimerized death domains [37] (**Figure 1.3**, Right). Ligand-binding to $p75^{NTR}$ leads to activation of downstream signaling events such as sphingomyelinase activation [38] and recruitment of TRAF-6, a mediator of tumor necrosis factor receptor activation [39], NRIF [40] and release of RhoGDI [41] (**Figure 1.2**).

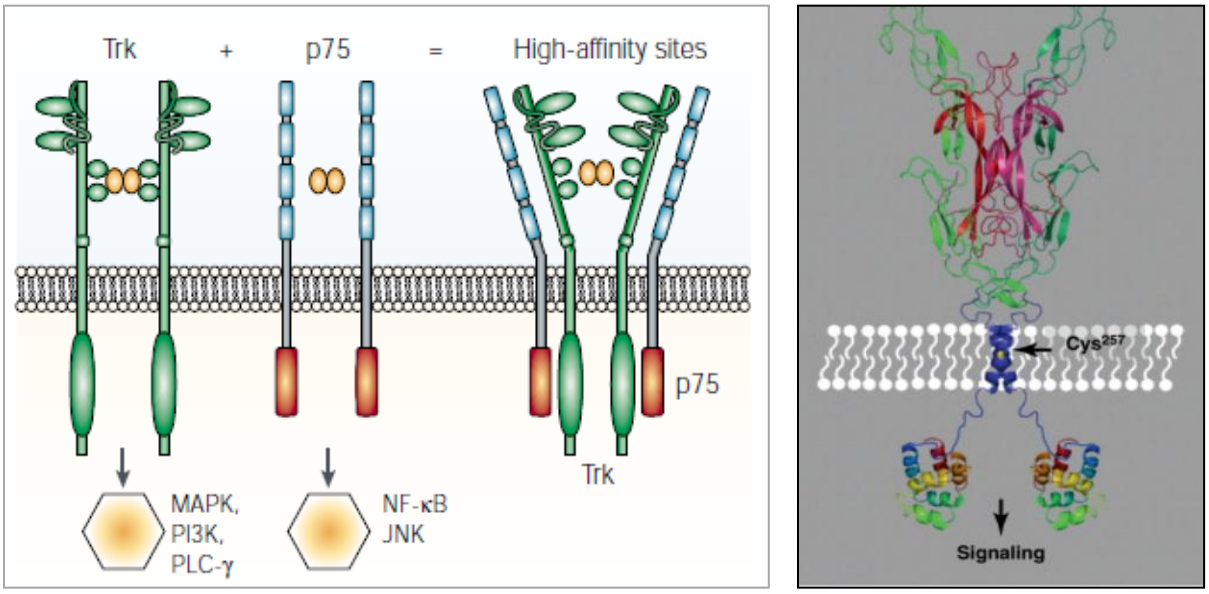


Figure 1.3: p75NTR activation. Left: p75NTR cooperates with receptor tyrosine kinases from the Trk family (TrkA, B and C) for high-affinity binding of neurotrophins (*Chao M.V., 2003*). Right: p75NTR activation in response to neurotrophin-binding (*Vilar M., 2009*).

Activation of TrkA and TrkB receptors can also occur via a GPCR mechanism, in the absence of NGF or BDNF, suggesting that receptor signaling may occur and persist inside neuronal cells on intracellular membranes [42]. It is of note that TrkA and TrkC instruct neurons to die in the absence of their ligands (NGF and NT-3 respectively), whereas TrkB does not induce cell death in the absence of its ligand BDNF[43].

Given the fact that different biological outcomes may occur at neurotrophin signaling, what eventually determines the cell fate is the type of the cell (neural, glia or non-neural) and the receptor or the ratio of receptors it expresses.

3. Neurotrophins and neurodegenerative diseases

Neurotrophins and their receptors have been reported as deregulated in many neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS). There has been a number of studies implicating NGF and

BDNF in the development of Alzheimer's disease (AD) [44]. NGF specifically prevents the death and stimulates the function of basal forebrain cholinergic neurons that undergo early and prominent degeneration in AD. Indeed, NGF levels in the basal forebrain region decline in AD [45] and BDNF mRNA and protein levels decrease in patients with AD [46], [47]. Also, the expression of TrkA, TrkB and TrkC receptors is reduced in the basal forebrain region of patients with AD [48].

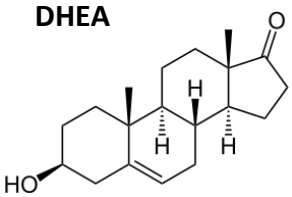
Parkinson's disease (PD) is characterized by limiting striatal dopamine concentrations due to apoptotic loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), which express NGF and TrkA in high levels [49]. Neurotrophins have shown great potential in animal models of PD, conferring protective and regenerative effects on the nigrostriatal dopaminergic neurons which undergo progressive degeneration in this disease [50]. In ALS, motor neurons are progressively lost and neurotrophins are implicated in this disorder, with a strong increase in their concentration and alterations in the receptors' phosphorylation [51]. Various neurotrophic factors have been investigated pre-clinically and clinically for the treatment of ALS, however no neurotrophic factors succeeded yet in a clinical phase III trial [52].

Overcoming the difficulties of neurotrophin delivery and pharmacokinetics in the nervous system, many efforts have been made in order to utilize the neuroprotective effects of neurotrophins and reverse the degeneration observed in disorders, such as AD [53], [54]. Indeed, activation of Trk receptors results in neuroprotective effects upon cortical, hippocampal, striatal, basal forebrain cholinergic and motor neurons after nerve injury [42], [55]. These observations are significant since cholinergic neurons in the basal forebrain degenerate in Alzheimer's disease; motor neurons undergo cell death in ALS; and striatal neurons in Parkinson's. Specifically for AD, a cell-based approach for NGF delivery has been promising in initial Alzheimer disease trials [56], [57].

4. Neurosteroids and DHEA

The term neurosteroid was introduced by Étienne-Émile Baulieu [58] and refers to steroids synthesized in the brain and influence brain functions by interacting with multiple cell surface receptors. Neurosteroids arise from cholesterol side-chain cleavage producing progesterone (PROG), pregnenolone (PREG), dehydroepiandrosterone (DHEA), and their sulfate esters and compounds, known as reduced metabolites of steroid hormones [59]. Neurosteroids are characterized by lipophilicity and their ability to cross the blood-brain Barrier (BBB) (peripherally synthesized steroids), which properties make them accumulate in the brain and have an impact on brain functions. Also, because of these properties, steroids offer great therapeutic opportunities because of their multiple effects in the nervous system, such as regulation of neurotransmission, promotion of neuronal viability, myelination, etc [60].

DHEA is an endogenous steroid hormone, one of the most abundant circulating steroids in humans [61]. It is produced in the adrenal glands, the gonads and the brain, by a well-studied biosynthetic process which includes a variety of enzymes, leading to the production of DHEA and its sulphated derivative, DHEAS [62]. DHEA is a metabolic intermediate in the biosynthesis of the androgen and estrogen sex steroids, however it has multiple additional functions as it potentially binds to many cell surface receptors [63], including neurotrophin receptors [64]. It has also been reported that DHEA is synthesized in neural and glial cells in the brain by the enzymes of the neurosteroidogenic pathway, making DHEA and DHEA(S) the main neurosteroids in the brain [65]. The concentration of DHEA and DHEA(S) varies throughout development, with high levels been detected at the early stages of development and adulthood, which decline during aging and pathological conditions (**Figure 1.4**). Studies investigating the role of neurosteroids in Alzheimer's disease have shown that there is significant decline of neurosteroid concentrations in individual brain regions of AD patients compared to age-matched without dementia controls [66]. Therefore, it is proposed that these molecules play a pivotal role in the development of nervous tissue, neural survival or apoptosis, in aging and neurodegenerative diseases [66], [67].



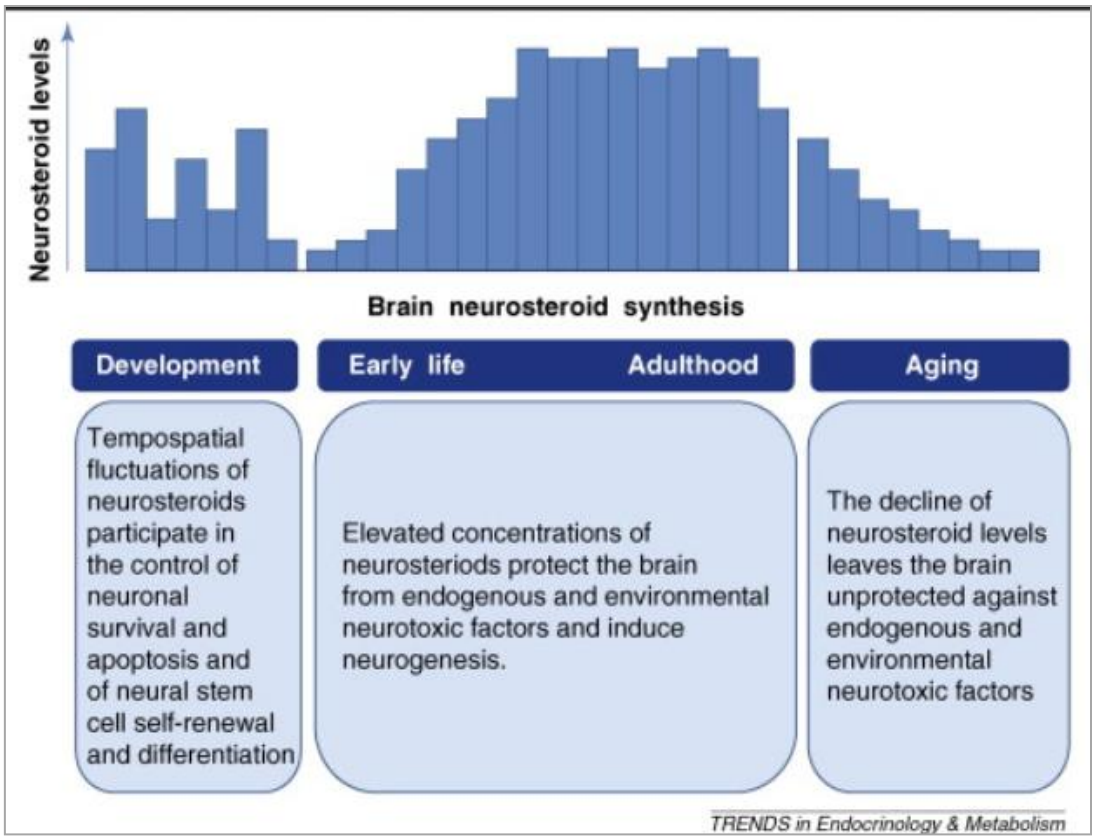


Figure 1.4 : **Changes in neurosteroidogenesis throughout lifespan** (*Charalampopoulos et al., 2008*).

5. DHEA and Neurotrophins: What is the link?

Since DHEA was found to exert protective effects in hippocampal and cortical neurons [68], [69], previous work in our laboratory proved that this protective role is also detected in adrenal medulla and is mediated by activation of pro-survival kinases PI3K/Akt and MEK1/2/ERK1/2, activation of the transcription factors NF-κB and CREB, which regulate the expression and activity of anti-apoptotic Bcl-2 proteins [70]. Later, Charalampopoulos et al. showed that DHEA-induced neuroprotection could be attributed to specific membrane DHEA binding sites [71]. Further studies in the laboratory focused on deciphering the signaling machinery that mediates the pro-survival effects of DHEA, where it was demonstrated that multiple parallel signaling events happened after DHEA binding to membrane sites, leading to cyto-protection [72]. In 2011, Lazaridis et al. provided for the first time, strong evidence that DHEA exerts its neuroprotective effects by directly interacting with NGF receptors, TrkA and $p75^{NTR}$, activating their downstream signaling pathways [64]. DHEA binds with high affinity (at nM level) either to TrkA receptor inducing phosphorylation of Shc, ERK1/2, and Akt kinases and thus regulating the effect of apoptotic Bcl-2 proteins, or to the $p75^{NTR}$ receptor which interacts with TRAF6, RIP2, and RhoGDI controlling neuronal apoptosis (**Figure 1.5**). Therefore, it seems rational to hypothesize that the relative abundance between the pro-survival receptor TrkA and the death receptor $p75^{NTR}$ may determine the cell survival or apoptosis [64]. A following study in our laboratory investigated the ability of DHEA to interact with the other two mammalian neurotrophin receptors (TrkB and TrkC) and whether it could also bind to invertebrate Trk related receptors (LTrk, ApTrk and AmphiTrk). The results showed that DHEA could bind to all Trk receptors, however with lower affinity compared to the designated neurotrophin ligands. DHEA could

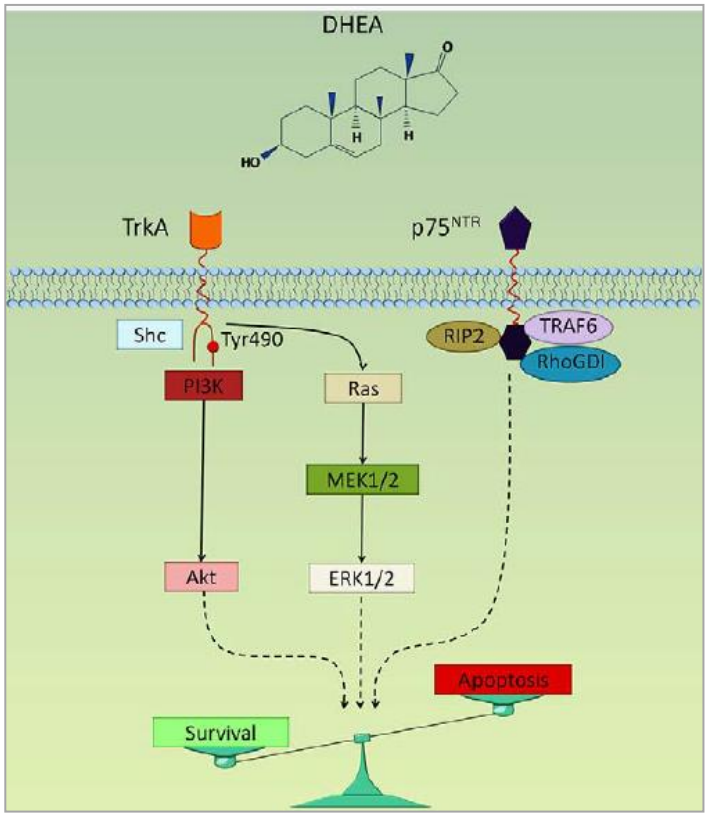


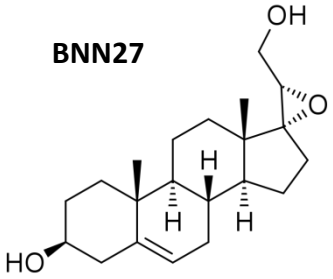
Figure 1.5: DHEA-driven signaling events. DHEA binds to TrkA and p75 leading to their activation producing prosurvival and apoptotic signals; the ratio of the receptors shall determine the cell fate eventually (*Lazaridis et al., 2011*).

induce the phosphorylation of all receptors except TrkB and activation of the ERK1/2 and Akt kinases, thus exerting anti-apoptotic effects [73].

6. Microneurotrophins

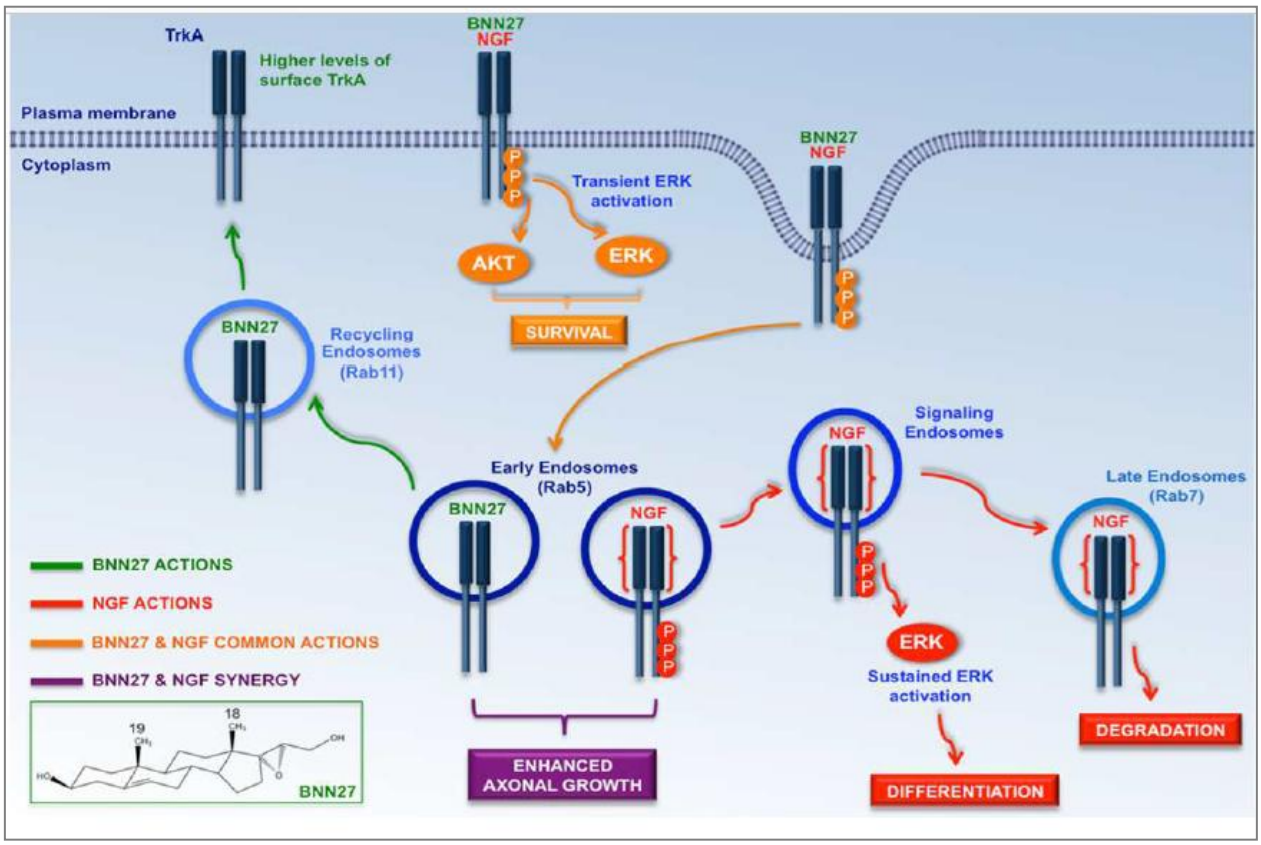
Although neurotrophins demonstrate multiple beneficial effects, their therapeutic potential is limited due to their polypeptidic structure and their large size, which disturbs their blood-brain barrier (BBB) penetration. Attempting to imitate their beneficial effects and in accordance with the finding that DHEA interacts with NGF receptors providing neuroprotective effects, DHEA could serve as a template molecule for designing analogs-agonists for NGF receptors, which could be used against neurodegenerative diseases where the amount of DHEA or NGF are limited [64]. Given that natural neurosteroids such as DHEA are metabolized into estrogens, androgens or progestins and interact with the respective receptors, the DHEA analogs synthetized by our collaborators [74] were modified at positions C3 and C17 and were evaluated for neuroprotective activity. These modifications improve the antiapoptotic-neuroprotective activities of these compounds and inhibit their interaction with the classical intracellular receptors of estrogens or androgens. The analogs are interacting with high affinity (K_d at nanomolar concentration) with DHEA binding sites, inducing antiapoptotic Bcl-2 proteins [74]. The spiro-epoxyneurosteroid derivatives 20, 23 and 27 were the molecules that stood out in this first trial and their properties were further studied in our laboratory.

BNN27



BNN27 was the first selected molecule for further evaluation of its properties. This small, BBB permeable DHEA-derivative was shown to prevent serum-deprived apoptosis in PC12 cells [74]. In 2016, Pediadiatakis et al. showed that BNN27 specifically interacts with and activates the TrkA receptor, inducing phosphorylation of TrkA tyrosine residues and down-stream neuronal survival-related kinase signaling. Also, BNN27 synergizes with NGF to induce axonal outgrowth and rescues from apoptosis NGF dependent and TrkA positive sympathetic and sensory neurons, *in vitro*, *ex vivo* and *in vivo* in NGF null mice, without promoting hyperalgesic events. Furthermore, they showed that BNN27 facilitated TrkA receptor translocation into membrane lipid rafts, providing evidence for the kinetics of BNN27-induced TrkA turnover (**Figure 1.6**) [75]. In another study of our laboratory [76], BNN27 was evaluated for its interaction with $p75^{NTR}$ and the following molecular effects. It was demonstrated that after physical interaction of BNN27 with $p75^{NTR}$ in specific amino-residues of its extracellular domain, there is recruitment of $p75^{NTR}$ to its effector protein RIP2 and the protein RhoGDI is simultaneously released. In Cerebellar Granule Neurons (CGNs) which express $p75^{NTR}$, but not TrkA receptors, it was shown that the activation of $p75^{NTR}$ by BNN27 rescued the cells from apoptosis by decreasing the phosphorylation of JNK pro-apoptotic kinase and reducing the cleavage of Caspase-3. Also, recent findings underline that BNN27 acting through NGF receptor

TrkA protects mature oligodendrocytes under demyelinating disorders-conditions [77], it targets both the neurodegenerative and inflammatory components of diabetic retinopathy [78], however it does not demonstrate neuroprotective activity in experimentally induced retinal detachment [79].

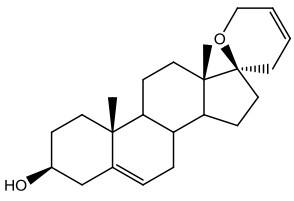


<u>Figure 1.6:</u> A proposed mechanism of action of BNN27 in a molecular level. BNN27 binds to TrkA receptor at nanomolar concentrations and induces TrkA activation and downstream signaling events. Also, it induces internalization and fast turnover of the receptor through endosomes. Similarities and differences in the mechanisms of action between NGF and BNN27 are depicted with different colours (*Pediaditakis et al., 2016*).

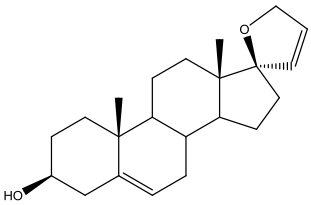
7. Aim of the study

Previous findings of our laboratory suggest that BNN27 exerts strong anti-apoptotic, neuroprotective actions via neurotrophin receptors, thus it may serve as a lead molecule to develop new, more effective BBB permeable neurotrophin-like small molecules (microneurotrophins) with potential applications in the treatment of neurodegenerative diseases [80]. Our collaborators in the Institute of Organic and Pharmaceutical Chemistry and Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, designed and synthesized the following novel microneurotrophin compounds:

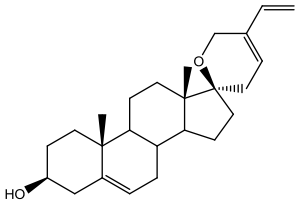
BNN218



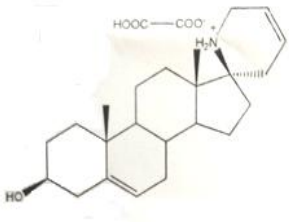
BNN219



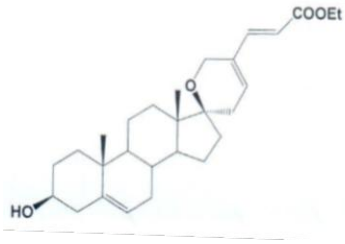
BNN237



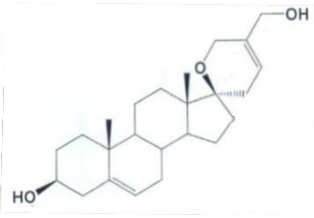
BNN238



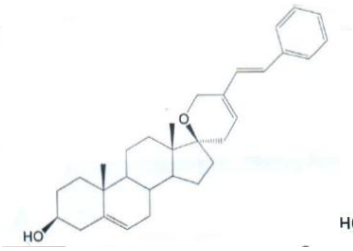
BNN396



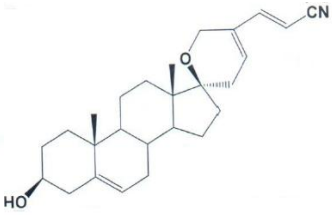
BNN397



BNN398



BNN403



In compounds BNN218 and BNN219 the size of the C17-spiro substituent was increased; BNN219 is substituted by 2,5-dihydrofuran (5-membered heterocyclic ring) while, BNN218 by a 3,6-dihydropyran (6-membered heterocyclic ring). Compound BNN237 is a derivative of BNN218 and more specifically, it bears a 5-vinyl, 3,6-dihydro pyran moiety at C17, instead of an unsubstituted 3,6-dihydropyran.

The present study focuses on evaluating the ability of these new microneurotrophin compounds to activate neurotrophin receptors TrkA and TrkB; i.e. to induce receptor transphosphorylation in specific tyrosine residues (Y490 and Y816 respectively) in specific time-

points. In parallel, the ability of the new compounds to rescue cells from apoptosis is being tested in the laboratory. The characterization of these two properties (activation of neurotrophin receptors and ability to rescue cells from apoptosis) in cell lines is the first step towards the investigation of the therapeutic potency of these microneurotrophins. The efficacy of the synthetic microneurotrophins as neuroprotective and neurogenic agents could be further tested *in vivo* in animal models of neurodegenerative disorders. Data provided by other teams in the laboratory for TrkA dependent cell survival and TrkB activation after treatment with analogs are also presented.

Materials and Methods

Chemical and biochemical reagents

Phosphatase Inhibitor Cocktail 50X (Cat. No. 524629, Millipore); Protease inhibitors (Cat. No. 539131, Millipore); anti-TrkA polyclonal rabbit IgG (Cat. No. 06-574, Millipore); Phospho-TrkA Y490 (Cat. No. 9141, Cell Signaling); Phospho-TrkA (Tyr785)/TrkB (Tyr816) (Cat. No. 4168, Cell Signaling); anti-TrkB (Cat. No. 33655, Abcam); anti-GAPDH (Cat. No. G8795, Sigma); DMEM (Cat. No. LM-D1099/500, Biosera); Goat anti-Rabbit HRP (Cat. No. 656120, Invitrogen); FBS (Cat. No. 10270-106, Thermo Fisher Scientific); Pen/Strep (Cat. No. 15140-122, Gibco); Culture flasks 25cm2 (Cat. No. 156367, Thermo Scientific); PBS 1X (Cat. No. LM-S2041/500, Biosera); Trypsin 0.5% (Cat. No. 15400-054, Gibco); DMSO (Cat. No. D5879, Sigma-Aldrich); Trypan blue 0.5% (Cat. No. T8154, Sigma); Turbofect (Cat. No. R0531, Thermo Fisher Scientific); NGF (Cat. No. 01-125, Millipore); BDNF (Cat. No. B-250, Alomone); PIERCE (Cat. No. 87788, Thermo Scientific); Tris-HCl (Cat. No. T5941, Sigma); 2-mercaptethanol (Cat. No. 63690, Fluka); SDS (Cat. No. 13771, SigmaAldrich); Glycerol (Cat. No. G6279, Sigma-Aldrich); acrylamide (Cat. No. A8887, Sigma); APS (Cat. No. 21.558-9, Sigma-Aldrich); TEMED (Cat. No. GE171312-01, Sigma-Aldrich); Protein marker (Cat. No. p77125, New England Biolabs); Trisma base (Cat. No. T6066, Sigma); Glycine (Cat. No. 33226, Sigma-Aldrich); Nitrocellulose membranes (Cat. No. 10600002, GE Healthcare Life Sciences); BSA (Cat. No. A2153, Sigma); Methanol (Cat. No. 32213-251, Honeywell); NaCl (Cat. No. S30140, Sigma); Tween 20 (Cat. No. P7949, Sigma); ECL SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Cat. No. 34580, ThermoScientific); Protein G-plus agarose beads (Cat. No. sc-2002, Santa Cruz Biotechnology).

Cell culture and cell transfection

HEK293 and CHO cells were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% heat-inactivated (56˚C for 30 min) fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin (full medium). In a humidified incubator at 37^{o}C, cells were provided with 95% oxygen and 5% CO_2. All procedures were performed in a laminar air flow hood in order to

maintain sterility conditions. NIH-3T3 cells stably transfected with TrkA or TrkB plasmid were kindly provided by Prof. Carlos F. Ibáñez (Karolinska Institutet) and were grown under the same conditions. In all experiments, 95-98% confluent cells were used. The maintenance of the culture involved change of growth medium every 2 days or split 1:4 with 0.05% trypsin when cells were confluent >80%. The procedure of splitting involved 1-2 washes with 1X PBS, incubation at 37^{o}C with 0.05% trypsin for 2 minutes, deactivation of trypsin by using full medium, centrifugation for 5 minutes at 1200 rpm, removal of supernatant and dilution of the cells in the proper volume of full medium. Freezing of the cells was performed when necessary with freezing medium [70% DMEM; 20% FBS; 10% DMSO] and cells were stored at -80^{o}C.
Cells were transfected with TurboFect (Fermentas) or Lipofectamin 2000 (InVitrogen) according to manufacturers' instructions. Transfected cells were typically used on the 2nd day after transfection.

Treatments with BNN analogs

Treatments with BNN analogs were performed using the following procedure: In a 12-well plate 150.000 cells were seeded for each condition that was tested one day before the treatments. The counting and the viability of the cells were performed by trypan blue exclusion test. Prior to counting and seeding the cells, the detachment from the culture flask was performed with a cell scraper, not with trypsin as it cleaves the extracellular domains of the receptors. The next day, full medium was removed from the wells and medium without serum was added (serum deprivation) for 4 hours. Afterwards, BNN analogs were diluted in the indicated concentrations (10^{-7}M or 10^{-8}M) for 30, 20 and 10 minutes. The control conditions in the experiments were: incubation with full medium (mild activation of receptors by serum proteins), addition of NGF at 100ng/ml (activation of TrkA), addition of BDNF at 100ng/ml or BNN20 at 10^{-7}M (activation of TrkB) and incubation with serum free (SF) medium (no activation). After treatment, cells were washed twice with ice-cold PBS, and suspended in 100 µL cold lysis buffer [50 mM Tris-HCl, 0.15 M NaCl, 1% Triton-X100, pH 7.4 or PIERCE lysis buffer] supplemented with protease and phosphatase inhibitors. The cell lysates were further used for immunoblotting or immunopercipitation as described afterwards.

Immunoblotting (IB)

After cell lysis, cell lysates were suspended in 2X SDS loading buffer [0.125M Tris-HCl; 4% 2-mercaptethanol; 4% SDS; 40% ddH2O; 0.3mM Bromophenol blue; 10% glycerol] and separated by 12% SDS-PAGE. The separation gel [Acrylamide 30%/Bis-acrylamide 0.8% w/v; Tris-HCl 1.5M pH=8.8; SDS 10%; APS 10%; TEMED; H2O] and the stacking gel [Acrylamide 30%/Bis-acrylamide 0.8% w/v; Tris-HCl 0.5M pH=6.8; SDS 10%; APS 10%; TEMED; H2O] were prepared by using equipment of Biorad. After sample heating (95^{o}C for 10 minutes) and loading to the gels, the tank was filled with Running Buffer [25mM Trizma Base; 192mM Glycine; 0.1% SDS] and the

electrophoresis was performed at 80V for 30 minutes and then at 110V for 3 hours. Afterwards, proteins were transferred to nitrocellulose membranes by electrophoresis at 300mA for 70 minutes in a tank filled with cold Transfer Buffer [25mM Tris-HCl; 192mM Glycine; 20% Methanol]. Membranes were blocked for 1 hour at Room Temperature (RT) with 5% BSA diluted in TBS-Tween20 0.1% [50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 0.1% Tween20] and were incubated with the corresponding primary antibodies overnight at 4^{o}C in a shaking platform. All primary antibodies were used in 1:1000 dilution at 5% BSA; TBS-Tween20 0.1%. The next day, membranes were washed 3-4 times for 15 minutes with TBS-Tween20 0.1% and then were incubated with the corresponding secondary antibody at 1:5000 dilution in 5% BSA, TBS-Tween20 0.1% for 1 hour at RT. After 3 washes of 15 minutes each, immunoblots were developed using the ECL Western Blotting Kit and were imaged with the Chemi Doc MPimager(Bio-Rad). Image-Lab software version 4.1(Bio-Rad) was used for image acquisition. When stripping of the antibodies was necessary, membranes were washed twice with TBS-Tween20 0.1% and were incubated in stripping buffer [2%SDS, 62.5mM Tris-HCl pH 6.8, 0.8% 2-mercaptethanol] for 25 minutes at 56^{o}C. Afterwards, the membranes were washed 5-6 times for 15 minutes and then were blocked and immunoblotted as already described. Analysis and quantification of band intensities was performed by Image J (Fiji) software.

Immunoprecipitation (IP)

When IP was performed, cell lysates were pre-cleared for 1 h with Protein G-plus Agarose beads. Afterwards, beads were discarded and the lysates were incubated with the appropriate primary antibody, overnight at 4^{o}C in a rotating wheel. The next day, Protein G-plus agarose beads were added to each sample and were incubated for 4 h at 4^{o}C in a rotating wheel. Beads were collected by centrifugation, washed twice with lysis buffer and re-suspended in SDS loading buffer. For immunoblot (IB) analysis, proteins were subjected to SDS-PAGE as previously described.

Statistical Analysis

All results are reported as the mean ±SEM. Comparison of two groups was performed using an unpaired t-test. Statistical analyses were performed using GraphPrism, version7 (GraphPad Software Inc.). A p-value of less than 0.05 was considered significant.

Results

I. Activation of TrkA by NGF in NIH-3T3/TrkA cells

In the following experiments, we used the cell line NIH-3T3 which was stably transfected with TrkA plasmid (from now on referred as NIH-3T3/TrkA) and the receptors were abundantly expressed. In order to examine the levels of TrkA expression and the receptor's response to NGF in this cell line, we firstly performed experiments where the cells were deprived of serum and supplemented with NGF for different time-points (10, 20 and 30 minutes). As control samples, we used CHO cells which do not express TrkA, PC12 cells which endogenously express TrkA, serum free (SF) cultured NIH-3T3 cells and NIH-3T3 cells cultured in full medium (with serum) (**Figure 2.1**).

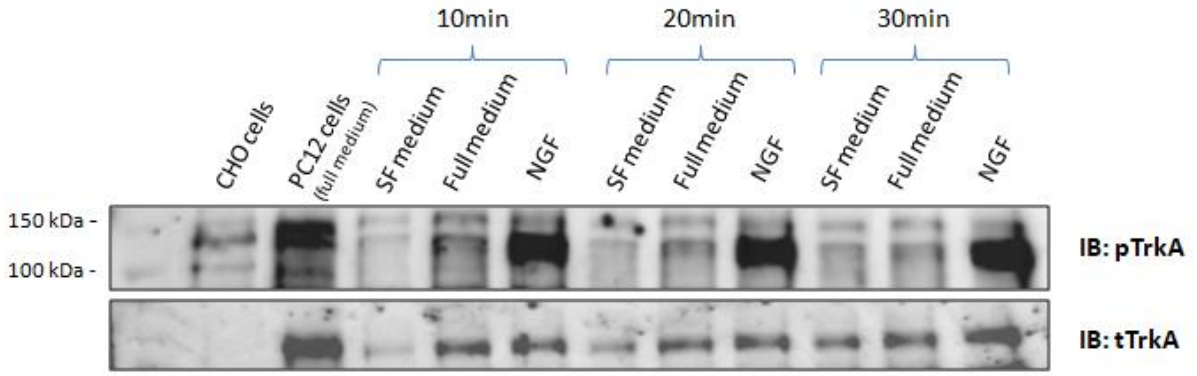


Figure 2.1: Activation of TrkA by NGF in NIH-3T3/TrkA cells at different time-points. All the cells used were starved from serum for 4 hours and then the culture medium was replaced with either full medium (DMEM+FBS+p/s), SF medium (DMEM only) or SF medium supplemented with 100ng/ml NGF for the indicated time-points. After the treatments, cell lysates were subjected to SDS-PAGE and immunoblotted with pY490 antibody, and then membranes were stripped and re-blotted with total TrkA antibody. The TrkA band is at 110-140kDa.

In Figure 2.1 we observed that CHO cells lack TrkA receptor as expected, however there is a weak signal in pTrkA membrane indicating that there might be unspecific binding of the antibody in that area. PC12 cells express TrkA receptor and the receptor is highly activated when the cells are grown in full medium. At the SF condition of the time-points 10 and 20 minutes, we observe that the total TrkA receptor is poorly detected most probably due to increased cell death. In the other conditions, total TrkA is efficiently detected. Full medium is activating the receptor slightly in all time-points. Treatment with NGF seems to activate the receptor very efficiently in all three time-points, allowing us to employ NGF treatment as a control experiment for activation of TrkA receptor in NIH-3T3/TrkA cells.

II. Activation of TrkA in NIH-3T3/TrkA cells by new BNN analogs at different time-points

In the following experiments we tried to evaluate the effect of the new analogs described in the introduction, on activating the neurotrophin receptor TrkA. For this purpose, we utilized a phosphorylation assay, in which NIH-3T3/TrkA cells were treated for a specific time-point with the analogs; then, they were lysed and subjected to SDS-PAGE and immunoblotting. The activation of the receptor was determined by its phosphorylation at the tyrosine residue 490 (Y490), consequently the first antibody used for each membrane was specific for this phosphorylated residue (anti-pTrkA). Afterwards, membranes were stripped and re-blotted for the total amount of the TrkA receptor (anti-tTrkA). In order to estimate the activation of the receptor, we measured the intensity of the TrkA band in both immunoblots and we calculated the ratio of phosphorylated receptor divided by the total amount of the receptor expressed. This ratio enables us to make comparisons between the samples and deductions about the TrkA activation potential of the new analogs.

The Western Blot membranes presented below are the most representative of every trial.

Activation of TrkA by new BNN analogs in 10-minute treatments

After 10-minute treatments with analogs to the NIH-3T3/TrkA cells we observe that there is no activation of the TrkA receptor in this time-point. CHO cells, which serve as a control sample, do not express TrkA, indicating the specificity and efficiency of the tTrkA antibody. It is clear that although there are good levels of TrkA expression (IB: tTrkA), none of the tested analogs or the serum could activate the TrkA receptor (IB: pTrkA). In this experiment, no statistical analysis is performed because of the low signal intensity in all biological and technical repeats.

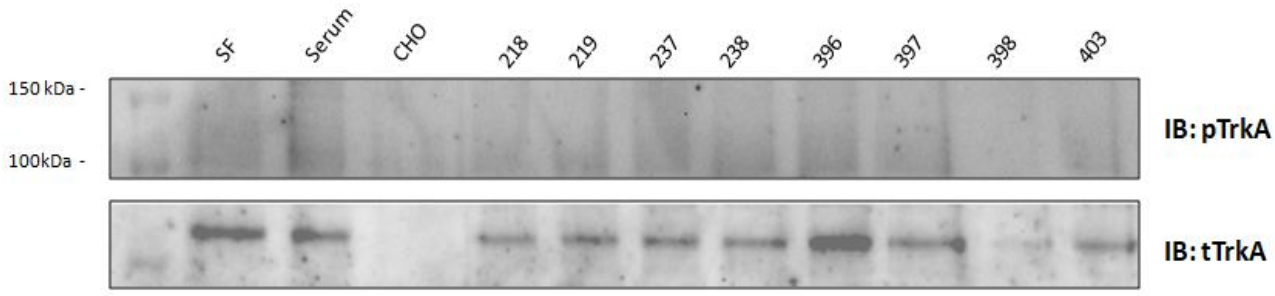


Figure 2.2: Activation of TrkA after 10-minute treatment with eight new BNN analogs. After 4 hours serum starvation, cell culture medium was replaced with either full medium (DMEM+FBS+p/s) or SF medium supplemented with 10^{-7} M of the new BNN analogs or 100ng/ml NGF, for 10 minutes. After the treatments, cell lysates were subjected to SDS-PAGE and immunoblotted with pY490 antibody, and then membranes were stripped and re-blotted with total TrkA antibody.

Activation of TrkA by new BNN analogs in 20-minute treatments

Activation of TrkA was tested after 20-minute treatment with the new analogs in NIH-3T3/TrkA cells (**Figure 2.3**). It is clear that serum and NGF activate the receptor, while there is variability on the activation by the analogs. CHO cells do not express TrkA receptor (IB: tTrkA), however using antibody for Y490 phosphorylation there is signal of strong intensity due to unspecific binding of the antibody. By measuring the band intensities (Image J software), we calculated the ratio pTrkA/tTrkA by dividing the each intensity measurement of the phosphorylated TrkA with the measurement of the total TrkA band. Afterwards, intending to normalize the data with the control (SF condition), we divided each condition's p/tTrka ratio with the ratio of SF condition (the normalized ratio is shown between the two membrane blots). From this experimental analysis, it is clear that the analogs which seem to have an effect on activating the receptor are BNN237, BNN397 and BNN403. NGF seems not to have activated the receptor efficiently maybe because of the repeated freeze/thaw cycles of the NGF vial. Another team in the laboratory was simultaneously working on the effects of the new analogs on cell apoptosis after 20-minute treatment; their results will be described in the next section.

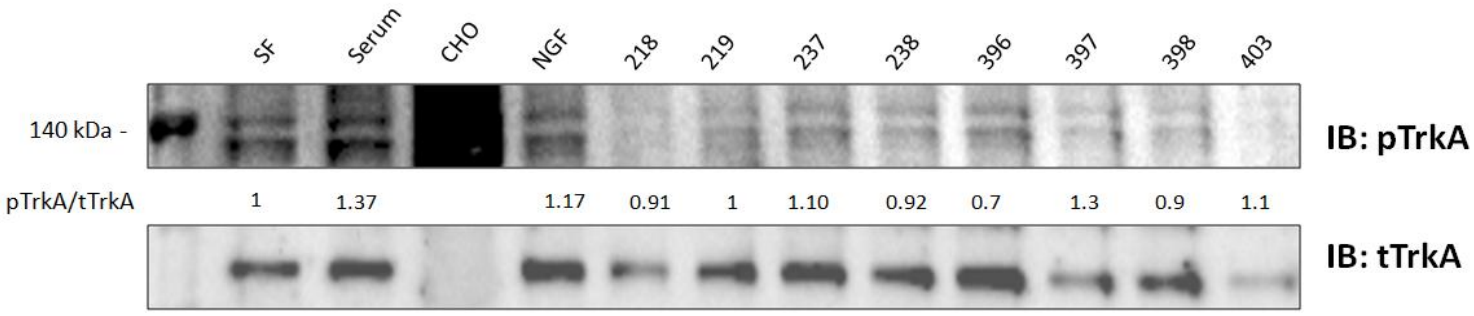


Figure 2.3: Activation of TrkA after 20-minute treatment with eight new BNN analogs. After 4 hours serum starvation, cell culture medium was replaced with either full medium (DMEM+FBS+p/s) or SF medium supplemented with 10^{-7} M of the BNN analogs or 100ng/ml NGF, for 20 minutes. After the treatments, cell lysates were subjected to SDS-PAGE and immunoblotted with pY490 antibody, and then membranes were stripped and re-blotted with total TrkA antibody. In this figure, the normalized (to SF) ratio pTrkA/tTrkA is also presented.

Activation of TrkA by new BNN analogs in 30-minute treatments

Activation of TrkA was tested after 30-minute treatment with the new analogs in NIH-3T3/TrkA cells (**Figure 2.4**). It seems that serum supplemented medium and NGF have activated the receptor (positive controls) and most of the analogs have a noticeable effect on the activation, too (IB: pTrkA). BNN219, BNN396 and BNN398 have a greater impact on the 30-minute activation, while BNN397, BNN218 and BNN403 give a lower signal. BNN237 and BNN238 do not seem to activate the receptor in this 30-minute trial. There are multiple explanations about the activation potential of BNN403 and BNN237 at different time-points, which will be discussed later. CHO cells, again in this experiment have unspecific binding for the antibody against phosphorylated receptor (IB: pTrkA), however they do not express TrkA (IB: tTrkA). The band intensities were analyzed for each condition and the ratio pTrkA/tTrkA was calculated, normalized to SF condition (control) and is presented below between the two membranes.

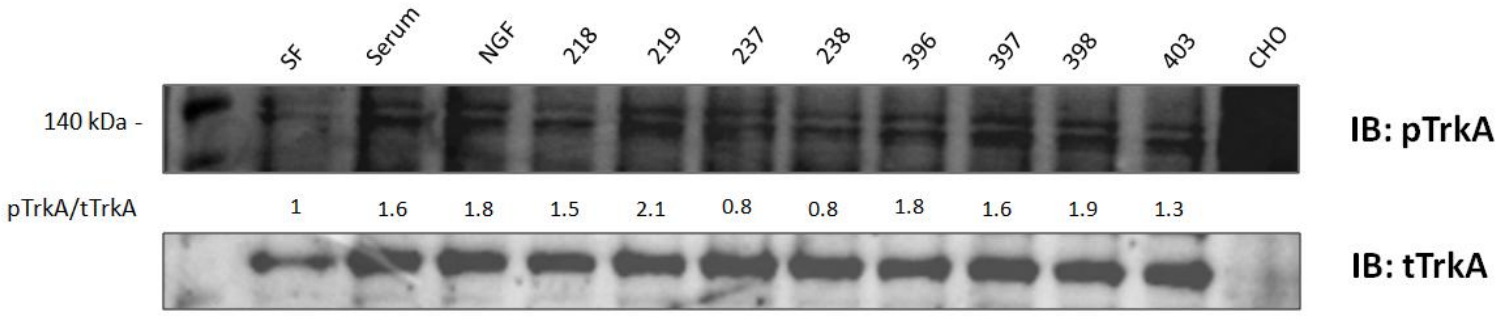


Figure 2.4: Activation of TrkA after 30-minute treatment with eight new BNN analogs. After 4 hours serum starvation, cell culture medium was replaced with either full medium (DMEM+FBS+p/s) or SF medium supplemented with 10^{-7} M of the new BNN analogs or 100ng/ml NGF, for 30 minutes. After the treatments, cell lysates were subjected to SDS-PAGE and immunoblotted with pY490 antibody, and then membranes were stripped and re-blotted with total TrkA antibody. In this figure, the normalized (to SF) ratio pTrkA/tTrkA is also presented.

III. Flow cytometry-based detection of cell apoptosis

Experimental data of this section are kindly provided by Maria-Anna Papadopoulou and Maria Kokkali.

PC12 cells were used for the cell apoptosis assay as they endogenously express the neurotrophin receptors TrkA and $p75^{NTR}$, they respond to NGF by extending neurites and acquiring a neuronal phenotype [81], properties suitable for our experiments. In order to investigate the pro-survival actions of the new analogs, cells were seeded in a 6-well plate, were starved from serum for 3 hours and then they were treated with serum free (SF), full medium (supplemented with serum), 100ng/ml NGF and analogs (at 10^{-7}M final concentration) for 48 hours. The cells were stained using the TUNEL assay (apoptotic cells are TUNEL positive cells) and the apoptosis was detected by flow cytometry analysis. Cells grown for 48 hours without serum (SF condition) had increased levels of apoptosis compared to the cells grown in full medium (serum condition). Treatment with NGF seems to rescue cells from apoptosis, while BNN218 and BNN398 seem to reduce the number of apoptotic cells, too. BNN403 exhibits a tension towards rescuing the cells from apoptosis (**Figure 2.5** and **Figure 2.6**). The experiments were done in triplicates and performing statistical analysis, the anti-apoptotic effects of the analogs are not statistically significant (more experimental trials should be performed), however the average values indicate the differences between the samples.

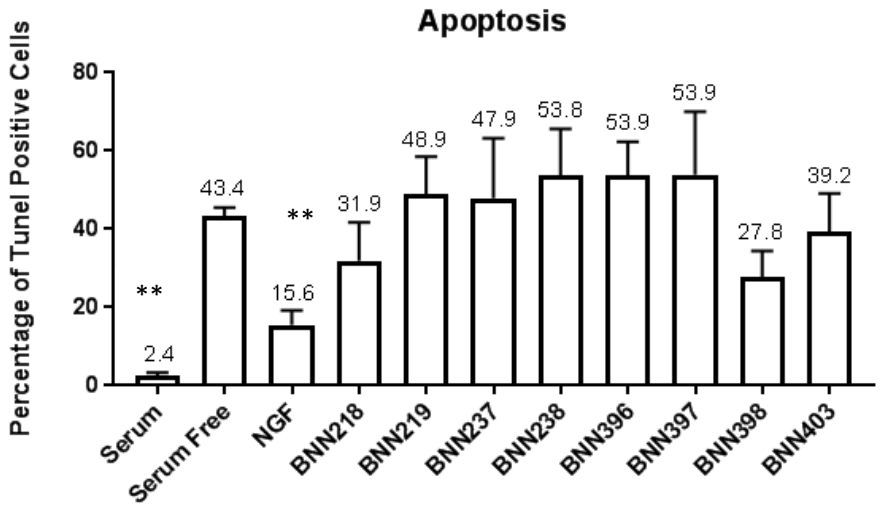
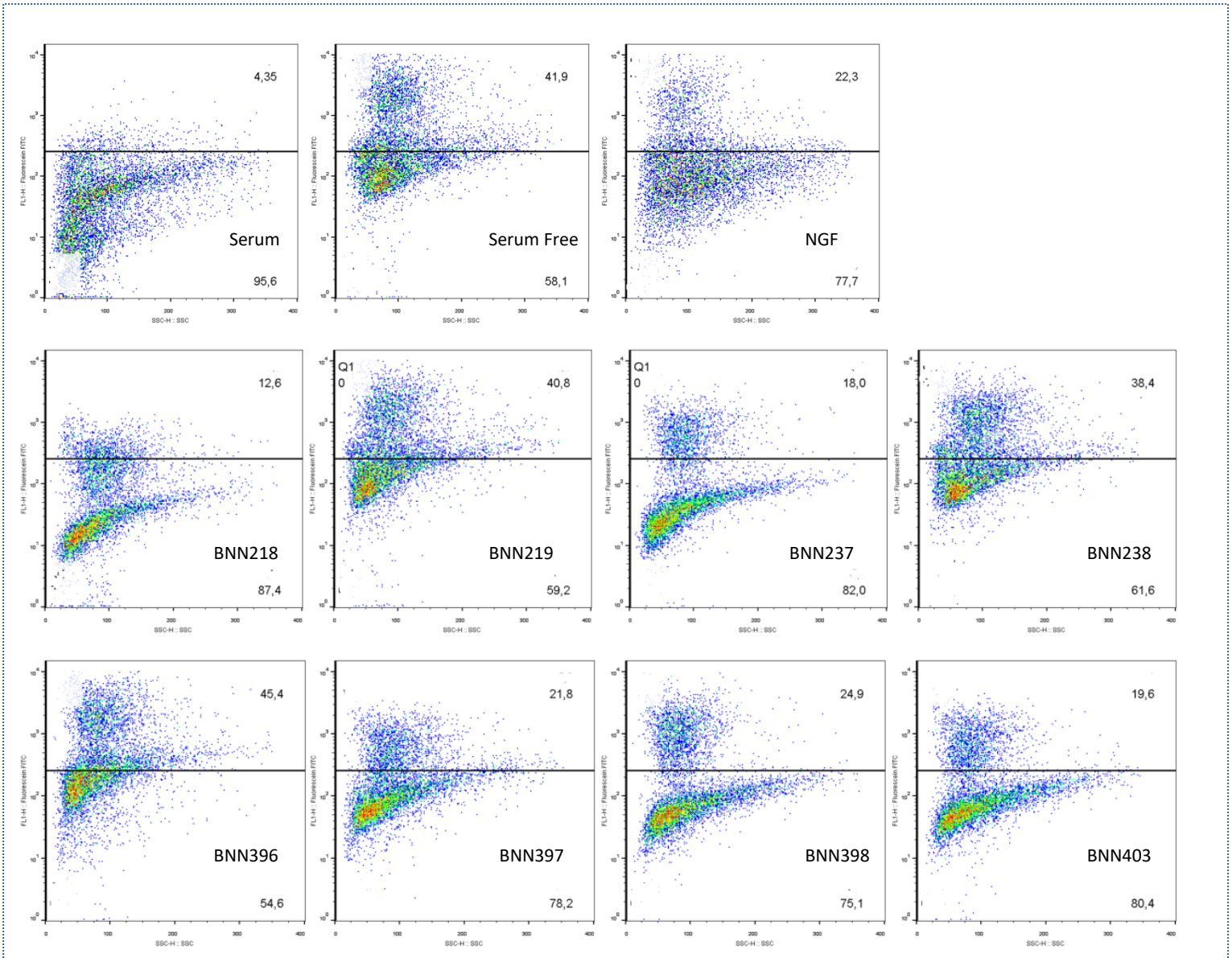


Figure 2.5: Percentage of apoptotic PC12 cells (TUNEL positive cells) analyzed by flow cytometry after 3-hour pre-starvation and treatment with analogs for 48 hours. TUNEL assay was used for the cell staining. Average values were calculated from n=3 experiments, statistical t-test analysis was performed between each sample and the serum free condition (**P<0.01).

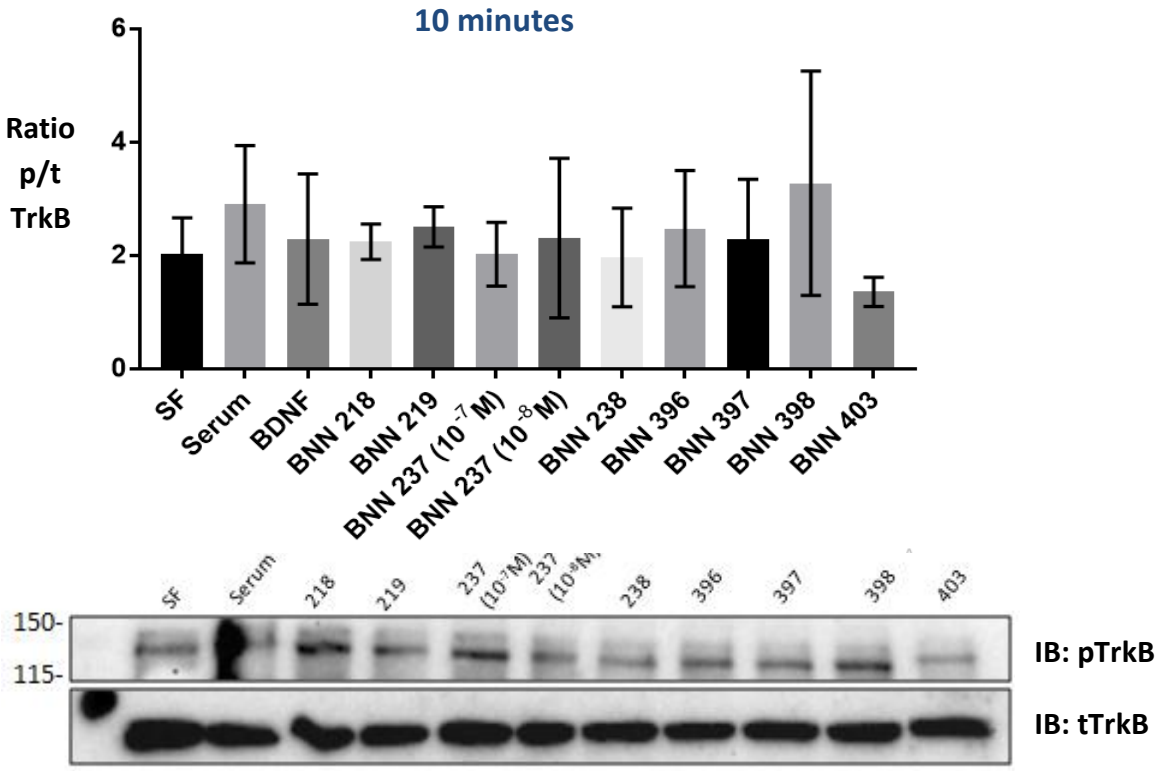


<u>Figure 2.6</u>: Dot plot pictures of the flow cytometry experiment in PC12 cells (every dot represents an individual cell) after 3-hour pre-starvation and treatment with analogs for 48 hours. TUNEL assay was used for the cell staining. Apoptotic cells are shown right above the axis in every diagram, while healthy cells are placed under the axis. The percentage of apoptosis in every sample is: *serum 4,35%, serum free 41,9%, NGF 22,3%, BNN218 12,6%, BNN219 40,8%, BNN237 18%, BNN238 38,4%, BNN396 45,4%, BNN397 21,8%, BNN398 24,9%, BNN403 19,6%.* These pictures are representative among the n=3 experiments.

IV. Activation of TrkB in NIH-3T3/TrkB cells by new BNN analogs at different time-points

Experimental data of this section are kindly provided by Mourat Vezir.

In this set of experiments we tried to evaluate the potential of the new analogs on activating the neurotrophin receptor TrkB. As described above, we utilized phosphorylation assays in NIH-3T3 cells stably transfected with TrkB receptor (NIH-3T3/TrkB). For specific time-points, cells were treated with the new analogs (at 10^{-7}M or 10^{-8}M) or 100ng/ml BDNF (as positive control of TrkB activation). As negative control, cells were grown in serum free (SF) medium or were grown in serum supplemented medium (full medium), as positive control. After treatments, were lysed and subjected to SDS-PAGE and immunoblotting. The activation of the receptor was determined by immunoblotting against TrkB phosphorylation at the tyrosine residue 816 (Y816) (anti-pTrkB). Afterwards, membranes were stripped and re-blotted for the total amount of the TrkB receptor (anti-tTrkB). It seems that none of the analogs in the 10-minute treatment activates the receptor. In the 20-minute treatment, BNN218 and BNN398 seem to induce TrkB activation, while in the 30-minute treatment BNN218, BNN237 and BNN397 have an effect on TrkB activation. The sample treated with BDNF do not activate TrkB receptor as expected, maybe because of multiple freeze/thaw cycles of the BDNF vial. In any case, the error bars do not allow us to reach a clear conclusion about the analogs and TrkB activation, so more experimental trials should be performed. The Western Blot membranes presented below are the most representative of every trial.



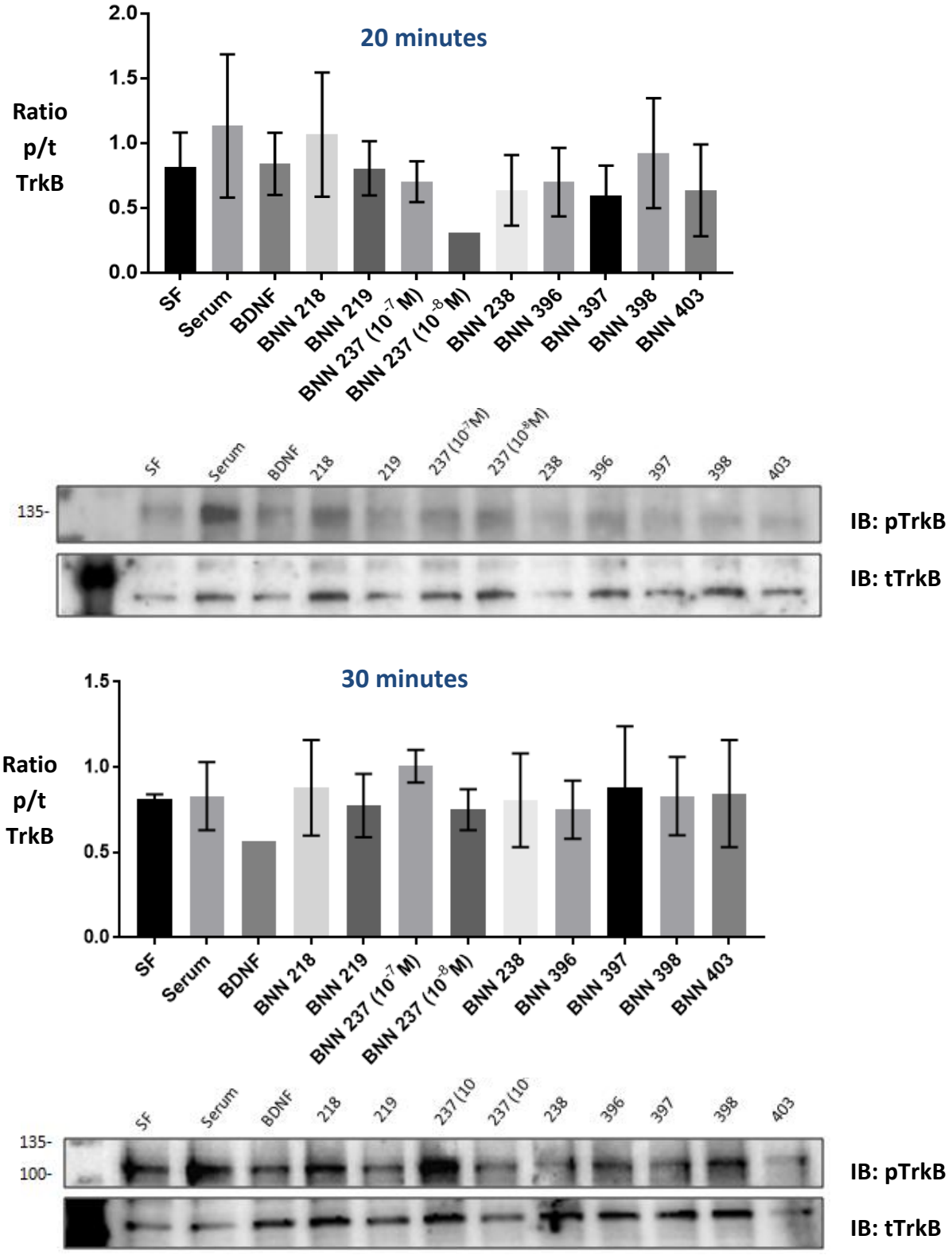


Figure 2.7: Activation of TrkB after 10, 20 and 30-minute treatment with eight new BNN analogs. After 4 hours serum starvation, cell culture medium was replaced with either full medium (DMEM+FBS+p/s) or SF medium supplemented with 10^{-7} M or 10^{-8} M of the new BNN analogs or 100ng/ml BDNF, for the indicated time-points. Cell lysates were subjected to SDS-PAGE and immunoblotted with pY816 antibody, then membranes were stripped and re-blotted with total TrkB antibody. The ratio p/t TrkB is also presented as the mean ±SEM in the graphs.

Discussion

It is well established that neurotrophins serve as essential components of the nervous system development, controlling cell survival, selection and differentiation. Defects in neurotrophic actions have been accused for promoting neurodegenerative conditions, such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). Nevertheless, due to their polypeptide nature and their inability to cross the blood-brain-barrier (BBB), their pharmacological potential is limited. Findings in our laboratory, suggest that dehydroepiandrosterone (DHEA), a lipophillic small molecule, exhibits neuroprotective effects via binding to NGF receptors, TrkA and $p75^{NTR}$, providing a useful pharmacological candidate with neurotrhophic properties [64], [70]. DHEA, however, is metabolized *in vivo* to estrogens, androgens and other metabolites, rendering its administration challenging. Attempting to eliminate these side-effects of DHEA, collaborators have recently developed synthetic C17-derivatives of DHEA (BNNs), that sustain the anti-apoptotic properties, but are deprived of androgenic/estrogenic actions [74]. These synthetic microneurotrophins have been recently shown to interact with both NGF receptors, TrkA and $p75^{NTR}$, providing promising evidence for their application as therapeutic agents [75], [76]. The current study focuses on evaluating the effect of newly synthesized microneurotrophin analogs on activating the NGF receptor TrkA.

The cell line NIH-3T3 stably transfected with TrkA receptor (NIH-3T3/TrkA) was primarily used in the experiments for TrkA activation. In the 10-minute treatment with the compounds, it is of note that none of the compounds activated the TrkA receptor, as NGF did (**Figure 2.1** and **Figure 2.2**). In the 20-minute treatments the analogs BNN237, BNN397 and BNN403 seem to induce TrkA phosphorylation (**Figure 2.3**), while in the 30-minute treatment BNN219, BNN396 and BNN398 have a greater impact on TrkA activation, but BNN397, BNN218 and BNN403 activate the receptor less efficiently (**Figure 2.4**). Results from the cell apoptosis assay (**Figure 2.5** and **Figure 2.6**) indicate that BNN218, BNN398 and BNN403 rescue cells from apoptosis after 48-hour treatment.

These results could be interpreted in multiple ways. Firstly, the activation of the TrkA by the new microneurotrophin compounds needs at least 20 minutes, as there is no phosphorylation signal in the 10-minute treatment (**Figure 2.2**). This observation is in agreement with previous findings from the microneurotrophin BNN27 which exerts its action at 15 minutes and it insists for 6 hours later; while NGF induced TrkA activation weakens after 30 minutes [75]. Observing the 20 and 30-minutes treatment (**Figure 2.3** and **Figure2.4**), it is clear that BNN237, BNN403 and BNN397 activate the TrkA receptor at 20 minutes and then the activation signal decreases. On the other hand, BNN219, BNN396 and BNN398 give a strong signal in the 30-minute activation while at 20 minutes the signal is insignificant. These differences between compounds could be attributed to the different TrkA activation potential of each analog and the different

kinetics of TrkA receptor dimerization that each compound induces. It is noteworthy that TrkA dimerization kinetics in cells treated by analogs may differ significantly from the kinetics that NGF induces [75]. BNN398 and BNN218 seem to stand out as a compound of interest, as they demonstrate satisfying TrkA activation signal at 30 minutes and, in parallel, rescue PC12 cells from apoptosis after 48 hour serum deprivation. BNN403 has interesting properties as it activates the TrkA receptor in 20 minutes, the signal decreases in 30 minutes but there is a tendency of rescuing PC12 cells from apoptosis (**Figure 2.5** and **Figure 2.6**). Although BNN219 and BNN396 give a remarkable signal in 30-minute TrkA activation, they do not rescue PC12 cells from apoptosis, which raises questions of other unknown functional characteristics of the compounds.

The analogs BNN218 and BNN398 which not only activate the TrkA receptor at 30 minutes, but they also rescue PC12 cells from apoptosis are likely to interact with TrkA receptor and induce cell survival signaling pathways. There is also probability that the analogs interact with $p75^{NTR}$ too (PC12 cells endogenously express $p75^{NTR}$ and TrkA), and the result of cell survival depends on the ratio of the two receptors; possibly TrkA which induces cell survival is expressed in higher levels than the pro-apoptotic receptor $p75^{NTR}$. In order to verify that cell survival is induced by binding of the analogs only to TrkA receptor, we should either silence $p75^{NTR}$ expression in PC12 cells (shRNA against $p75^{NTR}$) or conduct the cell survival experiment in NIH-3T3/TrkA cells (they do not express $p75^{NTR}$).

The analogs BNN219 and BNN396, in the 30-minute phosphorylation assays, it is clear that they induce TrkA activation; however in the cell survival assay they seem not to have a statistically significant effect in preventing apoptosis. In this case, these analogs might induce not only TrkA but also $p75^{NTR}$ activation; acting as partial agonists of TrkA they have less effective results in inducing cell survival but acting as full agonists of $p75^{NTR}$ they could induce cell apoptosis. To examine this scenario, silencing of $p75^{NTR}$ receptor in PC12 cells should be induced and if the induction of cell apoptosis is reversed, then we could assume that the interaction of the analogs with TrkA is dominant and therefore the cells eventually survive. If the cell apoptosis is not reversed by silencing $p75^{NTR}$, there is a possibility that the analogs interact with another receptor in the cell surface inducing cell apoptosis. Also, in order to prove that $p75^{NTR}$ is activated by these analogs, we could transfect cells with $p75^{NTR}$, make treatments with the analogs and run a co-immunoprecipitation assay in which we could test if $p75^{NTR}$ is bound to its interactor proteins (RIP2, TRAF6 or RhoGDI) and thus activated.

Analogs which do not produce interesting results in our phosphorylation and cell survival assays based on TrkA receptor, may interact with other neurotrophin receptors. Also, some analogs which have a potential for TrkA activation, such as BNN403 which induces a moderate TrkA activation and tends to rescue cells from apoptosis, may need more time in order to produce significant results in TrkA activation (> 30-minute treatments).

The analogs which produce significant results both in phosphorylation and cell survival assays should be considered as a priority for further investigation of their mechanisms of action. Firstly, it is crucial to specify the receptor to which each analog binds by silencing either TrkA or $p75^{NTR}$ and observing the consequential events. Further phosphorylation assays should be conducted to examine if TrkA phosphorylation occurs in other tyrosine residues (Tyr674/675 and Tyr785), or if the response of the receptor is time and dose dependent, as we examined only analog concentrations at 10^{-7}M for 10, 20 and 30 minutes. To evaluate the kinetics of each analog, we could employ binding assays and compare the efficacy of each analog with NGF, DHEA and BNN27. In order to reveal the exact binding site of each analog to the receptor, we could utilize NMR and pull down assays which verify the physical interactions. By receptor mutagenesis analysis, we could confirm which domains of the receptor are highly required for the analog binding and the downstream signal transduction.

Also, we should examine the activation of downstream signaling molecules (eg Shc, ERK1/2, Akt for TrkA or with TRAF6, RIP2, and RhoGDI for $p75^{NTR}$) in order to underline the signaling pathway that is induced after the analog treatment. Another interesting approach would be to employ internalization assays in order to assess whether the TrkA turnover is modulated after treatment with each analog and if this TrkA turnover pattern resembles the treatment with BNN27 or NGF [75]. The internalization of the complex NGF-TrkA in endosomes is known to induce signaling events from the endosome leading to neurite outgrowth [82], so we could examine if the axonal outgrowth is affected by the analog treatments.

The *in vitro* experiments on NIH-3T3/TrkA and PC12 cells should be confirmed in primary neuronal cell cultures in order to proceed to *ex vivo* trials. Sympathetic neurons are known to be NGF-dependent, expressing TrkA receptor [83], so we could utilize receptor's phosphorylation assays in order to define the TrkA activation potential of the analogs in this primary cell culture. We could also perform the cell survival assay before and after silencing of the receptor and we could identify the downstream affected signaling pathways.

In vivo experiments should confirm the *in vitro* findings about the neuroprotective potential of the analogs. In $ngf^{-/-}$ mouse embryos at the age of E13.5, the TrkA positive sensory neurons of Dorsal Root Ganglia (DRG) are led to naturally occurring programmed cell apoptosis. We could test if the treatment with analogs could rescue these cells from apoptosis at this stage of their life. The next step should be the *in vivo* testing of BNN analogs in experimental animal models of neurodegenerative diseases; such as 5xFAD for Alzheimer's disease, MPTP mouse and Weaver mouse models for Parkinson's disease and Cuprizone mouse models of Multiple Sclerosis and demyelinating diseases.

We hope that the new analogs will effectively mimic *in vitro* and *in vivo* the NGF activities which are mediated by TrkA receptor, such as inhibition of cell apoptosis or promotion of neurite

outgrowth in various neuronal cell types. If these actions are certified, the new analogs could be characterized as microneurotrophin agonists and their therapeutic application for neurodegenerative diseases shall be very promising.

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