Leo Jakman

Characterization of small-molecule GDNF mimics

Helsinki Metropolia University of Applied Sciences

Bachelor of Laboratory Services

Biotechnology

Thesis

17.12.2012

Helsinki Metropolia University of Applied Sciences

Author(s) Title Number of Pages Date	Leo Jakman Characterization of small-molecule GDNF mimics 32 pages 17.12.2012
Degree	Bachelor of Laboratory Services
Degree Programme	Laboratory Sciences
Instructor(s)	Yulia Sidorova, PhD Tiina Soininen, Head of Laboratory Sciences

Parkinson's disease (PD) is a neurodegenerative disease of the central nervous system characterized by motor impairment. The pathological hallmark and cause is the degeneration and death of the dopaminergic neurons in the structure of the brain known as substantia nigra pars compacta. Glial cell line-derived neurotrophic factor (GDNF), being a potent survival-promoting agent for dopaminergic neurons, has long been researched as a therapeutical agent in the treatment of PD. So far, the success has been limited. Many of the obstacles associated with GDNF could be overcome by GDNF mimics, small molecules functionally similar to GDNF but with better pharmacological properties. The purpose of this graduate study was to characterize four potential small-molecule GDNF mimics developed in research group led by Academy Professor Mart Saarma at the Institute of Biotechnology, University of Helsinki.

GDNF's neurotrophicity is attributed to the activation of intracellular signalling pathways such as the MAPK (mitogen-activated protein kinase) and the PI3K (the phosphoinositide 3-kinase) pathway. GDNF activates these pathways through the GDNF receptor complex consisting of a transmembrane receptor tyrosine kinase RET and a co-receptor known as GDNF family receptor α1 (GFRα1). Weather the potential GDNF mimics activate the same intracellular signalling pathways through the GDNF receptor complex and weather or not the molecules have survival promoting effects on dopaminergic neurons were the key questions this graduate study addresses. The methods used include a luciferase reporter gene-based system in cells expressing the GDNF receptor complex (developed by Sidorova *et al*.) for monitoring MAPK activation, phosphorylation assays based on western blot to study activation of the aforementioned cellular components associated with GDNF and finally, a survival assay utilizing primary dopaminergic neurons to study survival promoting effects of the potential GDNF mimics.

Of the four potential GDNF mimics one was conclusively shown to possess functional qualities similar to those of GDNF. As for the other three, data acquired were promising yet insufficient to make such claims and further experiments are required.

Keywords	GDNF, Parkinson's disease

Tekijä(t) Otsikko Sivumäärä Aika	Leo Jakman GDNF:n kaltaisten pienimolekyylisten yhdisteiden karakterisointi 32 sivua 17.12.2012
Tutkinto	laboratorioanalyytikko (AMK)
Koulutusohjelma	laboratorioala
Ohjaajat	tutkijatohtori Yulia Sidorova koulutusvastaava Tiina Soininen

Parkinsonin tauti on keskushermoston rappeumasairaus, joka aiheuttaa pääasiassa motorisia oireita. Taudin suurin yksittäinen aiheuttaja on mustatumakkeen dopamiinia tuottavien hermosolujen rappeutuminen. GDNF (glial cell line-derived neurotrophic factor) on neurotrofinen tekijä, joka estää näiden dopaminergisten solujen kuolemaa ja jota on pitkään tutkittu sovellettavaksi Parkinsonin taudin hoidossa. GDNF soveltuu kuitenkin huonosti lääkeaineeksi, ja parempia tuloksia voitaisiin saavuttaa toiminnallisesti GDNF:n kaltaisilla pienimolekyylisillä yhdisteillä. Tämän opinnäytetyön tavoitteena oli karakterisoida neljä tällaista yhdistettä, jotka on kehitetty akatemiaprofessori Mart Saarman johtamassa tutkimusryhmässä Biotekniikan instituutissa, Helsingin yliopiston erillislaitoksessa.

GDNF:n neurotrofisuus perustuu solunsisäisen signalointireittien, kuten MAPK:n (mitogeenien aktoivoima proteiinikinaasi) ja PI3K:n (fosfoinositidi-3-kinaasi) aktivoimiseen. GDNF aktivoi nämä GDNF-reseptorikompleksin välityksellä, joka koostuu solukalvon läpäisevästä RET-tyrosiinikinaasireseptorista ja solukalvoon ankkuroidusta GFRα1-apureseptorista (GDNF family receptor α1). Tässä opinnäytetyössä sanalla karakterisointi tarkoitetaan sen selvittämistä, kykenevätkö tutkittavat yhdisteet aktivoimaan samoja solunsisäisiä signalointireittejä kuin GDNF GDNF-reseptorikompleksin kautta ja mikä tärkeintä, onko näillä yhdistellä dopaminergisten hermosolujen kuolemaa estävä vaikutus. Tärkeimmät tekniikat olivat lusiferaasireportterigeenianalyysi MAPK-signalointireitin ja western blot -analyysi sekä tämän että PI3K:n ja GDNF-reseptorikompleksin komponenttien aktivaation määrittämiseksi. Lopuksi määritettiin hermosolujen kuolemaa estävä vaikutus primäärisillä dopaminergisillä neuroneilla *in vitro*.

Neljästä pienimolekyylisestä yhdisteestä yhden voitiin osoittaa toimivan GDNF:n tavoin tutkittujen ominaisuuksien osalta. Kolmen muun yhdisteen kohdalla tutkimus on kesken ja GDNF:n kaltaisuus on toistaiseksi osoittamatta.

Avainsanat	GDNF, Parkinsonin tauti

Contents

Abbreviatons

AKT	A protein kinase in the PI3K pathway
ARTN	Artemin, a GFL
BSA	Bovine serum albumin
BT1x	BT13, BT16, BT17 and BT18, the potential small-molecule GDNF mimics under investigation
DA	Dopamine, dopaminergic
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ERK1/2	Extracellular signal-regulated kinase 1 (p44 MAPK) and 2 (p42 MAPK) in the MAPK pathway
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor, a GFL
GFL	GDNF family ligand, see ARTN, GDNF, NRTN and PSPN
GFP	Green fluorescent protein
GFRα1-4	GDNF family receptor α 1, 2, 3 or 4
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, a buffer
MAPK	Mitogen-activated protein kinase (such as ERK1/p44 MAPK and ERK2/p42 MAPK) and the name of the intracellular signalling pathway
MW	Molecular weight
NRTN	Neurturin, a GFL
PBS	Phosphate buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase and the name of the intracellular signalling pathway
PSPN	Persephin, a GFL
RET	A receptor tyrosine kinase, from **RE**arranged during **T**ransfection
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline

TBST	A buffer containing 50 mM Tris-HCl (pH 7,4) 150 mM NaCl and 0,15 % Tween 20
TBSTX	A buffer containing 50 mM Tris-HCl (pH 7,4) 150 mM NaCl and 0,4 % Triton X-100
TH	Tyrosine hydroxylase, a marker for dopaminergic neurons
UPDRS	Unified Parkinson's Disease Rating Scale
WB	Western blot

1 Introduction

Several diseases such as Parkinson's, Alzheimer's and Huntington's occur as a result of progressive neurodegenerative processes in which neurons lose structure, function and as a result, die. The mechanisms underlying neurodegeneration are not well understood and existing therapies offer only symptomatic relief – known treatments do not reverse, stop or even slow down neuronal degeneration. Neurotrophic factors, a class of proteins crucial for the development and maintenance of neurons, promise to change this. Efforts are being made to harness the protective and restorative properties of neurotrophic factors in an attempt to battle neurodegeneration. One of the teams working in this field is the research group led by Academy Professor Mart Saarma in the Institute of Biotechnology at the University of Helsinki. This graduate study is based on experiments conducted as a member of this group during the year 2011 under the supervision of Dr. Yulia Sidorova.

One of the most promising candidates for the treatment of Parkinson's disease is the glial-cell-line-derived neurotrophic factor (GDNF). GDNF has been shown to promote survival and sprouting of dopaminergic neurons – the neuronal subtype that degenerates and dies in Parkinson's disease – both *in vitro* and *in vivo*. However, GDNF is far from an ideal pharmacological agent. Firstly, GDNF or cells producing GDNF must be delivered straight into a patient's brain through a surgical procedure. More importantly, the protein migrates poorly from the area of injection or production and does not necessarily reach its target cells. These and a number of other problems stem from the fact that GDNF is a protein and could very well be solved through the development of small molecules functionally similar to GDNF.

The purpose of this graduate study was the *in vitro* characterization of four synthetic molecules developed by the group of Prof. Saarma in hopes of establishing functional similarities between the four and GDNF. Utilizing cells expressing parts of, or the complete, GDNF receptor complex, three approaches outlined in chapter 3 were used.

2 Review of the literature

This section covers the key concepts necessary for understanding the work about to be presented and hopefully answers the questions why this research was conducted and why it was conducted in a specific way. In essence, the section describes the human movement and why it is compromised in Parkinson's disease, how GDNF and other neurotrophic factors factor in and why there is a need for small-molecule mimics.

2.1 The basal ganglia

Human movement is controlled by neural circuits that can be viewed as four distinct, interconnected subsystems. The first consists of the lower motor neurons and the local circuit neurons in the spinal cord and the brainstem. The local circuit neurons contribute to motor activity by controlling the lower motor neurons that, in turn, connect directly to musculature. Both the local circuit neurons and the lower motor neurons (although more rarely) are controlled by upper motor neurons that comprise the second subsystem. These neurons are found in the brainstem and the cerebral cortex and are essential for initiating voluntary movements. The upper motor neurons are further regulated by the third and fourth subsystems that are the cerebellum and basal ganglia. [1.]

The basal ganglia refer to a group of nuclei lying deep within the brain. The nuclei relevant for motor control include the corpus striatum and the globus pallidus at the base of the forebrain, the subthalamic nucleus in the ventral thalamus and the substantia nigra at the base of the midbrain (Figure 1). The corpus striatum, consisting of the caudate and the putamen, serves as an input to the basal ganglia. These structures receive projections from different regions of the brain, especially from the cerebral cortex. The way the inputs are organized in respect to the morphology of the striatal neurons suggests that the corpus striatum acts to integrate incoming signals. Further convergence is evident in the pallidum (the globus pallidus and the pars reticulata subdivision of the substantia nigra), where the corpus striatum itself projects. The pallidum is considered the output of the basal ganglia and connects eventually to the upper motor neurons. There is functionally important internal circuitry governing the output of the basal ganglia which is not described for the reason of relevance. [1.]

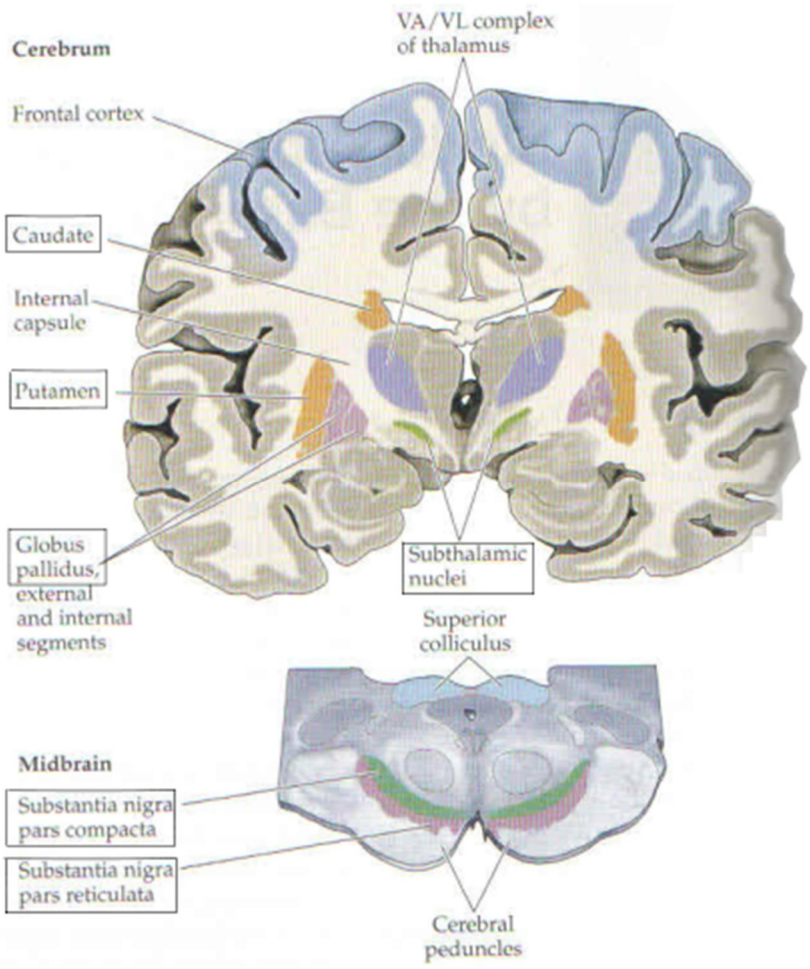

Figure 1. Left: Motor components of the human basal ganglia: the caudate and the putamen (the corpus striatum), the globus pallidus and the substantia nigra pars reticulata (the pallidum), the substantia nigra pars compacta and the subthalamic nucleus [1]. Right:

The main output of the basal ganglia is inhibitory. When excited, the corpus striatum inhibits the tonically active inhibitory cells in the pallidum which allows other inputs to excite the upper motor neurons. In a way, the basal ganglia circuitry acts as a gate that blocks unwanted movement. Huntington's disease is a hyperkinetic movement disorder in which striatal projections degenerate. The result is a reduction in the inhibitory outflow of the basal ganglia. This allows the upper motor neurons to become more easily activated and leads to the unwanted movement characteristic of Huntington's disease. In Parkinson's disease however the cellular population most severely affected are the dopaminergic neurons in the substantia nigra pars compacta. Dopamine provided by the substantia nigra pars compacta modulates the excitability of striatal neurons by cortical inputs. When nigral dopaminergic neurons degenerate, the striatum becomes less responsive to excitatory inputs from the cortex. Unlike in

Huntington's disease, the parkinsonian brain is characterized by an increase in the in-inhibitory outflow of the basal ganglia. Indeed, Parkinson's disease and associated movement disorders are hypokinetic. [1.]

2.2 Parkinson's disease

Parkinson's disease (PD) is the second most common progressive degenerative disorder of the central nervous system. The disorder is age-related with 85 % of newly diagnosed cases occurring in people over the age of 65 [2]. According to the Finnish Parkinson Association PD is fairly common in Finland with the incidence of 1-2/1000 on the population level and from over 70-year-olds as many as 2 % are affected. The significance of Parkinson's disease is increasing as is many parts of the world the population is aging.

PD is characterized by the impairment of motor function. The set of motor symptoms caused by the disease are collectively referred to as parkinsonism and include tremor at rest, rigidity, postural instability and difficulty in initiating and executing voluntary movement (akinesia, bradykinesia). Even though the most prominent signs of PD are motor, significant nonmotor symptoms commonly develop. These include behavioral and mental alterations (fatigue, depression, anxiety), cognitive deterioration to the point of dementia, sleep disturbances and dysfunctions in the autonomic and sensory nervous systems. PD is diagnosed chiefly on the basis of the clinical features and is commonly evaluated using the Unified Parkinson's Disease Rating Scale (UPDRS). [3.]

The vast majority of PD cases are idiopathic in etiology i.e. the cause is unknown. Enviromental toxins have been suggested as a causative agent. Indeed, chemicals such as 1,2,5,6-tetrahydropyridine (MPTP) and rotenone (a pesticide) have been linked with the development of PD. The remaining 5-10 % of PD cases are estimated to be caused by inheritable genetic mutations and often lead to an atypical clinical picture. Study of genetics behind PD has shed some light to pathological mechanisms underlying the disorder. These include disruptions in the proteolytic ubiquitin-proteasome system, oxidative stress and mitochondrial dysfunction. Buildup of toxic proteins has gained attention due to the fact that most PD patients develop intracytoplasmic eosinophilic inclusions called Lewy bodies the main component of which is α-synuclein. In fact, a mutation in the gene encoding α-synuclein was the first to be linked to parkinsonism. More research is needed to determine what causes the

majority of PD cases, whether it is the genetic or environmental factors, what they are and how they work together to induce pathogenesis. [2.]

Most prominent but by means not the only pathological feature of PD is the degeneration and death of dopaminergic (DA) neurons in the substantia nigra pars compacta found at the base of the midbrain. The resulting disruption of the nigrostriatal pathway and the subsequent deficiency of striatal DA are accountable for most of the motor symptoms. These symptoms manifest when the putaminal dopamine content has decreased 80 % (corresponds to 60 % loss of nigral DA neurons). Until the onset of symptoms dopamine deficiency is compensated by supersensitization of DA receptors. In addition to neuronal loss there is an increase in the number of nigral glial cells and loss of neuromelanin that gives substantia nigra its dark color. [3.]

As the cause underlying degeneration and death of dopaminergic neurons in PD is unknown, current treatments focus on symptomatic alleviation. This is achieved mainly through the use of levodopa, a dopamine precursor that is converted into dopamine in the central nervous system. Levodopa has revolutionized PD treatment but eventually causes severe side effects such as response fluctuations and dyskinesias (drug-induced involuntary movements). Other alternatives include DA agonists, dopamine releasers and surgical procedures such as lesioning and deep brain stimulation [3]. The efficacy of the latter two is based on successfully blocking the abnormal, pathological activity in affected neural circuits. [4]. Attempts to implant dopamine-producing cells or to regenerate the remaining dopaminergic neurons using neurotrophic factors offer to revolutionize the treatment of PD but are at least for now far away from clinical practice.

2.3 Neurotrophic factors

Neuronal survival, growth and differentiation are dependent on trophic support. Neurons receive this from the targets they innervate in the form of signaling molecules known as neurotrophic factors. [1.] These small secretory proteins are currently classified into four families: the neurotrophins, the neurokines (neurotrophic cytokines), the glial-cell-line-derived neurotrophic factor (GDNF) family ligands (GFLs) and the recently discovered family comprising cerebral dopamine neurotrophic factor (CDNF) and mesencephalic-astrocyte-derived neurotrophic factor (MANF). [5.]

The GDNF family of ligands consists of four members: GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). All four GFLs have survival promoting effects on several types of neurons including dopaminergic neurons. Other clinically important neuronal populations supported by one or more GFLs include motor and sensory neurons that are affected in motor neuron diseases and neuropathic pain, respectively. Knockout studies have shown GFLs are important also in the developing nervous system. Mice lacking GDNF, NRTN or their receptors show defects in the enteric and parasympathetic innervation. ARTN seems to be required for the development of sympathetic neurons. GDNF also has functions outside the nervous system in kidney development and spermatogenesis. [6.]

Neurotrophic effects of GFLs are mediated by a multicomponent cell surface receptor complex consisting of a signal-transducing and a ligand-binding unit. The signaling unit is a transmembrane receptor tyrosine kinase known as RET (from REarranged during Transfection). RET cannot bind a GFL directly. For this it needs a co-receptor (i.e. the ligand-binding unit) which is one of the four GDNF family receptor α proteins (GFRα1-4). GDNF has the highest affinity to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα4 (Figure 2). There is significant crosstalk, however. [6.] GDNF can bind to GFRα2 and GFRα3 and all GFLs are able to bind to GFRα1 [7].

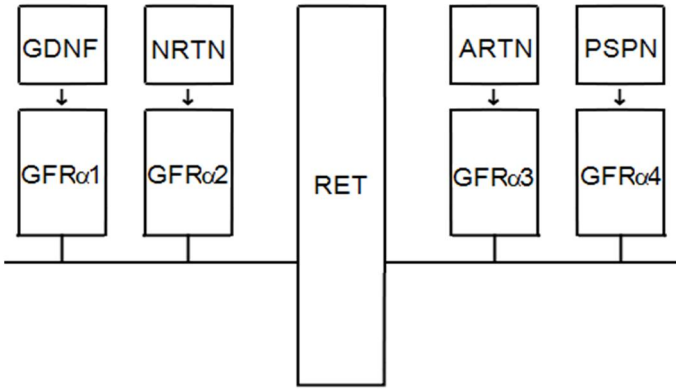

Figure 2. Components of the GFL receptor complex: the signal-transducing, transmembrane receptor tyrosine kinase RET shared by all GFLs and the ligand-binding co-receptors GFRa1-4 preferred by GDNF, NRTN, ARTN and PSPN, respectively. Crosstalk not shown.

As GFLs are homodimers, an active receptor complex consists of two units of RET associated with two units of GFRα receptors that bind one GFL molecule. It is unclear

whether a GFL is what allows the complex to assemble or if it merely stabilizes a pre-preformed GFRα-RET complex [8]. In either case, a functional GFL-GFRα-RET complex leads to transphosphorylation in the intracellular tyrosine kinase domains of the two RET units. Resulting conformational changes promote activation of several intracellular signaling cascades. [6.]

Neurotrophic properties of GDNF have been attributed to its activation of the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways [9] through the GDNF receptor complex (Figure 3). The PI3K cascade has been shown to promote cell survival in midbrain dopaminergic neurons, an effect that could be abolished by blocking this pathway [10]. Sustained and transient activation of the MAPK pathway have been linked to neuronal differentiation and proliferation, respectively [11].

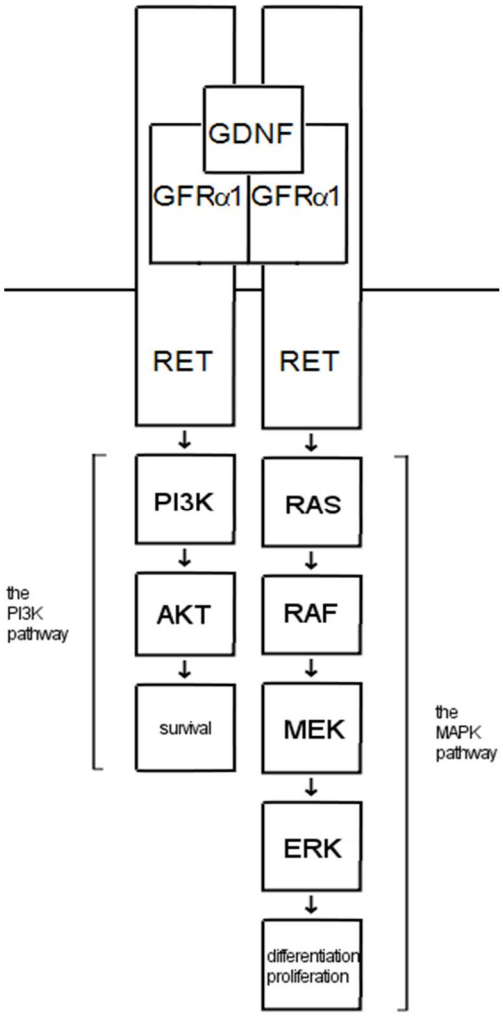

Figure 3. A simplified representation of two intracellular signaling pathways, MAPK and PI3K, activated by a functional GDNF receptor complex.

2.4 Neurotrophic factors - a cure for Parkinson's disease?

GDNF and other GFLs are known for their ability to support dopaminergic neurons. ARTN and PSPN have demonstrated neuroprotective effects in animal models of Parkinson's disease [12, 13]. GDNF has shown not only neuroprotective but also neurorestorative effects in several studies [14] as has NRTN [15, 16]. The onset of symptoms in Parkinson's disease can be attributed to the decreased dopamine content in the striatum. Interestingly from a therapeutic standpoint, initial striatal dopamine depletion is greater than suggested by the number of viable dopaminergic neurons left [17, 18]. This could indicate there is a loss of dopaminergic phenotype prior to cell death, something that could possibly be augmented using neurotrophic factors.

GDNF and NRTN have been subjected to clinical trial. Two small, open-label studies in which GDNF was infused directly into the putamen of PD patients showed promising results [19, 20, 21, 22]. More recently however, a randomized, double-blind phase II clinical trial failed to demonstrate a significant improvement in GDNF-treated patients compared to the placebo group [23]. However, the study has received a fair amount of criticism regarding the delivery strategy and the age of the patient population [24]. Moreover, whether the study had sufficient statistical power to detect the benefits of GDNF has been questioned [25, 26]. NRTN failed to show significant benefit in a clinical trial evaluated using the UPDRS motor score [27]. However, a benefit was seen 15 and 18 months post treatment while the endpoint in the study was 12 months. Based on what was learned, another study is on its way with a higher dosage, different delivery strategy and more statistical power to demonstrate efficacy.

The therapeutic potential of GDNF in treatment of PD is undermined by several issues, most importantly bioavailability. GDNF is unable to cross the blood-brain barrier and requires surgery for it to be delivered successfully into a patient's brain. Moreover, affinity to heparan sulfate proteoglycans in the extra-cellular matrix restricts diffusion of GDNF from the site of administration or secretion making it unable to reach the target, i.e. the dopaminergic neurons in substantia nigra pars compacta. Aside from bioavailability, there are several other issues to consider most of which arise from the fact that GDNF is produced as a recombinant protein in bacteria followed by modifications that render the protein biologically active. Not only is the practice expensive compared to manufacture of traditional pharmaceuticals, the properties of the product can also vary between batches. As for side-effects, recombinant GDNF has

the potential to induce inflammation and the formation of anti-GDNF antibodies. More-Moreover, RET is not the only receptor GDNF is able to signal through. GDNF can also activate the neural cell adhesion molecule and syndecan glycoproteins. [8.]

2.5 GDNF mimetics as an alternative to GDNF

An alternative to GDNF therapy is being pursued. Research group led by Academy Professor Mart Saarma is striving to develop small-molecule GDNF mimics, i.e. molecules functionally similar to GDNF. These low molecular weight GDNF receptor complex agonists would be able to cross the blood-brain barrier thus eliminating the need for surgery that is needed for successful GDNF delivery. Moreover, these molecules could be produced at a lower cost, with less variation between batches and with better pharmacological properties (in addition to molecular size and permeability) compared to GDNF. Finally, a GDNF mimic could be more specific than GDNF itself, targeting only RET through GFRα1. [8.]

The fact that GDNF is a dimer and signals through a multi-component receptor complex poses a question whether a small monovalent molecule can act similarly. Many receptor tyrosine kinases are thought to be activated when an extracellular bivalent ligand brings two receptor monomers together allowing the effective phosphorylation of the intracellular tyrosine kinase domains. It is also possible that the ligand merely induces a phosphorylation-promoting conformational change or stabilization in an inactive or partially active receptor dimer (or in the case of the GDNF receptor complex, a $(GFR\alpha)_2(RET)_2$ tetramer). Small molecules could act similarly by engaging so-called “hot spots” in preformed receptor complexes. [8.] This certainly seems plausible as a study showed that a GFRα1-binding monovalent small molecule can activate the GDNF receptor complex [28].

As discussed previously GDNF exerts its neurotrophic effects via the GDNF receptor complex by activating several intracellular signalling pathways, particularly the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. In order to find small-molecule alternatives to GDNF, thousands of chemicals were subjected to high-throughput screening using cell lines expressing components of the GDNF receptor complex and a luciferase-reporter-gene-based system for monitoring MAPK activation. Several molecules able to activate luciferase expression were identified. Based on the structures of these molecules a library of

similar compounds was designed *in silico*. This focused library was subsequently syn-synthesized and tested using the same reporter-gene-based method. Finally, four molecules (named BT13, BT16, BT17 and BT18) belonging to the focused library were found to activate luciferase expression. This graduate study focuses on the characterization of these four compounds to study potential functional similarities they share with GDNF.

3 Materials and methods

This chapter describes the methods used to achieve the aim of this graduate study, the characterization of four potential small-molecule GDNF mimics (BT13, BT16, BT17 and BT18). For clarity the methods (Table 1) were given short titles and are hereafter referred to as the luciferase assay (I), phosphorylation assay (II-a and II-b) and survival assay (III).

Table 1. An outline of methods used and the research questions they address.

		Method(s)	Cells	Research question	Variables
I	Luciferase assay	Luciferase reporter gene-based assay	StratαLUC, Nostratα	Compound-induced MAPK activation	GFRα1/no GFRα1, dose
IIa	Phosphorylation assay: ERK1/2 and AKT	Western blot	MG87RET + GFRα1	Compound-induced activation of ERK1/2 (MAPK pathway) and AKT (PI3K pathway)	Time, dose
IIb	Phosphorylation assay: RET	Immunoprecipitation, western blot	MG87RET + GFRα1	Compound-induced activation of RET	GFRα1/no GFRα1
III	Survival assay	Immunohistochemistry, computer-aided quantification	Primary dopaminergic neurons	Survial-prmoting effects of compounds	-

First, the reporter cells used to discover the four compounds were used to study dose-dependence in the compound-induced, MAPK-activation-associated luciferase expression. Also, the question was addressed whether GFRα1, the component of the

GDNF receptor complex, is required for the compounds' biological effect on reporter cell lines (I). Then, the compounds' ability to promote activation of the GDNF receptor complex and its downstream targets MAPK and PI3K was studied using direct phosphorylation assays. Activation of MAPK and PI3K pathways was investigated using antibodies specific to phosphorylated, i.e. active, forms of ERK1/2 and AKT – components of the MAPK and PI3K signalling cascades, respectively (IIa). Activation of the GDNF receptor complex was examined by immunoprecipitating its component RET and using immunoblot to determine the degree of phosphorylation (i.e. activation). In addition, requirement of the GFRα1 for compound-induced RET activation was investigated (IIb). Finally, the ability of the compounds to promote survival of primary embryonic midbrain dopaminergic neurons *in vitro* was tested using a computer-aided method based on tyrosine hydroxylase specific antibodies (a well-established marker for dopaminergic neurons) (III).

3.1 Materials and cell lines

Recombinant human GDNF was obtained from Amgen. Compounds BT13, BT16, BT17 and BT18 were supplied by collaborators.

All established cell lines used were derivatives of an MG87RET cell line. These murine fibroblasts stably express RET (long isoform) but not GFRα1. To increase the solubility of the compounds, the media contained 1 % dimethyl sulfoxide (DMSO) in analyses using established cell lines (luciferase and phosphorylation assays) or 0,1 % DMSO with primary cells (survival assay).

3.2 Luciferase assay

Luciferase assay was developed by Sidorova *et al*. to study GFL signaling through the GDNF receptor complex. The assay features two cell lines expressing the PathDetect ELK1 trans-reporting system (Stratagene) for monitoring MAPK pathway activation. In addition, the first cell line (StratαLUC) expresses both components of the GDNF receptor complex, human GFRα1 and long isoform of RET. The second cell line (NOstratα) expresses only RET. Both cell lines respond to GDNF (NOstratα only in the presence of soluble GFRα1). NOstratα cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS), 15 mM HEPES

(pH 7,2), 500 µg/ml Geneticin (Invitrogen), 100 µg/ml Normocin (InvivoGen) and 2 µg/ml puromycin. StratαLUC cells were cultured in NOstratα medium supplemented with 2 µg/ml blasticidin S.

PathDetect ELK1 trans-reporting system is based on two plasmids (Figure 4). The first plasmid constitutively expresses a fusion protein that consists of the activation domain of ELK1 transcription activator and the DNA binding domain (DBD) of another transcription activator, GAL4. The second plasmid contains GAL4 binding sites that control the expression of *Photinus pyralis* luciferase gene. Phosphorylation of ELK1 activation domain by ERK, component of the MAPK pathway, drives the interaction between the GAL4 DBD and its binding sites in the reporter plasmid leading to luciferase espression. Luciferase catalyzes a reaction that produces luminescence intensity of which correlates with the degree of MAPK pathway activation.

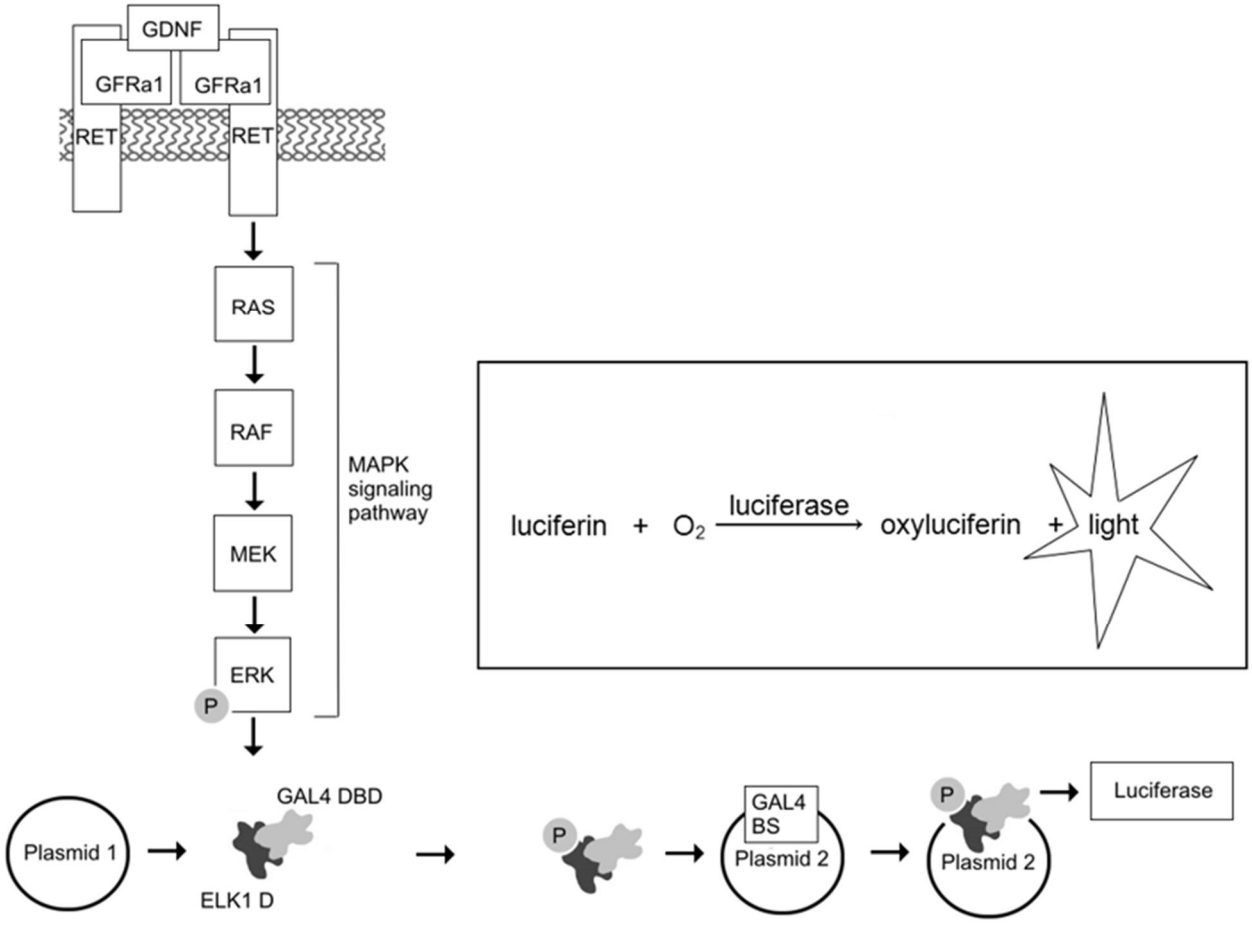

Figure 4. Principle of the luciferase assay. Phosphorylation of the fusion protein expressed by plasmid 1 allows its DNA binding domain (DBD) to interact with GAL4 binding sites in plasmid 2 leading to luciferase expression. Luciferase catalyses the oxidation of luciferin – a reaction that produces light. Adapted from Sidorova et al. [7].

The conditions in which compounds' ability to activate luciferase expression was tested vary slightly. To test BT13 and BT16 cells were seeded in medium containing no selective antibiotics as is recommended (DMEM containing 10 % FBS, 15 mM HEPES, pH 7,2 and 1 % DMSO). With BT17 and BT18 cells were grown overnight in their respective maintenance mediums (see above) which was replaced 1-2 h prior to proceeding with the analysis.

When analysing compounds BT13 and BT16, StratαLUC and Nostratα cells were seeded in 20 000 cells/well density on 96-well cell culture plates (Greiner Bio-One) in DMEM containing 10 % FBS, 15 mM HEPES (pH 7,2) and 1 % DMSO. On the following day medium was replaced with DMEM containing 15 mM HEPES, 1 % DMSO (negative control) and compounds BT13, BT16 (1-50 µM) or GDNF (25 ng/ml, positive control).

To analyse BT17 and BT18, cells were seeded in maintenance medium (containing selective antibiotics) which was replaced on the following day with DMEM containing 10 % FBS, 15 mM HEPES (pH 7,2) and 1 % dimethyl sulfoxide (DMSO). Half of this medium was replaced with DMEM containing 15 mM HEPES, 1 % DMSO (negative control) and compounds BT17, BT18 (1-50 µM) or GDNF (50 ng/ml).

Cells were incubated overnight in a humidified 5 % CO_2 athomsphere at +37 °C and lysed with 20 µl/well Passive Lysis Buffer (Promega) on a shaker at RT for 15 min. 10 µl of lysate and 20 µl of Luciferase Assay Substrate (Promega) were mixed on Black IsoPlates (PerkinElmer) on ice. A 1450 MicroBeta luminescence counter (PerkinElmer) was used to measure luminescence (RT). Results from the second run were used. Data are presented as M ± m where M represents the average of at least three repeats and m the standard deviation.

3.3 Phosphorylation assays

Phosporylation assays regarding AKT, ERK1/2 (of the PI3K and MAPK pathways, respectively) and RET were conducted using the MG87RET cell line maintained in DMEM supplemented with 10 % FBS, 100 µg/ml Normocin and 2 µg/ml puromycin and transfected with human GFRα1 prior to analyses.

Assaying phosphorylation of a given protein can be accomplished using antibodies specific to the phosphorylated form of this protein. Thus, it is important to preserve the protein in a phosphorylated state. This was accomplished with a combination of ice and phosphatase inhibitors (sodium fluoride or NaF, sodium orthovanadate or Na_3VO_4) to slow down reactions and to block phosphatase activity, respectively.

The proteins were then resolved and visualized using western blot. To be able to determinate phosphorylation induced by compounds or even GDNF, the overall protein content in the lysates must be similar. To monitor this, a protein known as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was stained. The gene encoding GAPDH is considered a housekeeping gene and is stably and constitutively expressed in most tissues making is suitable for this application [29].

In order to investigate RET phosphorylation, it was necessary increase its concentration through immunoprecipitation. Precipitation is achieved with the help of Sepharose beads covalently coupled with protein G. Protein G binds immunoglobulins, in this case anti-RET antibodies, and allows RET to precipitate along with the heavy, insoluble Sepharose.

3.3.1 Phosphorylation assay: ERK1/2 and AKT

MG87RET cells were seeded 100 000-120 000 cells/well on 12-well cell culture plates (Greiner Bio-One) in DMEM containing 10 % FBS and 100 µg/ml Normocin. Cells were grown overnight and transfected with human GFRα1 (or green fluorescent protein, GFP for transfection control) using Lipofectamine 2000 (Invitrogen). The Lipofectamine reagent and DNA were diluted in Opti-MEM I Reduced Serum Media (Invitrogen) and combined according to manufacturer's instructions. Cells were incubated with DNA-Lipofectamine mixture diluted 1:5 in DMEM containing 10 % FBS and 100 µg/ml Normocin for 4-5 h. After this the transfection mixture was replaced with DMEM containing 10 % FBS andn100 µg/ml Normocin (unless otherwise stated).

Expression of GFP was confirmed on the following day using fluorescent microscopy (Figure 5). Cells were starved for 4-5 h in serum-free DMEM containing 15 mM HEPES and 1 % DMSO (negative control) and treated with compounds (1-50 µM) or GDNF (50-100 ng/ml, positive control) for 1-90 min (unless otherwise stated). Immediately

after, the cells were placed on ice and washed with ice-cold PBS containing 1 mM NaF and 1 mM Na_3VO_4. The cells were then suspended in 100 µl/well 2x Laemmli buffer, boiled for 10 min and stored in -20 °C.

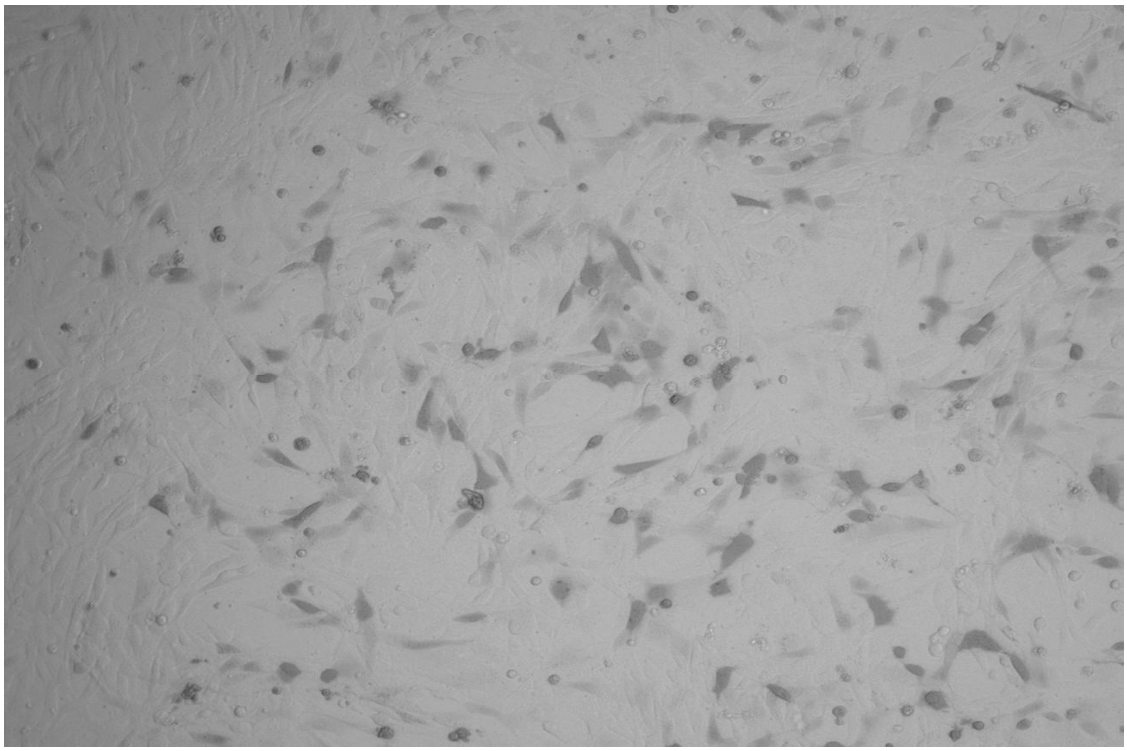

Figure 5. Green fluorescent protein (GFP) was used as a transfection control and to estimate transfection efficiency. Fluorescent MG87RET cells (darker) expressing GFP superimposed over cells under visual light.

For western blot, samples were first boiled for 2 min and centrifuged (16 000 rcf, 2 min). Proteins were separated using 12 % SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), transferred onto a nitrocellulose membrane, blocked with 10 % non-fat milk in TBST (50 mM Tris-HCl, pH 7,4, 150 mM NaCl, 0,15 % Tween 20) for 15 min at RT and incubated overnight at +4 °C with rabbit antibodies against phospho-AKT (Cell Signaling) or phospho-ERK1/2 (Cell Signaling, 4695) diluted 1:1000 in TBST containing 5 % BSA (bovine serum albumin). Membranes were washed with TBST and incubated for 45 min at RT with horseradish peroxidase or HRP-conjugated anti-rabbit antibodies (Amersham) diluted 1:3000 in TBST containing 3 % milk. Membranes were washed three times for 15 min at RT with TBST and proteins were visualized using Pierce ECL substrate (Thermo Scientific) or, if necessary, with SuperSignal ELISA Femto substrate (Thermo scientific). Images were taken using LAS-3000 imager (Fujifilm).

To confirm equal loading, membranes were stripped in buffer containing 62,6 mM Tris-HCl pH 6,7, 2 % SDS and 0,2 % 2-mercaptoethanol for 20 min at 60 °C, rinsed with PBS (phosphate buffered saline) and washed 3 times for 10 min with TBST. Membranes were blocked as previously described, incubated for 1 h at RT or overnight at +4 °C with mouse antibodies against GAPDH (Millipore) diluted 1:2000 - 1:6000 in TBST containing 3 % milk. Membranes were washed, incubated for 45 min at RT with HRP-conjugated antibodies against mouse (Dako). Membranes were washed and proteins visualized as described above.

3.3.2 Phosphorylation assay: RET

MG87RET cells were seeded 100 000-125 000 cells/well on 6-well cell culture plates (Greiner Bio-One) in DMEM containing 10 % FBS and 100 µg/ml Normocin. Cells were transfected on the following day with human GFRα1 or GFP using Lipofectamine 2000 (Invitrogen) as described earlier in chapter 3.3.1. Cells were starved for 4-5 h in serum-free DMEM containing 15 mM HEPES and 1 % DMSO (negative control) before stimulating with compounds BT13 and BT18 (5-50 µM) or GDNF (100 ng/ml, positive control) for 15 min.

Cells were placed on ice, washed with ice-cold PBS containing 1 mM NaF and 1 mM Na_3VO_4 and lysed for approximately 1 h on a shaker with 1 ml/well modified RIPA (radioimmunoprecipitation assay) buffer (10 % glycerol, 1 % Triton X-100, 1 % NP-40, 0,25 % Na-deoxycholate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, Mini Protease Inhibitor Coctail, Roche). Cell debris was precipitated by centrifugation (16 000 rcf, 10 min) and the lysate was incubated on a vertical rotator overnight at +4 °C with 1 µg/ml goat anti-RET (long isoform) antibodies (Santa Cruz Biotechnology) and 40 µl 1:1 protein G Sepharose (Invitrogen) – modified RIPA buffer mixture. Beads were washed three times (2 500 rcf, 5 min) with ice-cold TBS containing 1 % Triton X-100. Samples were boiled for 10 min in 100 µl/sample 2x Laemmli buffer and stored in -20 C.

For western blot, samples were boiled and centrifuged as described in chapter 3.3.1 before resolving proteins using 7,5 % SDS-PAGE. After blotting and blocking (also described in chapter 3.3.1), membranes were incubated overnight at +4 °C or 2 h at RT with mouse antibodies against phosphotyrosine (Millipore) diluted 1:1000 in TBST containing 3 % milk, washed and incubated for 45 min at RT with HRP-conjugated anti-

mouse antibodies (Dako). Membranes were washed, proteins visualized and mem-membranes then stripped, washed and blocked as described in chapter 3.3.1.

RET was stained as a loading control. Membranes were incubated overnight at +4 °C or 2 h at RT with goat anti-RET (long isoform) antibodies (Santa Cruz Biotechnology) diluted 1:500 in TBST containing 3 % milk, washed and incubated for 45 min with anti-goat antibodies (Daco) diluted 1:1000 in TBST containing 3 % milk. Membranes were washed and proteins visualized as described in chapter 3.3.1.

3.4 Survival assay

Preparation of plates, isolation and plating of midbrain neurons was performed by Zheng Congjun in accordance with a method developed by Dr. Andressoo [30]. 3 mm^2 microislands on 4-well plates (Nunc) were coated with 1 mg/ml poly-L-ornithine (Sigma) in 0,15 M borate buffer (pH 8,7), washed with PBS and air-dried.

3.4.1 Isolation and plating of midbrain cells

Approximately 1 mm^2 of the midbrain floor was extracted from E13.5 NMRI mice in Dulbecco's PBS at RT and washed 3 times with Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS). The tissue was incubated with 0,5 % trypsin in Ca^{2+}/Mg^{2+}-free HBSS for 20 min at 37 °C before adding 50 % FBS and 2 mg/ml DNAse (Roche). Cells were dissociated by pipetting, washed twice (1200 rcf, 5 min) with culture medium (Neurobasal Medium, Invitrogen, supplemented with B-27, Invitrogen, 2 mM L-glutamine and 100 µg/ml Primocin, InvivoGen). 24 000 cells were plated per microisland in 4 µl of culture medium. Cells were allowed to attach for 1-2 h before the addition on 500 µl of culture medium containing 0,1 % DMSO (negative control) and compounds (0,003-2 µM) or GDNF (30 ng/ml, positive control).

3.4.2 Tyrosine hydroxylase staining

Dopaminergic neurons were visualized using antibodies against tyrosine hydroxylase (TH) on 5th day *in vitro*. Cells were fixed with 4 % paraformaldehyde (PFA) in PBS for 15 min at RT, washed three times with PBS and permeabilized with TBSTX (50 mM Tris-HCl, pH 7,4, 150 mM NaCl, 0,4 % Triton X-100) three times for 15 min. After blocking for 1 h at RT with TBSTX containing 10 % horse serum, cells were incubated overnight with sheep antibodies against tyrosine hydroxylase (Chemicon) diluted 1:500 in TBSTX containing 1 % horse serum. Cells were washed three times with TBSTX and

incubated for 1 h at RT with Cy3-conjugated antibodies against sheep immunoglobulins (Jackson Immunoresearch) diluted 1:500 in TBSTX containing 1 % horse serum. Cells were washed with TBSTX for 15 min three times, once with PBS and once with H_2O before mounting in Gelvatol mounting medium.

3.4.3 Quantification of TH-positive cells

Microislands (Figure 6) were photographed with the Lumar V12 fluorescence stereomicroscope using a Cy3 filter (Zeiss) . Non-specific staining on the edges of the microislands and major distortions such as those caused by air bubbles were manually deleted using ImageJ software (version 1.43u).

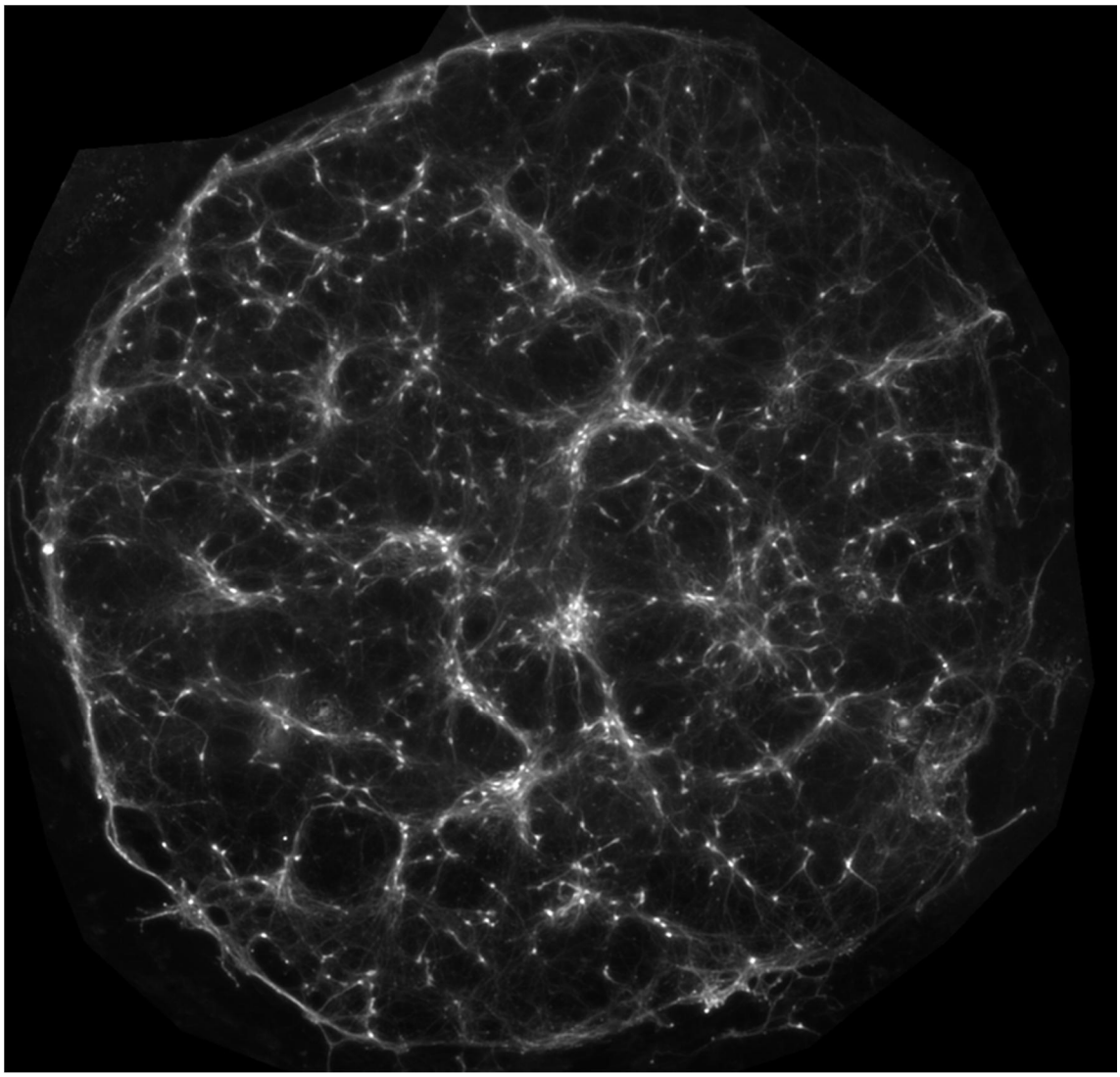

Figure 6. A microisland under fluerescent light showing TH-positive cells.

TH-positive cells were counted using Image-Pro Plus (version 5.1.2.59, Media Cybernetics) in a blinded manner so that the quantification data were matched with

sample information only after completion of the counting. Counting was based on pixel intensity and size which were manually adjusted to create objects matching cell bodies (Figure 7).

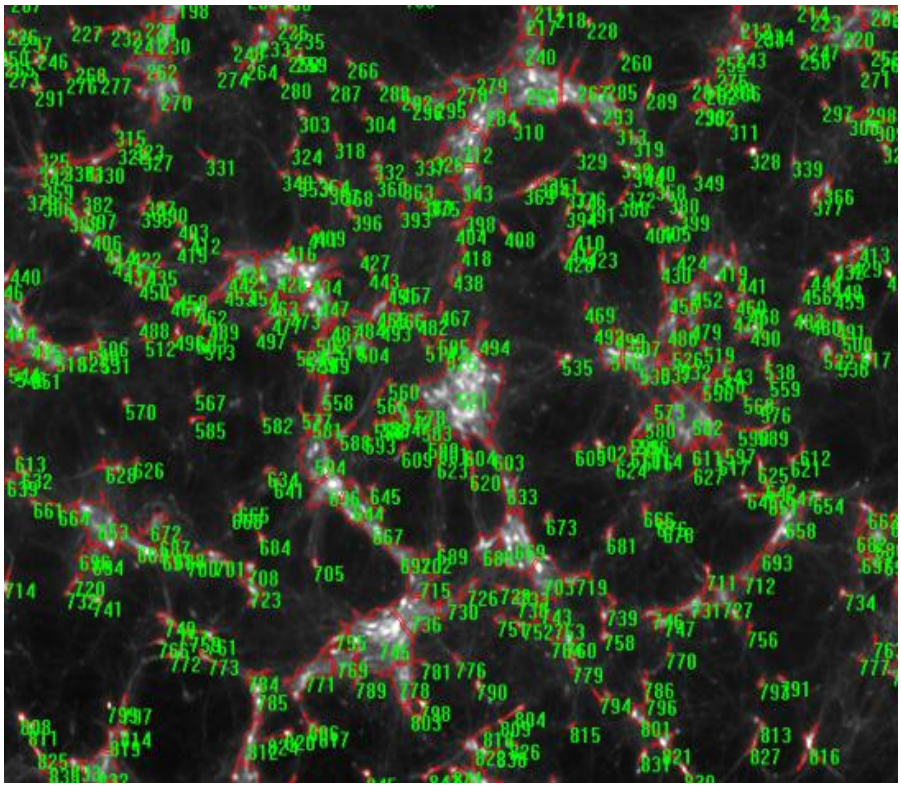

Figure 7. Computer-aided count of TH-positive cells after manual adjustments.

Effect on survival was determined using paired Student's t-test ($p < 0,05$). Results from three individual experiments with a minimum of four repeats per treatment group were used.

4 Results and discussion

The purpose of this graduate study was to characterize four synthetic molecules potentially functionally similar to GDNF. These compounds (BT13, BT16, BT17 and BT18) were developed the research group led by Academy Professor Mart Saarma using reporter cell lines (StratαLUC, NOstratα) described in detail in chapter 3.2. The first phase in this study was to confirm that the compounds do indeed induce the same cellular response in the reporter cells as GDNF does and do so dose-dependently. To do this, cells expressing the complete GDNF receptor complex i.e. RET and GFRα1 (the StratαLUC cell line) together with the luciferase reporter gene-based MAPK pathway

monitoring system were used to study compounds' ability to promote MAPK activation. In addition, cells expressing the reporter gene system and RET only (NOstratα cell line) were used to investigate whether or not compound-induced activation requires the presence of GFRα1.

4.1 Luciferase assay

All four compounds activated luciferase expression dose-dependently both in StratαLUC and in NOstratα cells (Figure 8). This suggests that GFRα1 is not necessary for compound-induced activation of luciferase expression. However, the presence of GFRα1 appears to increase the response significantly.

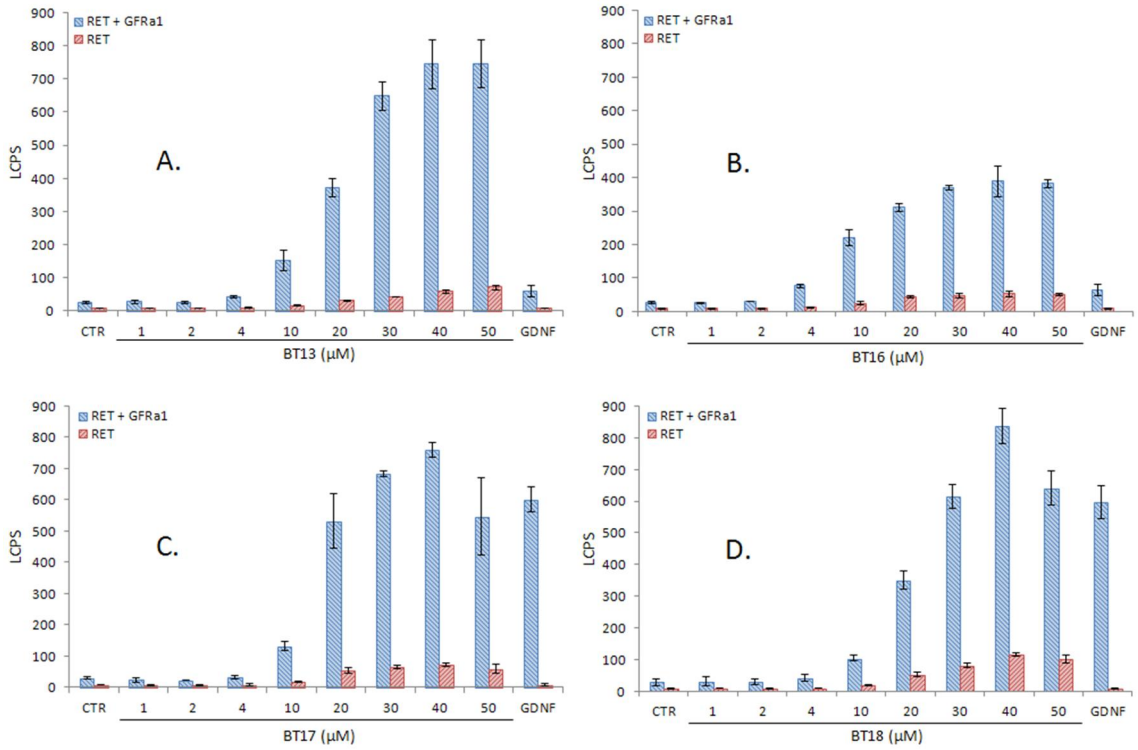

Figure 8. Luciferase assay shows dose-dependent activation of luciferase expression by compounds BT13 (A), BT16 (B), BT17 (C) and BT18 (D) both in StratαLUC (RET + GFRα1) and NOstratα (RET only) cells. StratαLUC cells responded to GDNF (positive control, 25 ng/ml panels A and B, 50 ng/ml panels C and D) whereas Nostratα cells did not (as determined using t-test, $p < 0,05$). CTR – untreated cells, LCPS – luminescence counts per second.

When compounds BT13 and BT16 were tested the GDNF concentration of 25 ng/ml stimulated luciferase activity approximately twice as much compared to untreated cells (Figure 8, panels A and B) whereas with BT17 and BT18 the GDNF-induced signal was

20 times higher than that of the untreated cells (Figure 8, panels C and D). This dis-discrepancy might be explained by a pipetting error, lower concentration (25 ng/ml instead of 50 ng/ml), loss of biological activity or the combination of these three factors. GDNF did however serve its purpose as a positive control as the response in both experiments was statistically significant compared to untreated cells (t-test, $p < 0,05$). As expected, GDNF was unable to produce significant activation of luciferase expression without its co-receptor.

Luciferase assay proved to be an excellent tool for screening purposes. It is not only sensitive and fast but also cost and labor efficient. However, the evidence it provides is indirect and the observed activation of luciferase expression by compounds might be contributed to factors unrelated to the ones mediating the GDNF-induced response. Thus, direct phosphorylation assays were employed to investigate the compounds' ability to activate RET and its downstream targets AKT and ERK1/2.

4.1 Phosphorylation assay: ERK1/2 and AKT

Ability of the compounds to activate intracellular signaling cascades MAPK and PI3K was studied by direct phosphorylation assay of their components ERK1/2 and AKT, respectively, using GFRα1-transfected MG87RET cells (see chapter 3.1.) and western blot. Activation was determined by comparing the degree of phosphorylation of these proteins in cells treated with compounds (1-50 µM) or GDNF (50-100 ng/ml, positive control) to phosphorylation in untreated cells (negative control). GAPDH was stained as a loading control.

To estimate the optimal stimulation time for dose-dependence assays, cells were exposed to compounds for 1, 5, 15, 45 and 90 minutes (Figure 9) unless otherwise stated - failure to adhere to these time-points occurred in experiments regarding compounds BT13 (Figure 9, panel A) and BT18 (Figure 9, panel D). Cells were also treated with GDNF using same time-points in order to compare time-dependent activation induced by GDNF and by compounds.

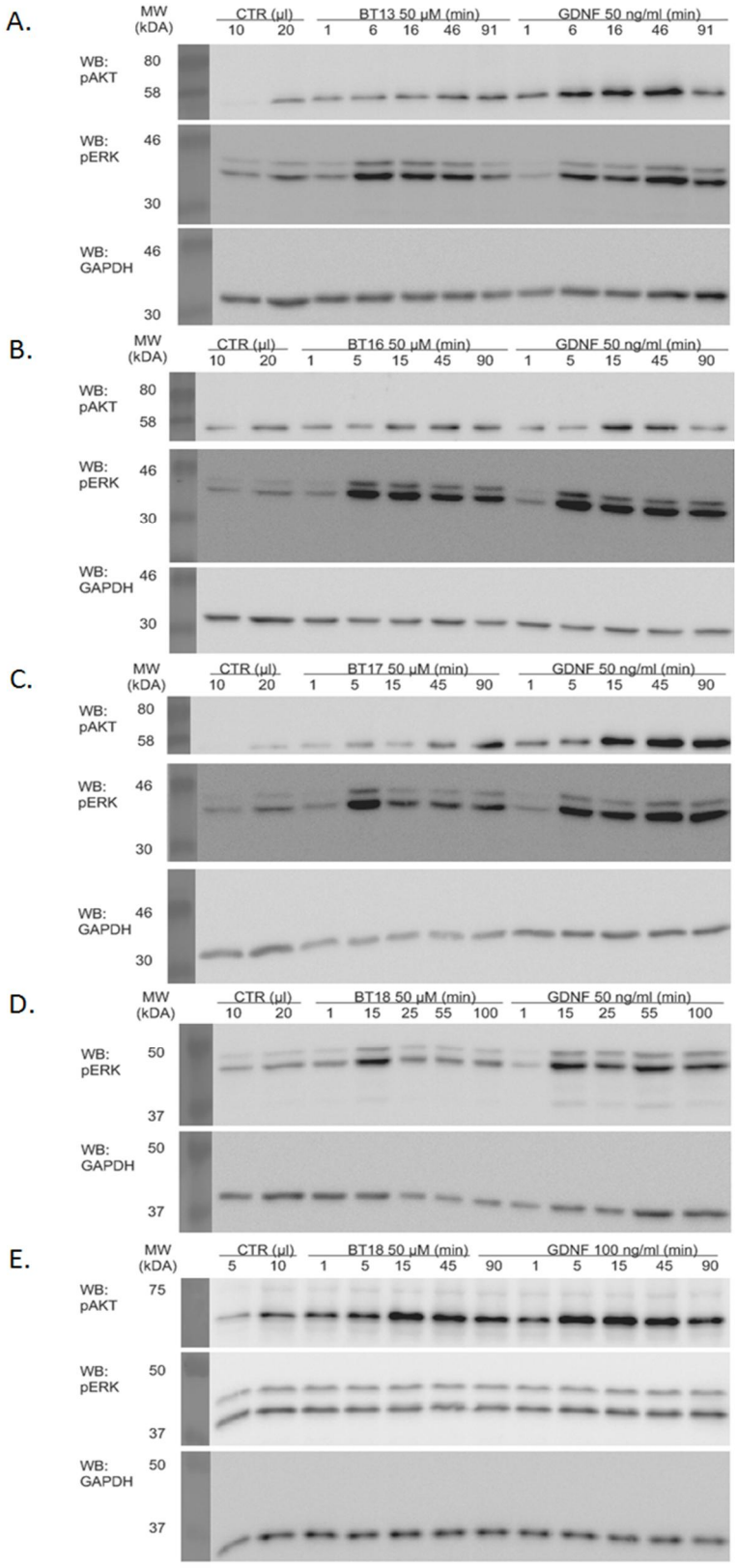

Figure 9. Compounds BT13 (A), BT16 (B), BT17 (C) and BT18 (D, E) activate AKT and ERK1/2 time-dependently. Western blotting (WB) of GFRα1-transfected MG87RET fibroblasts stimulated with compounds (50 µM) or GDNF (50 ng/ml, positive control) for 1 – 90 min (unless otherwise stated). CTR – untreated cells, MW – molecular weight.

Compounds BT13 (Figure 9, panel A), BT16 (Figure 9, panel B) and BT17 (Figure 9, panel C) activated ERK1 (upper band) and ERK2 (lower band). BT18 did so initially (Figure 9, panel D), but when the experiment was repeated due to a missing time-point, the compound produced no observable ERK1/2 activation (Figure 9, panel E). The same occurred with GDNF (positive control) in this experiment, which contradicts results from a multitude of published studies and as well as data acquired in this study. One possible reason for this inconsistency is the transfection procedure which was different from the procedure used in all other experiments. In this experiment, the transfection reagent was incubated overnight with the cells instead of 4-6 hours as recommended by the manufacturer. This might have influenced the outcome as Lipofectamine 2000 has been reported to activate ERK1/2 [Runeberg-Roos, P., unpublished observations]. High baseline activity could very well mask any effect produced by compounds and GDNF. The other possible explanation is the quality of antibodies that were from a different batch. It was suspected that these antibodies recognize total ERK1/2 instead of phosphorylated forms of these proteins. The intensity of stained pERK1/2 (or total ERK1/2, if antibodies were flawed) was indeed elevated in all cells in the experiment and demanded unusually low exposure time for detection.

Most of the data indicate that activation of ERK1/2 by GDNF peaks between 1 and 5 minutes. This is most evident in panel B (Figure 9) where ERK1/2 activation clearly decreases after the 5 minute peak. Panel A (Figure 9) shows the same decrease although not as clearly due to unequal loading. In panel D (Figure 9) the 5 minute time-point is missing but the decrease after 15 minutes is consistent with conclusions based on images shown in panels A and B. Surprisingly data in panel C (Figure 9) indicates that levels of pERK1/2 continue to stay elevated even after 45 minutes. ERK1/2 activation induced by BT13, BT16 and BT17 (panels A, B and C in Figure 9) also peaked between 1 and 5 minutes indicating that they activate ERK1/2 with similar time-dependence to GDNF. Whether or not BT18 (panels D and E in Figure 9) does so cannot be concluded due to the missing 5 minute time point and aforementioned problems with the transfection.

All four compounds (BT13, BT16, BT17 and BT18, Figure 9, panels A to E) activated AKT. Activation induced by BT13, BT16 and BT17 apparently keeps increasing with time and was at its highest after 90 minutes (panels A to C, Figure 9). Compound BT18 behaved differently with activation reaching its peak after 15 minutes after which the signal began to drop (panels D and E, Figure 9). GDNF-induced AKT activation

appears to reach its peak between 5 and 45 minutes as data indicate in panels A and E or between 15 and 45 minutes as suggested by data shown in panels B and C (Figure 9). Notably, AKT activation by GDNF decreases between 45 and 90 minutes whereas with compounds BT13, BT16 and BT17 the activation increases (panels A, B and C in Figure 9). Time-dependence in AKT activation by compound BT18 (panel E, Figure 9) appears similar to that induced by GDNF.

Combined data from luciferase and phosphorylation assays were considered enough to conclude MAPK activation by all four compounds (BT13, BT16, BT17 and BT18). Therefore, focus was given to dose-dependence in compound-induced AKT activation. Stimulation time of 90 minutes was selected based on the results from time-dependence assays.

All four compounds (BT13, BT16, BT17 and BT18) activated AKT in a dose-dependent manner (Figure 10) although the loading is questionable due to problems with GAPDH detection. Fading of chemiluminescence during visualization was unexceptionally rapid. Interpretation becomes problematic if the substrate is exhausted during visualization. However, the chance of unequal loading producing such a consistent pattern of dose-dependency seems unlikely.

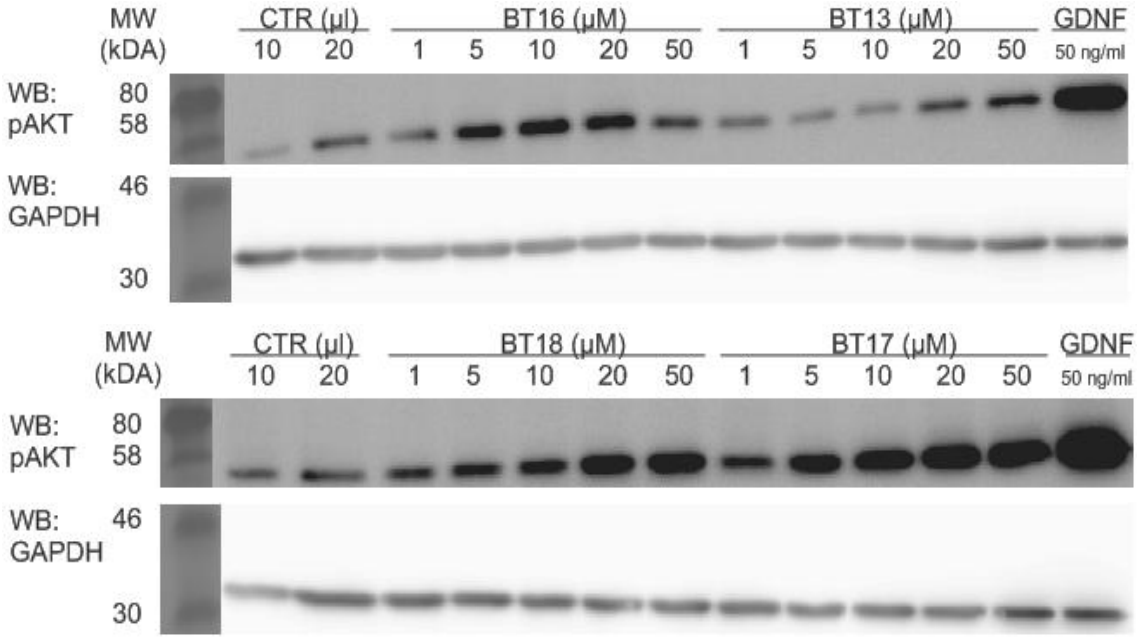

Figure 10. AKT is activated dose-dependently by compounds BT13, BT16, BT17 and BT18. Western blotting (WB) of GFRα1-transfected MG87RET cells stimulated with varying concentrations of compounds (1-50 µM) or GDNF (50 ng/ml, positive control) for 90 min. CTR – untreated cells, MW – molecular weight.

Compound BT16 exhibits signs of toxicity. Data presented in Figure 10 (upper panel) indicates that the concentration of 50 µM was less efficient in activating AKT than that of 20 µM. The higher concentration might have caused cell damage rendering the cells unable to respond to stimuli normally.

It was concluded that all four compounds activate not only MAPK but also AKT indicating they promote the activation of the antiapoptotic PI3K pathway. It was of interest to determine whether or not the compounds do so by activating RET and if so, is GFRα1 required.

4.2 Phosphorylation assay: RET

The involvement of RET in compound-induced activation of MAPK and PI3K pathways was studied using MG87RET cells transfected with GFP or GFRα1. RET was immunoprecipitated and analyzed using western blot. Degree of RET phosphorylation in cells treated with compounds (5-50 µM) or GDNF (75-100 ng/ml, positive control) was compared to that in untreated cells (negative control). RET was stained as a loading control. Comparisons were also made between cells transfected with GFRα1 and cells transfected with GFP to study the compounds' ability to signal through RET without GFRα1. RET phosphorylation assays were limited to compounds BT13 and BT18 due to unavailability of BT16 and BT17. Moreover, some of the data points are inconclusive owing to unequal loading that could not be adjusted despite of numerous attempts.

Compound BT13 induced RET phosphorylation in GFRα1-transfected MG87RET cells (Figure 11, panel A). At least 10 and 25 µM concentrations of BT13 produced a notable phosphorylation compared to untreated cells. Whether or not concentrations of 5 and 50 µM also activated RET cannot be concluded because of the differences in amount of loaded protein. Because of this issue the BT13-induced RET phosphorylation in GFP-transfected cells is even less clear. Yet at least concentration of 50 µM seems to induce phosphorylation compared to GDNF that is not able to function without GFRα1 and can be regarded as a negative control.

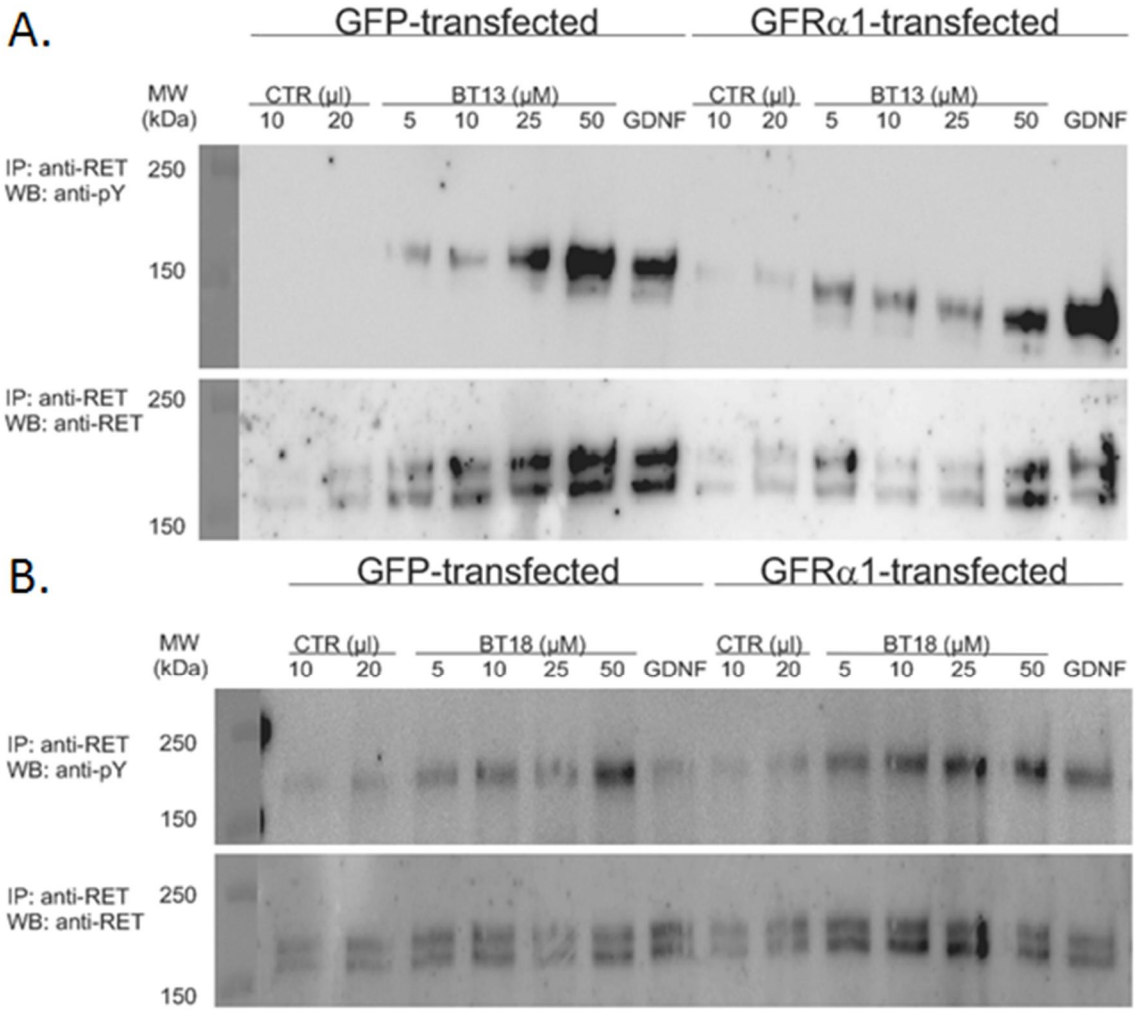

Figure 11. Compound BT13 induces RET phosphorylation. Western blot of GFP/GFRα1-transfected MG87RET fibroblasts treated for 15 min with varying concentrations of BT13 or GDNF (positive control, 75 ng/ml, panel A, 100 ng/ml, panel B. CTR – untreated cells. MW – molecular weight. IP – immunoprecipitation. pY – phosphotyrosine. WB – western blot.

Compound BT18 appears to phosphorylate RET in concentration of 50 µM both in GFP-transfected (compared to GDNF) and in GFRα1-transfected cells (Figure 11, panel B). No solid conclusion can be made from data points corresponding to other concentrations.

To conclude, results from RET phosphorylation assay indicate compounds BT13 and BT18 activate RET. This and data from ERK1/2 and AKT phosphorylation assays suggests functional similarity between BT13, BT18 and GDNF. However, in accordance to data from luciferase assays, compounds seem to be able to activate RET independent of GFRα1. It was concluded that the combined results from

phosphorylation assays warrant assaying compounds' effect on survival of DA neu-neurons.

4.3 Survival assay

Ability of compounds to promote survival of dopaminergic neurons was studied using primary murine (NMRI) embryonic (E13,5) midbrain neurons. Cells were cultured for 5 days *in vitro* (5 DIV) after which they were stained using tyrosine hydroxylase staining and counted in a blinded manner. Number of TH-postive cells after 5 DIV treated with compounds (0,003 µM – 5 µM) or GDNF (30 ng/ml, positive control) was compared to the number TH-positive cells in the untreated groups (negative control). Comparisons were made between three independent experiments with at least four repeats per treatment group using two-tailed, paired Student's t-test to determine statistically significant difference between the groups ($p < 0,05$). Due to limitations on time and material only results for six concentrations of BT13 and three concentrations of BT18 were acquired (Figure 12). For other concentrations and compounds BT16 and BT17, there was not enough statistical power to determine effect on survival (data not shown).

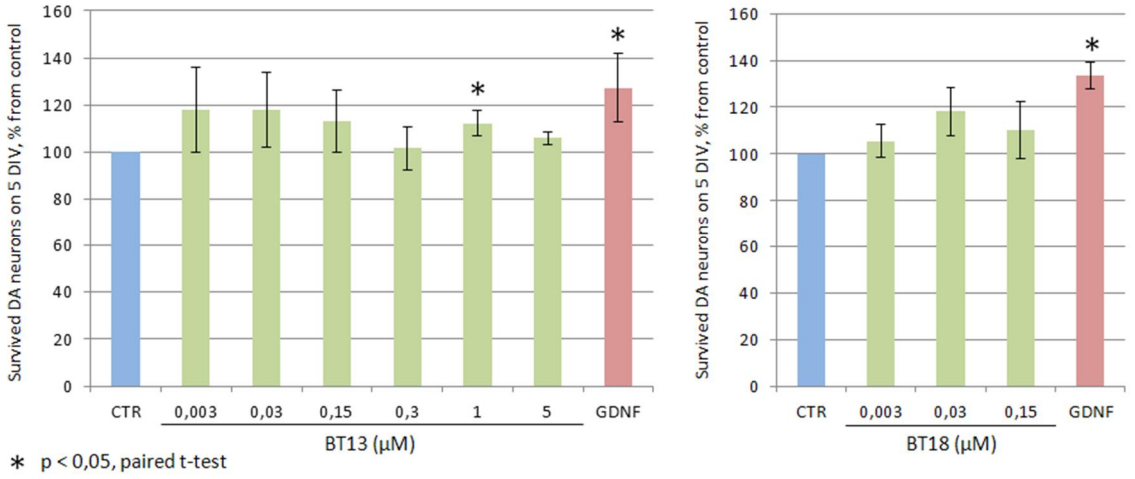

Figure 12. BT13 significantly promotes survival of dopaminergic embryonic neurons in 1 µM ($p < 0,05$) whereas no significant survival promoting effect was observed with BT18 on any of the tested concentrations. Concentration of GDNF was 30 ng/ml. CTR – untreated cells.

Compound BT13 significantly promotes the survival of primary embryonic dopaminergic neurons in 1 µM concentration (Figure 12, left panel). No such effect could be determined for the other concentrations used. Moreover, no survival promoting effect by BT18 in tested concentrations could be shown (Figure 12, right panel). However, BT18 concentration of 0,03 µM came very close ($p = 0,0504$). Further

experimenting is necessary to establish possible survival promoting effects of com-compounds BT16, BT17 and BT18.

5 Conclusions and perspectives

The purpose of the work presented was to show functional similarities between GDNF and four synthetic, potential small-molecule GDNF mimics (BT13, BT16, BT17 and BT18). Methods and the order in which the methods were employed were chosen with time and cost efficiency in mind. First, the compounds were tested using the luciferase assay designed to show activation of the MAPK pathway that is known to mediate GDNF's neurotrophic effects. Treatment with all four compounds led to an increase in luciferase expression suggesting compound-induced MAPK activation. This called for further experiments that constitute the second and third phase of this study. During the second phase, phosphorylation immunoassays were used to investigate whether compounds activate not only MAPK but also another important pathway, the PI3K cascade. As compound-induced activation of both signaling pathways was shown, it was important to determine the involvement of RET and GFRα1, the components of the GDNF receptor complex. This constitutes the third phase of this study. Because the compounds BT16 and BT17 were not available, RET phosphorylation assays were limited to BT13 and BT18 which did fortunately display some, yet not conclusive, activation of RET (also without GFRα1, however, supporting GFRα1-independent activation seen in luciferase assay). Data compiled from the luciferase and phosphorylation assays suggesting warranted for the final and perhaps the most important part of this study, which focused on investigating the possible survival promoting effects of the compounds BT13 and BT18 (as BT16 and BT17 were not available). Exposure to compound BT13 was found to significantly increase survival of primary DA neurons *in vitro*. BT18 did not show such an effect based on data that was acquired. To conclude, the aims set for this study were mostly met.

Results indicate that the compound BT13 and GDNF share functional similarities, most importantly the survival promoting effect on cultured dopaminergic neurons. The reason behind pursuing the development of small-molecule alternatives to GDNF is to ultimately be able to treat Parkinson's disease. Results from this study are certainly promising but an aeon away from any such clinical applications. Further studies are

required to address a multitude of issues including efficacy *in vivo* and numerous pos-possible unwanted effects on cell populations throughout the body expressing RET or RET and GFRα1.

References

1 Purves, D, Augustine GJ, Fitzpatrick D, Hall WC, LaMantia A-S, McNamara JO, White LE (2008). Neuroscience. 4th ed. Massachusetts: Sinauer Associates, inc.

2 Toulouse A, Sullivan AM (2008). Progress in Parkinson's disease – where do we stand? Prog.. Neurobiol. 85(4):376-392.

3 Fahn, S (2003). Description of Parkinson's disease as a clinical syndrome. Ann. N.Y. Acad. Sci. 991:1-14.

4 Hamani, C, Lozano, AM (2003). Physiology and pathophysiology of Parkinson's disease. Ann. N.Y. Acad. Sci. 991:15-21.

5 Aron L, Klein R (2011) Repairing the parkinsonian brain with neurotrophic factors. Trends Neurosci. 34(2):88-100.

6 Airaksinen, MS, Saarma, M (2002).The GDNF family: signalling, biological functions and therapeutic value. Nat. Rev. Neurosci. 3(5):383–394.

7 Sidorova YA, Mätlik K, Paveliev M, Lindahl M, Piranen E, Milbrandt J, Arumäe U, Saarma M, Bespalov MM (2010). Persephin signaling through GFRalpha1: the potential for the treatment of Parkinson's disease. Mol. Cell Neurosci. 44(3):223-232.

8 Bespalov M, Saarma M (2007). GDNF family receptor complexes are emerging drug targets. Trends Pharmacol. Sci. 28(2):68-74.

9 Takahashi M (2001). The GDNF/RET signaling pathway and human diseases. Cytokine Growth Factor Rev. 12(4):361-373.

10 Wang, HJ *et al*. (2007) Role of PI3-K/Akt pathway and its effect on glial cell line-derived neurotrophic factor in midbrain dopamine cells Acta Pharmacol. Sin. 28(2):166-172.

11 Marshall, CJ (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell. 80(2):179-185.

12 Rosenblad C, Grønborg M, Hansen C, Blom N, Meyer M, Johansen J, Dagø L, Kirik D, Patel UA, Lundberg C, Trono D, Björklund A, Johansen TE (2000). *In vivo* protection of nigral dopamine neurons by lentiviral gene transfer of the novel GDNF-family member neublastin/artemin. Mol. Cell Neurosci. 15(2):199-214.

13 Åkerud P, Holm PC, Castelo-Branco G, Sousa K, Rodriguez FJ, Arenas E. (2002). Persephin-Overexpressing Neural Stem Cells Regulate the Function of Nigral Dopaminergic Neurons and Prevent Their Degeneration in a Model of Parkinson's Disease Mol. Cell Neurosci. 21(2):205-222.

14 Grondin, R and Gash, DM (1998). Glial cell line-derived neurotrophic factor (GDNF): a drug candidate for the treatment of Parkinson's disease. J. Neurol. 245(11 Suppl. 3): 35-42.

15 Liu WG, Lu GQ, Li B, Chen SD (2007). Dopaminergic neuroprotection by neurturin-expressing c17.2 neural stem cells in a rat model of Parkinson's disease. Parkinsonism Relat. Disord. 13(2):77-88.

16 Liu WG, Wang XJ, Lu GQ, Li B, Wang G, Chen SD (2007). Dopaminergic regeneration by neurturin-overexpressing c17.2 neural stem cells in a rat model of Parkinson's disease. Mol. Neurodegener. 2:19.

17 Dauer, W, Przedborski S, (2003). Parkinson's Disease: Mechanisms and Models. Neuron. 39(6):889-909.

18 Airavaara M, Voutilainen MH, Wang Y, Hoffer B (2011). Neurorestoration. Parkinsonism Relat. Disord. 18(Suppl. 1): 143-146.

19 Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat. Med. 9(5):589-595.

20 Slevin JT, Gerhardt GA, Smith CD, Gash DM, Kryscio R, Young B (2005). Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. J. Neurosurg. 102(2):216-222.

21 Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS (2005). Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. Ann. Neurol. 57(2):298-302.

22 Slevin JT, Gash DM, Smith CD, Gerhardt GA, Kryscio R, Chebrolu H, Walton A, Wagner R, Young AB. Unilateral intraputamenal glial cell line-derived neurotrophic factor in patients with Parkinson disease: response to 1 year of treatment and 1 year of withdrawal. J. Neurosurg. 106(4):614-620.

23 Lang AE, Gill S, Patel NK, Lozano A, Nutt JG, Penn R, Brooks DJ, Hotton G, Moro E, Heywood P, Brodsky MA, Burchiel K, Kelly P, Dalvi A, Scott B, Stacy M, Turner D, Wooten VG, Elias WJ, Laws ER, Dhawan V, Stoessl AJ, Matcham J, Coffey RJ, Traub M (2006). Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann. Neurol. 59(3):459-466.

24 Yasuhara T, Shingo T, Date I. Glial cell line-derived neurotrophic factor (GDNF) therapy for Parkinson's disease. Acta Med. Okayama. 61(2):51-56.

25 Hutchinson M, Gurney S, Newson R. GDNF in Parkinson disease: an object lesson in the tyranny of type II. J. Neurosci. Methods. 163(2):190-192.

26 Matcham J, McDermott MP, Lang AE. GDNF in Parkinson's disease: the perils of post-hoc power. J. Neurosci. Methods. 163(2):193-196.

27 Marks WJ Jr, Bartus RT, Siffert J, Davis CS, Lozano A, Boulis N, Vitek J, Stacy M, Turner D, Verhagen L, Bakay R, Watts R, Guthrie B, Jankovic J, Simpson R, Tagliati M, Alterman R, Stern M, Baltuch G, Starr PA, Larson PS, Ostrem JL, Nutt J, Kieburtz K, Kordower JH, Olanow CW (2010). Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomised, controlled trial. Lancet Neurol. 9(12):1164-1172.

28 Tokugawa K, Yamamoto K, Nishiguchi M, Sekine T, Sakai M, Ueki T, Chaki S, Okuyama S (2003). XIB4035, a novel nonpeptidyl small molecule agonist for GFRα-1. Neurochem. Int. 42(1):81–86

29 Barber RD, Harmer DW, Coleman RA, Clark BJ (2005). GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol. Genomics. 21(3):389-395.

30 Planken A, Porokuokka LL, Hänninen AL, Tuominen RK, Andressoo JO (2010). Medium-throughput computer aided micro-island method to assay embryonic dopaminergic neuron cultures *in vitro*. J. Neurosci. Methods. 194(1):122-131.