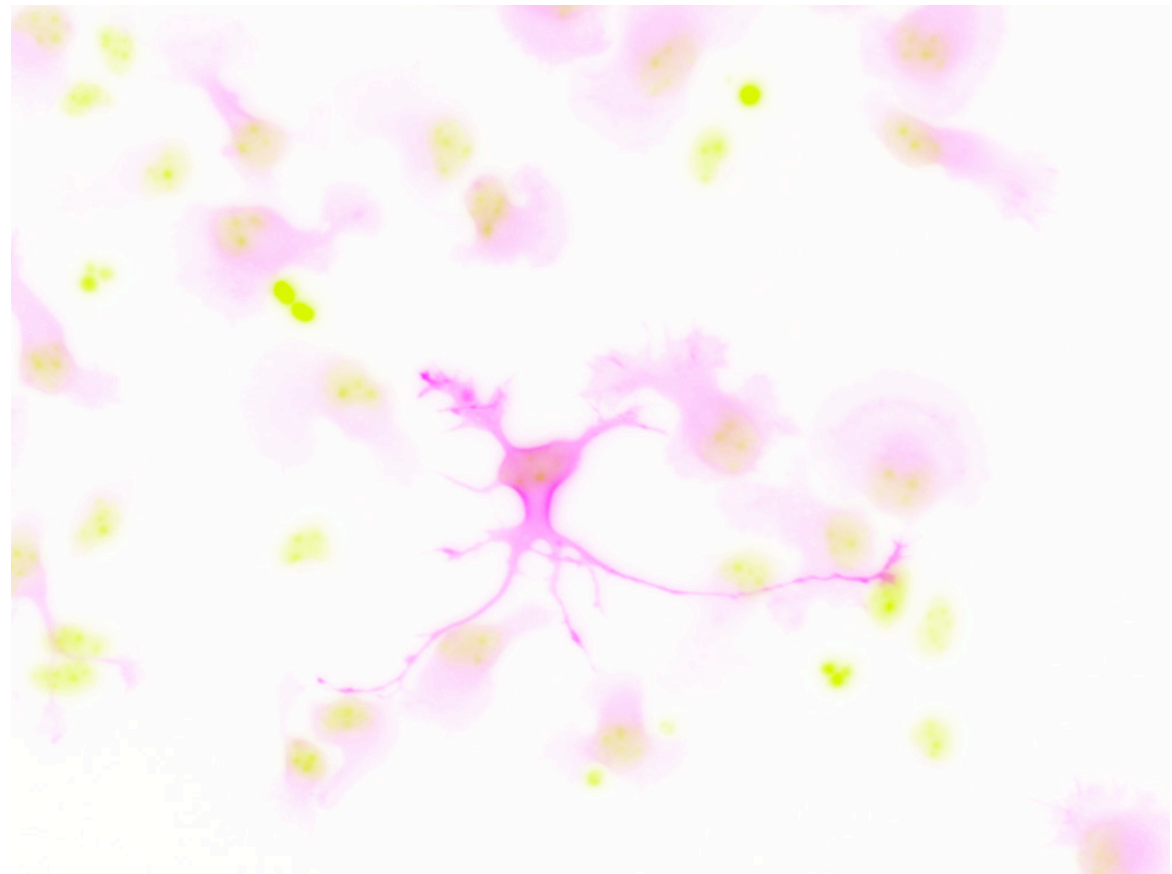


Thesis for doctoral degree (Ph.D.)  
2010

# CONTROL OF NEURONAL SURVIVAL, MIGRATION AND OUTGROWTH BY GDNF AND ITS RECEPTORS



Maurice Perrinjaquet

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# **CONTROL OF NEURONAL SURVIVAL, MIGRATION AND OUTGROWTH BY GDNF AND ITS RECEPTORS**

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Institutet**

Stockholm 2010

The cover image illustrates cultured MGE neurons immunostained for GABA (purple) and counterstained with DAPI (yellow).

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*E widme die Arbeit der Erinnerung  
a ds Grossmuetti ond ds Mili.*

## ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) is the prototypical member of a family of growth factors that are indispensable in nervous system development and maintenance. GDNF signals by binding to a multi-component receptor complex comprised of the ligand-binding subunit GFR $\alpha$ 1 and the signaling subunit RET or NCAM. While initial interest in GDNF was merely focused on its potential therapeutic effects in Parkinson's disease, it has rapidly become evident that the mechanisms by which GDNF and its receptors regulate diverse physiological events are incomplete. Thus, we have demonstrated new insights into the control of neuronal survival, migration, and outgrowth by GDNF and its receptors.

To begin clarifying the complexity of GDNF signaling, we examined RET intracellular tyrosine residues which become phosphorylated upon receptor activation, and serve as docking sites for downstream signaling effectors. The functions of most of these phosphorylated tyrosine residues are still unknown. In paper I, we identified the protein-tyrosine phosphatase SHP2 as a novel direct interactor of RET and as the first effector known to bind to phosphorylated Tyr<sup>687</sup>. Furthermore, we found that activation of protein kinase A (PKA) by forskolin reduced the recruitment of SHP2 to RET, negatively affecting ligand-mediated neurite outgrowth. Together, these findings establish SHP2 as a novel positive regulator of RET function and reveal Tyr<sup>687</sup> as a critical platform for integration of RET and PKA signals.

To continue to understand GDNF signaling diversity, we examined RET signaling in a clinical context. Most patients with medullary thyroid carcinoma (MTC) and type 2A multiple endocrine neoplasia (MEN2A) exhibit cysteine residue mutations in the juxtamembrane region of RET which result in unexplained oncogenic activation. Thus, in paper II we identified and described the self-association determinants of the RET transmembrane (RET-TM) domain underlying such oncogenic activation by mutations found in these patients. We found that strong propensity for RET-TM self-association underlies - and may be required for - dimer formation and oncogenic activation by juxtamembrane cysteine mutations localized in close proximity to the plasma membrane in MTC and MEN2A syndromes.

To further dissect the many facets of GDNF signaling, we alternatively investigated the functions of GDNF and GFR $\alpha$ 1 independent of RET. GDNF promotes the differentiation and migration of GABAergic neuronal precursors from the medial ganglionic eminence (MGE). These functions are dependent on GFR $\alpha$ 1, but are independent of the two known receptor partners RET and NCAM. In paper III, we revealed that soluble GFR $\alpha$ 1 is able to promote GABAergic differentiation and migration, but requires endogenous GDNF production. Furthermore, we showed that MET signaling inhibition promoted the same physiological response as GDNF. Finally, we justified the existence of a novel transmembrane receptor for the GDNF/GFR $\alpha$ 1 complex and uncovered an unexpected interplay between GDNF/GFR $\alpha$ 1 and HGF/Met signaling in the early diversification of GABAergic MGE interneuron subtypes.

## LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

- I. **Maurice Perrinjaquet**, Marçal Vilar and Carlos F. Ibáñez  
Protein-tyrosine phosphatase SHP2 contributes to GDNF neurotrophic activity through direct binding to phospho-Tyr<sup>687</sup> in the RET receptor tyrosine kinase  
*J Biol Chem*, 2010, 285:31867-31875
- II. Svend Kjaer, Kei Kurokawa, **Maurice Perrinjaquet**, Chiara Abrescia and Carlos F. Ibáñez  
Self-association of the transmembrane domain of RET underlies oncogenic activation by MEN2A mutations  
*Oncogene*, 2006, 25:7086-7095
- III. **Maurice Perrinjaquet**, Dan Sjöstrand, Annalena Moliner, Sabrina Zechel, Fabienne Lamballe, Flavio Maina and Carlos F. Ibáñez  
Met signaling in GABAergic neurons of the medial ganglionic eminence restricts GDNF activities in cells expressing GFR $\alpha$ 1 and a novel transmembrane receptor  
*Submitted manuscript*

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## LIST OF ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
ARTN	Artemin
BDNF	Brain-derived neurotrophic factor
C	Cysteine
CGE	Caudal ganglionic eminence
CNS	Central nervous system
CREB	Cyclic AMP response element binding
DA	Dopaminergic
DIV	Day(s) <i>in vitro</i>
E	Embryonic day
ECD	Extracellular domain
ENS	Enteric nervous system
ERK	Extracellular signal-regulated kinase
FMTCT	Familial medullary thyroid carcinoma
FRS2	Fibroblast growth factor receptor substrate 2
GAB	Growth factor receptor-bound protein 2-associated binder
GABA	Gamma-aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GFLs	GDNF family ligands
GFR $\alpha$	GDNF family ligand receptor alpha
GPI	Glycosylphosphatidylinositol
GRB	Growth factor receptor-bound protein
HGF	Hepatocyte growth factor
ICD	Intracellular domain
LGE	Lateral ganglionic eminence
MEN2A	Multiple endocrine neoplasia type 2A
MET	Mesenchymal epithelial transition factor
MGE	Medial ganglionic eminence
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NRTN	Neurturin
PI3K	Phosphoinositide 3-kinase
PLC $\gamma$	Phospholipase C gamma
PNS	Peripheral nervous system
PSPN	Persephin
PTB	Phosphotyrosine binding domain
PTP	Protein tyrosine phosphatase
RET	Rearranged during transfection
RMS	Rostral migratory stream
RTK	Receptor tyrosine kinase
SCG	Superior cervical ganglion
SH2	Src homology 2 domain
SHC	SH2-containing transforming protein 1
SHP2	SH2-containing protein tyrosine phosphatase 2
STAT3	Signal transducer and activator of transcription 3
TGF- $\beta$	Tumor growth factor beta
TM	Transmembrane
Y	Tyrosine





# PROLOGUE

## Why did I study GDNF?

My interest to focus my doctoral research on GDNF transpired after reading a report on a phase I clinical trial of Parkinson patients treated with GDNF (Gill 2003). This safety open-label trial, in which all five patients showed improvements in standard scores of movement and motor skills, inspired me to pursue hands-on fundamental research dissecting the intracellular pathways mediated by GDNF in neuronal survival, outgrowth, and differentiation. As I embarked on this research, Amgen, the company holding the patent for the clinical use of GDNF, decided to halt all future clinical trials after a review of the phase II trial. They reported that there was patient safety concerns as few patients clinically improved (even placebo controls) while other patients' conditions severely worsened. Two participants in the study even developed neutralizing antibodies against GDNF. This was Amgen's second clinical trial failure, after an initial double-blind safety trial showed extensive side effects suffered by patients treated with GDNF without any clinical improvement (Nutt 2003). Amgen sadly refused all patients, even Gill's patients still under treatment, the right to continue taking GDNF to dampen their symptoms of Parkinson's disease. Regrettably, this halt then lead to a lawsuit between both parties. Interestingly, post-mortem data of these clinical trials have shown that GDNF did not efficiently reach the target tissues – as GDNF cannot cross the blood-brain barrier. From this data emerged an important understanding of GDNF and drug delivery methods: GDNF must be delivered in a different manner to precisely reach target tissues, and deeper insights into the physiological neurotrophic activities of GDNF on human dopaminergic neurons were needed. As a result, Amgen decided to make their pre-clinical GDNF available to scientists to examine GDNF's mechanisms of action. Our laboratory was a recipient of Amgen's GDNF, which was not only used in my studies, but was more importantly the motivating foundation of my doctoral research.

The results of my research endeavors are presented in this thesis.

# INTRODUCTION

## GDNF FAMILY LIGANDS AND THEIR RECEPTORS

Glial cell line-derived neurotrophic factor (GDNF) was first discovered in 1993 by scientists at the company Synergen in a screen to discover novel secreted neurotrophic factors enhancing survival of primary dopaminergic (DA) neurons isolated from rat embryonic midbrains (Lin 1993). The protein was initially purified from conditioned media of the rat B49 glial cell line and characterized as a disulfide-linked homodimeric glycoprotein with neurotrophic activities specific to dopaminergic neurons (Lin 1994). Human GDNF cDNA encodes a 211 amino acid residue prepropeptide precursor that is further processed by furin-type endopeptidases to yield a predicted 30 kDa mature homodimeric molecule formed by two identical 134 amino acid subunits (Lonka-Nevalaita 2010). The two monomers are associated in a head-to-tail orientation displaying anti-parallel left-right symmetry (Eigenbrot 1997).

GDNF is the prototypical member of the GDNF family ligands (GFLs) that also include Neurturin (NRTN), Artemin (ARTN), and Persephin (PSPN) (Fig.1). NRTN was isolated from conditioned media of the Chinese hamster ovarian (CHO) cell line, as it displayed the ability to promote the survival of cultured sympathetic neurons from the superior cervical ganglion (SCG) (Kotzbauer 1996). PSPN was thereafter cloned by degenerate PCR (Milbrandt 1998) and ARTN was found by multiple database searches based on its homology to mature NRTN (Baloh 1998). The GFLs are distant members of the transforming growth factor-beta (TGF- $\beta$ ) superfamily, which are characterized by a common three-dimensional fold containing a cysteine knot (Eketjall 2004). GDNF shares only a 23% sequence similarity with TGF- $\beta$  superfamily members and differs from them as it distinctly signals through a receptor tyrosine kinase instead of a serine/threonine kinase (Massague 1998, Saarma 2000).

Although many growth factors bind directly to their receptor kinase, the GFLs are unique in that they must first bind to one of the four GDNF family receptor alphas (GFR $\alpha$ 1–4) (Fig. 1). GFR $\alpha$ 's are glycosylphosphatidylinositol (GPI)-anchored membrane proteins that were originally discovered by expression cloning and screening of GDNF binding proteins (Jing 1996, Treanor 1996). GFR $\alpha$  proteins can be cleaved by phospholipases and are functional in soluble form (Paratcha 2001). While GDNF preferentially binds to GFR $\alpha$ 1, there is some promiscuity within the family as GDNF can also bind to both GFR $\alpha$ 2 and GFR $\alpha$ 4 (Fig. 1) (Airaksinen 2002, Sidorova 2010).

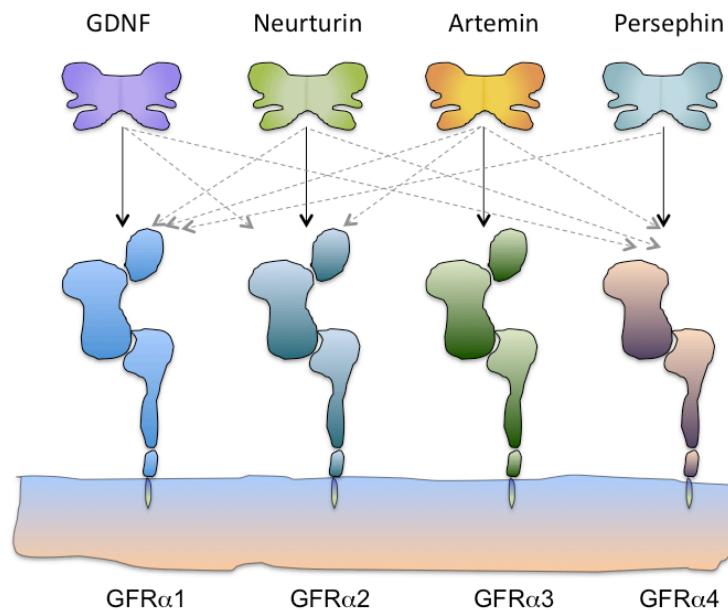


Figure 1: The GDNF family ligands and their respective GFR $\alpha$  receptors. Dashed lines represent known interactions while a full line represents the preferred binding receptor for each ligand.

In order to signal across the cell membrane, the GDNF/GFR $\alpha$  complex must bind to a transmembrane receptor protein - either the receptor tyrosine kinase RET or the neural cell adhesion molecule NCAM (Durbec 1996, Trupp 1996, Paratcha 2003). These GFR $\alpha$  co-receptors are understood to determine both RET ligand binding specificity and to facilitate RET activation (Scott 2001). The current model argues for GDNF binding first to GFR $\alpha$ 1 and subsequently to RET although some evidence argues in favor of GDNF binding to a preformed GFR $\alpha$ 1/RET complex (Fig. 4) (Eketjall 1999, Cik 2000, Simi 2010). All four members of the GFLs signal through this multi-receptor complex comprised of the respective ligand, the corresponding GFR $\alpha$  co-receptor binding subunit, and finally the signaling subunit RET or NCAM.

Both the GFR $\alpha$  co-receptor and the signaling receptor are linked as dimers, resulting in a 2:2:2 stoichiometric signaling receptor; although the tridimensional structure of this hetero-hexameric complex still awaits confirmation by high-resolution crystallization techniques (Leppanen 2004, Sjostrand 2007, Parkash 2009, Simi 2010).

## **GDNF, GFR $\alpha$ 1 AND RET IN DEVELOPMENT**

### **Embryonic development: Findings from the knock-outs**

Studies using knock-out mice for GDNF, GFR $\alpha$ 1, and RET have demonstrated the important roles that these molecules play in embryonic development. Most strikingly, *Gdnf*<sup>-/-</sup>, *Gfra1*<sup>-/-</sup> and *Ret*<sup>-/-</sup> mice die perinatally due to kidney agenesis or severe dysgenesis and display a severe phenotype in the peripheral nervous system (PNS) (Schuchardt 1994, Moore 1996, Sanchez 1996, Cacalano 1998, Enomoto 1998). In the enteric nervous system (ENS), they have incomplete innervations or a total lack of enteric neurons in the small and large intestine. In the autonomic nervous system, both the parasympathetic and sympathetic nervous systems are severely affected due to the complete genetic ablation of *Gdnf* or its receptors. RET-deficient mice display impaired sympathetic neuron precursor migration and axonal growth (Durbec 1996; Enomoto 2001). GDNF knock-out animals have a 35% loss of superior cervical ganglion (SCG) neurons (Moore 1996). Interestingly, ablation of GFR $\alpha$ 1 expression does not affect sensory neuron numbers (Cacalano 1998, Enomoto 1998). The central nervous system (CNS) was essentially unaffected by the deletion of these proteins, with the exception of a 22% loss of spinal motoneurons in the *Gdnf* and the *Gfra1* null mice (Airaksinen 1999). For most researchers the lack of phenotype in the ventral midbrain was a major disappointment because it suggested that these proteins were not essential for the embryonic development of DA neurons *in vivo*. As a result, a deeper understanding of the temporal pattern of expression of GDNF and its receptors was required.

### **Brain development: From primary cultures to conditional knock-outs**

Since the early discovery of GDNF's neurotrophic activities on primary midbrain dopaminergic neurons, other brain regions were subsequently investigated for expression of GDNF and its receptors. GDNF and GFR $\alpha$ 1 were found to be strongly expressed in the olfactory bulb and in the olfactory neuroepithelium (Trupp 1997, Maroldt 2005). Within the olfactory bulb, GDNF was suggested to act as a diffusible chemoattractant to neuronal progenitors migrating along the rostral migratory stream that originated from the subventricular zone of the lateral ventricle in an NCAM-dependent manner (Paratcha 2006). In primary hippocampal and cortical neurons, GDNF has been shown to promote axonal growth via binding to NCAM (Paratcha 2003, Nielsen 2009). In the hippocampus, a region devoid of RET

expression, GDNF is known to promote neuronal synapse formation through trans-homophilic binding between GFR $\alpha$ 1 proteins expressed in both pre- and post-synaptic terminals (Ledda 2007). Due to the neonatal lethality of *Gdnf*, *Gfra1*, and *Ret* null mice, very little was understood about the physiological role of these proteins in postnatal brain development and adult plasticity until the advent of the GFLs conditional knock-out mice.

Recent publications of conditional knock-out mice have expanded the current knowledge of the physiological roles that GDNF and its receptors play *in vivo*. This genetic technology relies on the ability of the Cre recombinase of bacteriophage P1 to catalyze the excision of the DNA flanked by *loxP* recognition sequences (Sauer 1988). In conditional knock-outs, spatially controlled gene deletion is obtained by breeding genetically modified mice carrying alleles of the gene of interest flanked by *loxP* sites together with mice expressing the Cre recombinase from the locus of specific genes expressed in the target tissue. Therefore, embryonic lethality can be rescued in these animals through genuine expression of GDNF and its receptors in tissues/organs essential for embryonic development (such as kidneys, ureter and enteric neurons) while gene expression in targeted tissue is ablated. In light of the reported dopaminotrophic activities by GDNF, the first tissue targeted by the conditional ablation of RET was the ventral mesencephalon, with a focus on the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) (Lin 1993, Stromberg 1993). Surprisingly, excision of RET in cells expressing the dopamine transporter DAT showed that RET was dispensable for the maintenance of DA neurons (Jain 2006). In contrast, another study found that *Ret* ablation caused progressive and late loss of DA neurons in the SNpc with severe degeneration of the nigrostriatal DA terminals (Kramer 2007). In one year old mice, a significant 25% loss of tyrosine hydroxylase (TH) fibers was observed compared to age-matched control mice; a result which increased further to 38% in two year old animals. Interestingly, both studies used the *DAT-Cre* mice to excise the floxed *Ret* allele. Although their results lead to different conclusions in aged mice, both studies concur that RET is dispensable for the development and early maturation of the DA nigrostriatal system.

Scientists had long questioned GDNF's physiological role in ventral mesencephalon development and maintenance; since both *Gdnf* and *Gfra1* null mice suffer no DA nigrostriatal system deficits at birth (Moore 1996, Enomoto 1998, Baloh 2000). Instead of inactivating the gene of interest in the tissue of interest during embryogenesis, as was devised for both *Ret* conditional knock-outs, a different strategy was used for the deletion of the *Gdnf* conditional allele. A tamoxifen-

inducible CRE recombinase (*Esr1-Cre*) with ubiquitous expression was employed to regulate temporal gene inactivation (Hayashi 2002, Vooijs 2001). To obtain the conditional *Gdnf* mouse, heterozygous *Gdnf* mice were first crossed with *Esr1-Cre* mice, and their progeny was further crossed in the *Gdnf*<sup>F/+</sup> floxed allele to obtain the desired *Gdnf*<sup>F/-</sup>; *Esr1-Cre* genotype (Pascual 2008). The conditional *Gdnf* allele was excised in all cells of the body following tamoxifen injection in two month old animals; histological analysis was performed seven months later. Surprisingly, 60-70% of the dopaminergic neurons in the SN and the VTA were lost in these animals; a phenotype which was accentuated in the locus coeruleus with the absence of noradrenergic neurons (Pascual 2008). These results re-established GDNF's critical role in the survival and maintenance of adult catecholaminergic neurons. Additionally, these results indicated that a loss of GDNF could be an important contributor in the pathophysiology of neurodegeneration observed in Parkinson disease (Ibanez 2008).

### **Cortical interneuron development: GFR $\alpha$ 1 in GABAergic specification?**

While the human cerebral cortex accounts for ~80% of the total volume of the brain, interneurons represent about 25% of all neurons in the cortex (Hendry 1987, Meinecke 1987, Swanson 1995). For proper network activity to be achieved, the cerebral cortex maintains a very tightly synchronized regulation by the coordinated actions of two types of neurons, excitatory glutamatergic projection neurons and inhibitory local-circuit GABAergic interneurons (Wonders 2006). These neurons are born in distinct proliferative zones of the cerebral cortex and migrate long distances to reach their final position within the network (Corbin 2001). Projection neurons are born in the cortical ventricular zone and migrate via radial migration into the cortical plate. On the other hand, GABAergic interneurons originate in the transient neurogenic zone of the subpallial telencephalon and migrate tangentially into the neocortex between embryonic day 12.5 (E12.5) and E15.5 in the mouse (Fig. 2) (Marin 2003, Levitt 2004). Cortical GABAergic interneuron neurogenesis has been shown to occur in both the medial (MGE) and the caudal ganglionic eminences (CGE); whereas the lateral (LGE) ganglionic eminence gives rise to interneurons destined to migrate through the rostral migratory stream (RMS) to the olfactory bulb (Anderson 1997, Wichterle 1999, Anderson 2001, Nery 2002, Metin 2006). Cortical GABAergic interneurons make up a highly heterogeneous cell population classified by their morphologies, electrophysiological properties, ion channels, neuropeptides, and calcium binding properties (Markram 2004).

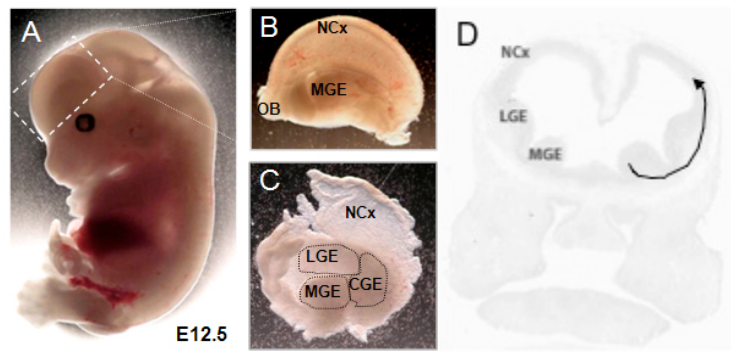


Figure 2: Ganglionic eminences of the developing telencephalon in an E12.5 mouse embryo (A and B). Schematic delineating the medial, lateral, and caudal ganglionic eminence borders (MGE, LGE, CGE) are shown in panel C. Tangential migration route of GABAergic interneurons, born in the MGE and traveling to the Neocortex (NCx), is depicted in a coronal section of the mouse head (D).

Many growth factors have been shown to be motogens for migrating interneurons from the MGE including: semaphorins, hepatocyte growth factor (HGF), neurotrophins, and neuregulin-1 (NG-1) (Marin 2001, Powell 2001, Polleux 2002, Flames 2004). Although combinatorial codes of transcription factors have been found to delineate different progenitor domains in the SVZ of the MGE, it remains unclear as to exactly how interneuron diversity is achieved (Flames 2007, Fogarty 2007, Liodis 2007, Miyoshi 2007).

GDNF was shown to promote differentiation and tangential migration of GABAergic cortical neurons isolated from the MGE (Pozas 2005). These GDNF functions are understood to be independent of the two known transmembrane receptors, RET and NCAM, but are dependent upon  $GFR\alpha 1$ .  $GFR\alpha 1$  expression was detected in a subset of postmitotic GABAergic precursors located in the ventral MGE, whereas *Gdnf* mRNA was found in the MGE and in the neocortex. Very low levels of *Ret* mRNA were observed in the MGE, signifying that a different transmembrane receptor was used by GDNF in these cells (Pozas 2005). In a subsequent study,  $GFR\alpha 1$  was further shown to be required for the correct allocation of parvalbumin (PV) expressing interneurons in the cortex, using a mutant mouse line that rescues the lethality of the *Gfra1* null animals (Canty 2009). In these “cis-only” mice, *Gfra1* cDNA is expressed under the *Ret* locus in order to ablate *Gfra1* solely in cells that do not express RET (Enomoto 2004). Cis-only mice displayed a severe PV interneuron loss in discrete regions of the cortex, while other cortical regions remained unaffected by the loss of  $GFR\alpha 1$ . These results indicated that  $GFR\alpha 1$  may guide the development of a subset of PV-expressing interneurons; and therefore may contribute to the diversification and later allocation of distinct cortical interneuron subtypes.



## CONSTITUTIVE AND GDNF-MEDIATED RET SIGNALING

### RET: Structural features for signaling purposes

RET (rearranged during transfection) was originally identified as a novel oncogene activated by DNA rearrangement using the murine NIH 3T3 cell line transfected with human lymphoma DNA (Takahashi 1985, Tahira 1990). The *Ret* gene was subsequently cloned, containing 21 exons localized to chromosome 10, and was shown to encode a receptor tyrosine kinase (RTK) expressed in many tumor cell lines (Takahashi 1987). The RET extracellular domain (ECD) is comprised of four cadherin-like motifs containing a calcium binding site between the second and third cadherin-like domains (CLD, Fig. 3), which is followed by a cysteine-rich domain (CRD) of ~120 residues containing a highly conserved group of 14 cysteines adjacent to the transmembrane (TM) domain (Fig. 3) (Airaksinen 1999, Anders 2001). The RET TM domain spans from residue 636-657, however nothing was known of its potential role in functional regulation of RET outputs until the publication of our investigation presented in paper II. The RET intracellular domain (ICD) contains two tyrosine kinase (TK) subdomains separated by a kinase insert. Alternative splicing of 3' sequences of exon 19 leads to three RET isoforms: RET9, RET51, and a rare RET43 (Myers 1995). RET9 (1072 amino acids) and RET51 (1114 amino acids) diverge in their tail sequence downstream of glycine 1063 (Santoro 2004). The RET9 ICD contains 16 tyrosine (Y) residues but lacks Y1090 and Y1096, which are present in RET51.

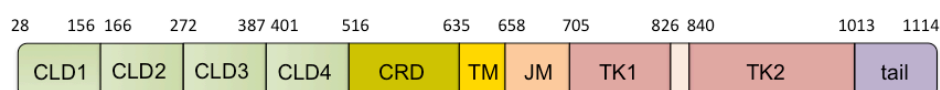
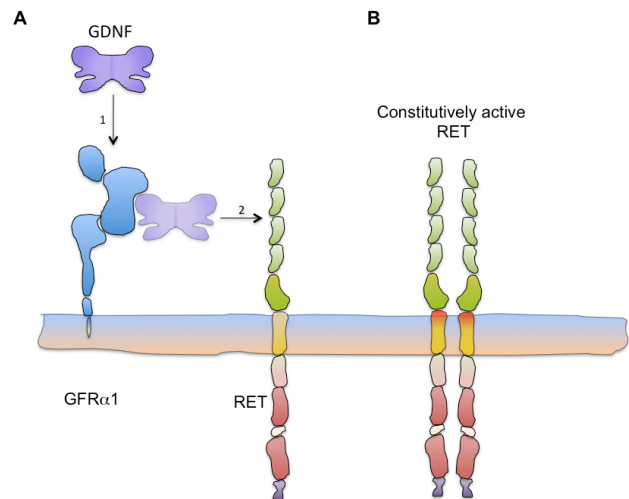


Figure 3: Schematic representation of RET structural domains. Amino acid residue numbers are allocated according to the human RET51 isoform.

### RET activation: Tyrosyl phosphorylation as a kick start

RET activation is achieved by two means: either upon ligand/GFR $\alpha$  receptor complex interaction or when carrying a point mutation rendering dimeric RET to be constitutively active (Fig. 4) (Takahashi 2001). Receptor dimerization promotes *trans*-phosphorylation of intracellular tyrosine residues which then serve as docking sites for various adaptor proteins containing either Src homology 2 (SH2) domains or phosphotyrosine binding domains (PTB) (Pawson 1995).

Figure 4: RET activation is achieved either by interaction with the GDNF/GFR $\alpha$ 1 complex (A) or by constitutive activation due to mutations in extracellular juxtamembrane cysteine residues shown in red (B).



At least 14 of the 18 intracellular tyrosine residues have been found to be phosphorylated in human RET51; as identified by phosphopeptide mapping or mass spectrometry including: Y687, Y752, Y806, Y809, Y826, Y900, Y905, Y928, Y981, Y1015, Y1029, Y1062, Y1090, and Y1096 (Liu 1996, Kawamoto 2004, Knowles 2006). The tyrosines Y900 and Y905 are both found within the kinase domain activation loop. Tyr<sup>905</sup> has been shown to be required for full RET activation and downstream signaling (Coulpier 2002). Site-specific adaptor proteins have also been identified for some of these tyrosine residues such as: Tyr<sup>905</sup>, Tyr<sup>981</sup>, Tyr<sup>1015</sup> and Tyr<sup>1096</sup>. When phosphorylated, these become binding sites for Grb7/10, Src, PLC $\gamma$ , and Grb2, respectively (Pandey 1996, Encinas 2004, Borrello 1996, Alberti 1998). While Tyr<sup>752</sup> and Tyr<sup>826</sup> can bind directly to the STAT3 transcription factor (Schuringa 2001), Tyr<sup>1062</sup> has been revealed as a multi-docking site for Shc, FRS2, IRS, and Dok (Fig. 5) (Arighi 1997, Kurokawa 2001, Melillo 2001, Grimm 2001). Subsequently, Shc was found to recruit Grb2 which further associates with Gab2 and SHP2; thereby arguing for a multi-protein scaffolding recruitment of phosphorylated Y1062 (Beset 2000). SHP2 is a protein-tyrosine phosphatase containing two SH2 domains followed by a C-terminal phosphatase domain, and its most N-terminal SH2 domain has been shown to bind distinct phospho-Tyr residues in activated RTKs (Chan 2008). Germline mutations in *Shp2* lead to the Noonan and LEOPARD syndromes characterized by cardiac abnormalities and dysmorphic facial features (Tartaglia 2001). Tyr<sup>687</sup> remained uncharacterized until our investigation described its significance which is presented in paper I.

## RET intracellular pathways: Beyond neurotrophic survival

Tyrosine Y1062 has often been conceptualized as the motor of RET activation, while the other tyrosine residues act more like signaling modulators of RET. Knock-in animals, carrying the silencing mutation of Y1062F, display a similar phenotype as RET null mutants demonstrating the importance of this residue in RET signaling during development (Jijiwa 2004, Jain 2006). *In vitro*, Tyr<sup>1062</sup> activates various intracellular signaling pathways including: the PI3K/Akt, Ras/Erk, Jnk, and p38MAPK (Fig. 5). Downstream activation of these pathways consequentially leads to transcriptional induction by the CREB and NF- $\kappa$ B transcription factors (Hayashi 2000). Extensive bodies of work have been devoted to correlate each signaling pathway to a specific biological response such as: proliferation, survival, migration, cell cycle progression, and neurite outgrowth (Gustin 2007). However, most tyrosines have revealed redundant physiological responses. For instance, the recruitment of Grb2 by Tyr<sup>1096</sup> activates both the PI3K and the MAPK signaling pathways in a similar pattern to Y1062 (Degl'Innocenti 2004, Jain 2006). Moreover, Serine S696 was shown to be phosphorylated by PKA promoting Rac-GEF activity through Jnk resulting in lamellipodia formation of neuroblastoma cell lines (Fukuda 2002). Knock-in animals carrying a mutation of this serine residue into an alanine displayed a severe migratory defect in enteric neural crest cells thereby lacking enteric neurons in the distal colon (Asai 2006).

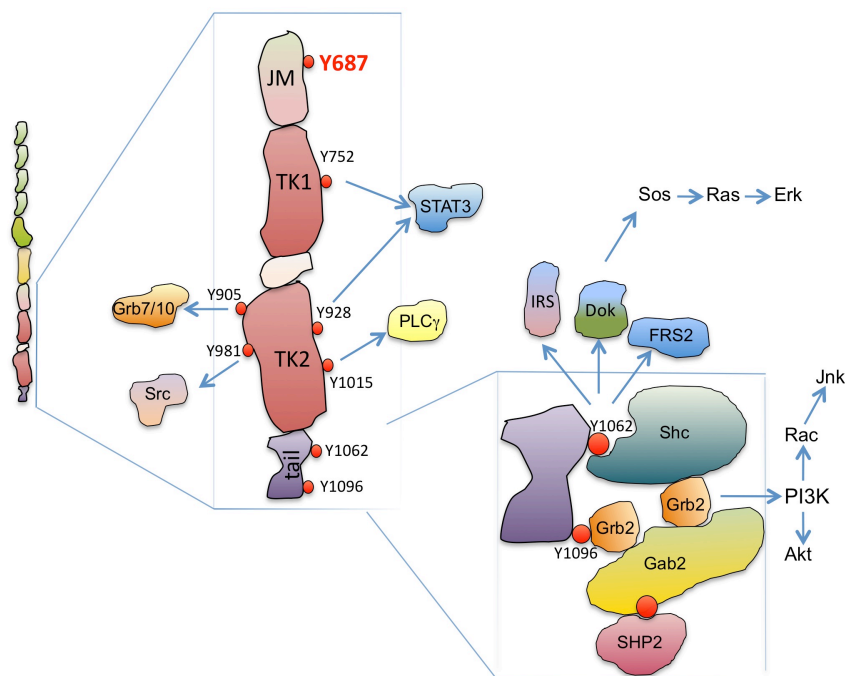


Figure 5: Adaptor proteins known to bind to phosphorylated intracellular tyrosine residues of RET and their respective downstream signaling pathways. Note that the juxtamembrane tyrosine 687 remains uncharacterized.

## **GDNF and RET in disease**

As described in the prologue, GDNF has been extensively studied for its neurotrophic function in support of nigrostriatal dopaminergic neurons in humans (Grondin 1998, Hurelbrink 2001). Extensive efforts on various animal models of amyotrophic lateral sclerosis (ALS), spinal cord injury, and cerebral ischemia have demonstrated the regenerative potential of GDNF on nerve degeneration (Mohajeri 1999, Wang 2002, Suzuki 2007, Cao 2003, Zhang 2009, Kilic 2003).

*Ret* mutations however have been more commonly associated with human diseases. Receptor tyrosine kinases (RTKs) are key regulators of crucial cellular processes such as growth, differentiation, migration, proliferation, and apoptosis (Robinson 2000). Subtle changes in the regulation of RTK activity often result in aberrant signaling which is known to be critically involved in human cancers (Ullrich 1984, Zwick 2001). RET is no exception in the RTK family. Single point mutations lead to the formation of active dimers, inducing constitutive activation of the tyrosine kinase and consequently generating oncogenic transforming potential (Asai 1995). These mutations are found in the RET extracellular domain (ECD) on cysteine residues C609, C611, C618, C620, C630 and C634 and result in a free unpaired cysteine able to form intermolecular disulphide bonds (Santoro 1995, Lai 2007).

Gain-of-function dominant *Ret* mutations have been found in patients with types 2A and 2B multiple endocrine neoplasia (MEN) as well as and sporadic and inherited medullary thyroid carcinoma (respectively MTC, FMTC) (Manie 2001). MEN2A is characterized by MTC and pheochromocytomas; the clinical MEN2B phenotype also includes oral neuromas, ganglioneuromatosis, skeletal abnormalities, and is characterized by a mutation in the residues A883 or M918 (Donis-Keller 1993, Mulligan 1993, Mulligan 1994). Loss-of-function mutations of the *Ret* allele have been found in Hirschsprung's disease (HSCR), which is a congenital developmental condition characterized by incomplete innervations of the distal gut (Edery 1994, Kashuk 2005). Although they were not causal of HSCR, two germline *Gdnf* mutations leading to the P21S and the I211M residue substitutions have been reported (Salomon 1996, Martucciello 1998, Martucciello 2000). Approximately 90% of all HSCR cases are directly linked to a *Ret* gene mutation (Lantieri 2006).

## **AIMS**

The principal objective of this thesis was to explore the signaling mechanisms activated by GDNF that control critical cellular events such as: neuronal survival, growth, migration, and differentiation.

Specifically:

1. Evaluate the role of the juxtamembrane tyrosine residue 687 of RET in promoting neuronal survival and neurite outgrowth in SCG neurons.
2. Determine the self-association mechanism of the RET transmembrane domain underlying oncogenic MEN2A mutations.
3. Elucidate the role of GDNF and GFR $\alpha$ 1 signaling in GABAergic differentiation and cell migration of cortical interneuron precursors of the medial ganglionic eminence.

## COMMENTS ON MATERIALS AND METHODS

The materials and methods used in this thesis have been described in the papers I-III. Please refer to the corresponding methods section of each paper for a detailed account. The following comments briefly characterize the experimental methodology for clarification.

### NEURONAL SURVIVAL

In paper I, we studied the role of RET-mediated neuronal survival in sympathetic neurons isolated from superior cervical ganglia (SCG). Dissociated neurons were microporated with the RET plasmid of interest together with dsRed for subsequent visualization in an Axiovert 200M inverted fluorescence microscope (Zeiss). After the first day in culture (DIV1), individual neurons were imaged and their precise spatial position was recorded in order to follow their daily survival using the OpenLab software (Improvision). Axonal fragmentation followed by cell soma collapse was evaluated to quantify cell death (Fig. 6). Subsequent TUNEL staining confirmed apoptotic cell death.

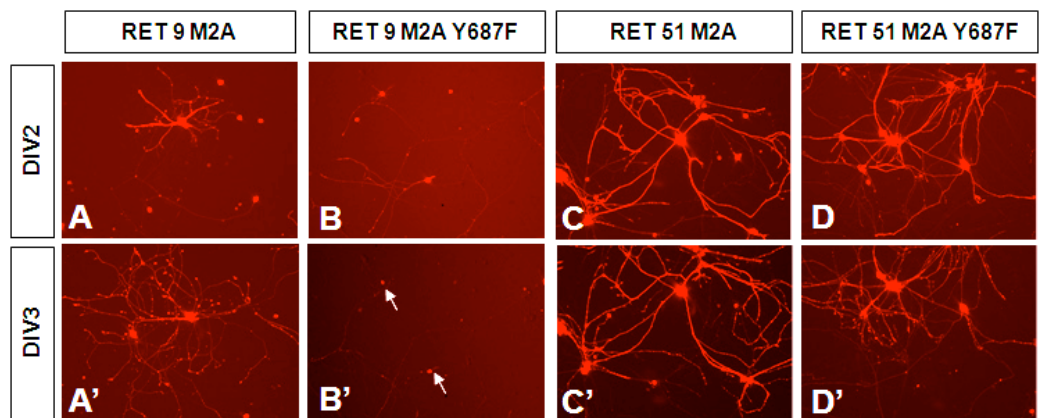


Figure 6: SCG neurons expressing specific RET constructs together with dsRed following 2 and 3 days in culture, respectively. Axonal fragmentation is observed in panel B'.

## NEURITE OUTGROWTH

### RET-mediated neurite outgrowth in sympathetic neurons

In paper I, we examined the role of tyrosine residue Y687 on neurite outgrowth of SCG neurons. Dissociated neurons were microporated with respective plasmids together with dsRed, plated and imaged 30 hours later. The longest neurite of each neuron was traced and quantified with OpenLab (Fig. 7A). A representative composite image of all traced neurites within a single well was also included in the paper.

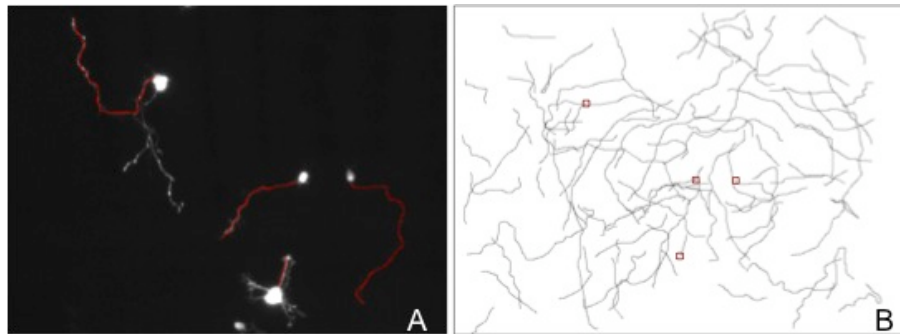


Figure 7: Neurite traces of SCG neurons are shown in panel A. Red squares in panel B represent the cell soma of neurons shown in panel A. A representative composite image of all traces within a well is shown in panel B, note that both panels are different in scale.

### GABAergic neurite outgrowth of MGE neurons

In paper III, we quantified GABAergic neurite outgrowth of neurons isolated from the medial ganglionic eminence (MGE). Cells were dissociated and incubated in their respective treatment conditions for 48 hours before fixation, staining with anti-GABA antibodies and counterstained with DAPI. Pictures were taken with an Axiovert 200M inverted microscope using OpenLab. Neurite outgrowth counts were performed using the ImageJ software (NIH) on the GABAergic staining micrographs, setting cell perimeter threshold above 18000 and cell circularity below 0.5. These parameters designate a differentiated GABAergic bearing neurites to be longer than thrice the cell body diameter and excluding undifferentiated circular cells (Fig. 8).

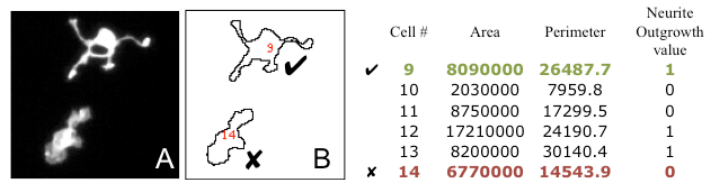


Figure 8: GABAergic neurite outgrowth counts. GABAergic immunostainings are depicted in panel A. Cell perimeter threshold measurements using ImageJ are shown in panel B. The table illustrates the value obtained for cell perimeter and area. Note that cell number 9 (as indicated by ✓ in panel B) has extended neurites meeting neurite outgrowth threshold requirements, whereas cell number 14 (as identified by ✗) does not. Not all cells presented in the table are visible in panel A.

## NEURONAL MIGRATION

In paper III, we analyzed the migration of cultured MGE, LGE and CGE GABAergic neurons. Freshly dissected tissue was dissociated and neurons were subsequently plated on a pre-coated PDL filter of a Boyden ChemoTx (Neuroprobe) chamber with a 5 µm pore size in a 96-well format. Dissociated cells were resuspended and plated in the top chamber in a 50 µL drop as shown (Fig. 9, panel A). Cell migration was induced by adding growth factors or blocking antibodies in the lower compartment of the chamber. Cultures were incubated for 48 hours, after which the cells remaining on the top of the filter were gently scrapped away with a cotton tip. The filters were then fixed in 4% PFA and stained with DAPI. The lower side of the filters was imaged with an Axiovert 200M inverted fluorescence microscope (Zeiss) in the DAPI channel and quantified using the ImageJ software (NIH) (Fig 9B).

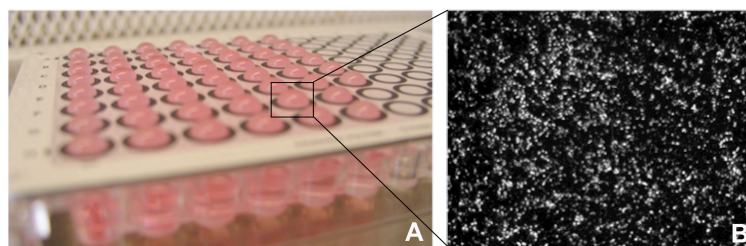


Figure 9: A representative Boyden chamber used in the study is shown in panel A. Cell migration counts were performed on DAPI counterstainings of the filter depicted in panel B.



# RESULTS AND DISCUSSION

## PAPER I

Upon activation, the RET tyrosine kinase receptor undergoes autophosphorylation of intracellular tyrosine residues, which then serve as docking sites for downstream signaling effectors carrying Src homology (SH2) or phosphotyrosine-binding domains (PTB). Previous studies have indicated that at least 14 of the 18 tyrosine residues present in the intracellular region of RET can become phosphorylated. Of the remaining residues, only four have actually been shown to directly bind to a downstream effector in response to GDNF. The function of the majority of phosphorylated tyrosine residues in RET, including Tyr<sup>687</sup>, remains unknown. Tyr<sup>687</sup> is located in the juxtamembrane region of the RET receptor, a domain known to exhibit regulatory functions in other catalytic receptors; signifying that Tyr<sup>687</sup> may be important for RET activation or for its downstream effects.

In the first paper of this thesis, we evaluated the role of the juxtamembrane tyrosine residue Y687 of RET in GDNF-mediated signaling. Primarily, we aimed to identify the novel adaptor protein binding this phosphorylated tyrosine residue and subsequently, to understand the biological significance of this interaction. To achieve this, we prepared a T7 phage display cDNA library using differentiated PC12 cells. For the screening of the library, we employed as bait a synthetic phosphopeptide derived from human RET encompassing phospho-Y687 and flanked with 10 amino acid residues. The screen retrieved a phage clone encoding the N-terminal SH2 domain of the protein tyrosine phosphatase SHP2. We further showed that this phage clone was bound to the phospho-Y687 bait and was not bound to baits encompassing other RET phospho-Tyr residues. This result therefore argued for a direct interaction between RET and SHP2. In contrast, SHP2 has been shown to bind indirectly to RET at tyrosine residue Y1062 with the help of Gab2/Grb2 (Fig. 5) (Besset 2000). Noticeably, the SHP2 immunoreceptor tyrosine-based inhibitor motif (ITIM) includes a valine residue at the -2 position (I/V/L/S-x-pY-x-x-L/V), which is reminiscent of the RET target sequence VSpY<sup>687</sup>SSS (Songyang 1993, Liu 2006). We further showed that in the mammalian COS cell line, full length SHP2 requires the phosphorylation of Y687 to be bound to RET in a peptide pulldown assay using agarose beads coated with either the phosphorylated or the non-phosphorylated peptides used as baits.

Next, we analyzed the SHP2 binding determinants in RET using site-directed mutagenesis of selected residues. Endogenous SHP2 was immunoprecipitated in COS cells transfected with plasmids expressing the constitutively active form of RET, carrying the C634R mutation found in MEN2A patients. Both MEN2A-RET 51 and 9 isoforms were pulled down to similar levels with SHP2. In this background, we showed that the mutation of the tyrosine 687 into a phenylalanine (hereafter termed Y687F) failed to recover any RET protein in the SHP2 immunoprecipitates; thereby validating Y687 as novel site for SHP2 binding. Unexpectedly, mutation of tyrosine 1062 into a phenylalanine also abrogated the RET/SHP2 interaction, even though Y1062 is known to be a binding site for the multi-adaptor scaffolding complex Gab2/Grb2/SHP2 through Shc (Besset 2000). These results suggested that SHP2 might not be recruited to these two sites independently. Instead SHP2 may be forming a single signaling complex and binding both sites at the same time (Fig. 10); thus acting as a regulatory complex for RET signaling output.

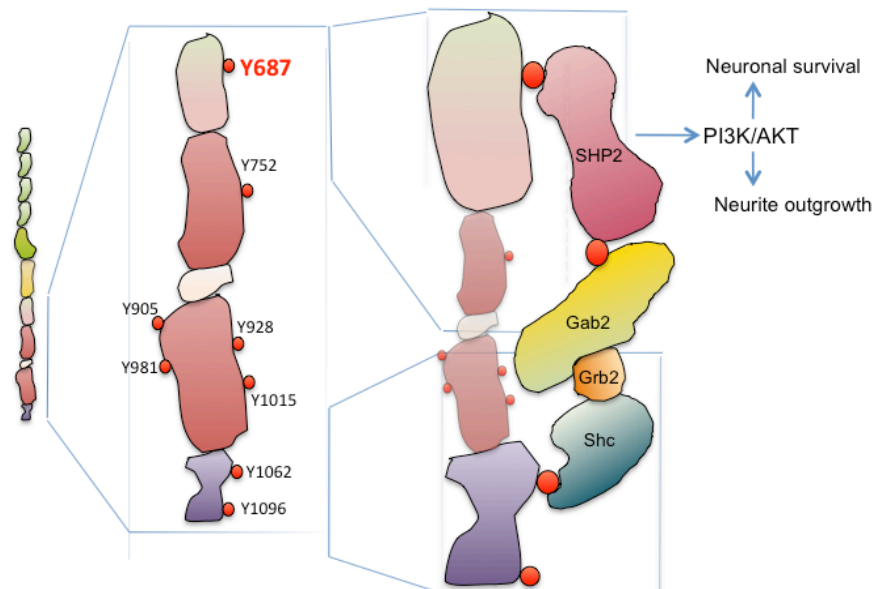


Figure 10: Theoretical schematic of SHP2 recruitment to both tyrosine 687 and 1062, illustrating SH2-domain interactions with their respective phosphorylated tyrosine targets. Note the dimeric SHP2 molecule interacting with two phospho-Tyr targets simultaneously.

To further investigate this hypothesis, we analyzed the influence of the Y687F mutation on the multi-adaptor assembly site Tyr<sup>1062</sup>. If SHP2 were to be part of a multi-adaptor scaffolding complex binding directly to Y687 and indirectly to Y1062, then Shc or Gab2 immunoprecipitation studies would not only reveal intact levels of either protein binding to RET in the Y687F mutant compared to the wild-type, but also lower levels of SHP2. Indeed, association of SHP2 with Shc and Gab2 was diminished in the mutant, whereas RET levels were unaffected by the mutation. This data strongly argues that Tyr<sup>687</sup> is necessary for SHP2 recruitment to Y1062, and regulates multi-adaptor complex assembly.

While mapping the SHP2 binding determinants in RET, we found that the mutation of the serine residue S696 into an alanine strongly potentiated the binding of SHP2 to RET. This serine residue was shown to have an epistatic interaction with Y687 in lamellipodia formation of SK-N-MC cells (Fakuda 2002). When inserted into the Y687F mutant, the S696A mutation displayed a total loss of binding to SHP2 - arguing for a direct interaction between these two phosphorylation sites of RET. We further showed that forskolin treatment, an activator of adenylate cyclase, impaired SHP2 binding to RET upon GDNF stimulation in the MN1 cell line where both molecules are endogenously expressed. Forskolin has been shown to induce the phosphorylation of S696 of RET through PKA (Fakuda 2002). From these experiments, we concluded that the phosphorylation of serine 696 negatively regulates the binding of SHP2 to tyrosine 687 in RET. Unfortunately, the juxtamembrane was not included in the crystallized structure of the RET intracellular domain as recently published; however we advocate for a close proximity between S696 and Y687 with a possible reciprocal interference when phosphorylated (Knowles 2006).

To understand the role of RET Y687 in GDNF-mediated signaling, we generated clonal fibroblast cell lines stably expressing either wild-type or the RET Y687F mutant. Although we found no major changes in the phosphorylation levels of RET, the Akt pathway - a downstream pathway of RET and PKA - was however strongly reduced in the mutant and never reached the levels of wild-type RET. Other downstream pathways such as Erk, Jnk, and Src were unaffected by the mutation, suggesting that RET signaling through Y687 may have a role in Akt-mediated biological events such as cell differentiation and survival. Consequently, we examined the role of mutation Y687F in neuronal differentiation of PC12 cells. We found a major reduction of RET-mediated neuronal differentiation in the Y687F mutant using the RET MEN2A background, after both 3 and 6 days of differentiation induction. We confirmed this result by employing GDNF-mediated differentiation instead of the constitutively active receptor. In accordance with our earlier data, inferring a negative role of PKA activity in SHP2 binding to RET, we observed that pre-treatment of PC12 cells with forskolin significantly reduced neuronal differentiation mediated by GDNF in the RET wild-type construct but not in either the Y687F mutant or the S696A mutant.

Finally, we evaluated the physiological role of tyrosine 687 in primary cultures of sympathetic neurons. Although SCG neurons endogenously express RET and GFR $\alpha$ 1, we microporated the constitutively active form of RET to bypass the need of a ligand that would activate the endogenous receptors. We found that the Y687F mutation in both RET isoforms significantly reduced neurite outgrowth at DIV1, in agreement with our results obtained in PC12 cells. Since the Akt pathway is a mediator of neuronal survival, we expected to observe an increase in cell death of neurons expressing the RET mutant. We therefore set up a neuronal survival assay, in which SCG neurons were left to survive solely on the expression of the microporated constitutively active RET construct. SCG neurons were first microporated with the different RET constructs, incubated with minimal amounts of NGF for one night for cell recovery, and then cultured in  $\alpha$ -NGF blocking antibody for a couple of days. From DIV3 on, we found that neurons expressing RET9 M2A Y687F exhibited axonal degeneration followed by cell soma collapse and were unable to sustain neuronal survival. Subsequent analysis with TUNEL staining confirmed that these neurons were apoptotic.

Taken together, our results have identified SHP2 to be the first effector protein to bind to the phosphorylated tyrosine 687 of RET. Our evidence assigns a central role for Tyr<sup>687</sup> in GDNF neurotrophic outputs and substantiates the evidence that its juxtramembrane location serves as a critical spatial integrator for the regulation of both RET and PKA signaling.

## PAPER II

Patients with medullary thyroid carcinoma (MTC) and type 2A multiple endocrine neoplasia (MEN2A) contain cysteine residue mutations in the extracellular juxtamembrane region of RET. These mutations cause the formation of covalent receptor dimers linked by intermolecular disulfide bonds between unpaired cysteines, followed by oncogenic activation of the RET kinase. The close proximity to the plasma membrane of the affected cysteine residues prompted us to investigate the possible role of the RET transmembrane domain (RET-TM) in receptor-receptor interactions underlying dimer formation.

In the second published article, we determined precisely how the RET-TM engages in homo-dimer formation by unpaired cysteine residues in the context of mutations found in MEN2A and MTC patients. Receptor tyrosine kinases, such as RET, have a single-pass transmembrane domain that is thought to stabilize ligand-induced dimerization and to

maintain receptors in close proximity in the plasma membrane. Gain of function *Ret* mutations typically found in human diseases involve cysteine residues in close proximity with the extracellular transmembrane of RET. MEN2A and MTC patients carry such mutations in juxtamembrane cysteine residues. Although the cyteine-rich domain (CRD) of RET spans over 120 residues, most MEN2A and MTC mutations are found within the 25 residues of the plasma membrane, prompting us to examine the role of the transmembrane domain in self-association and oncogenic activation (Fig. 11).

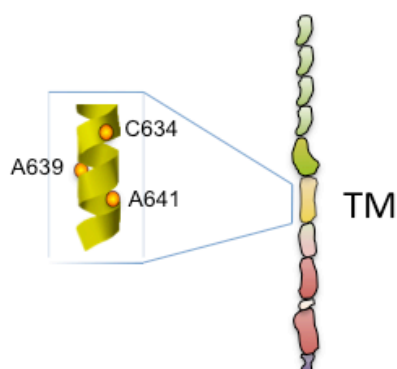


Figure 11: Schematic highlighting amino acid residues within the RET transmembrane (TM) domain that self-associate upon oncogenic activation in the the MEN2A syndrome. 87% of MEN2A patients patients carry a mutation in cysteine 634 634 (Cuccuru, 2004).

To study the propensity of the RET-TM to self-associate we used the reporter ToxCAT system, which allowed us to quantify the efficiency of TM-TM interactions in a biological membrane. This reporter is based on the capacity of the ToxR transcriptional activator of the *Vibrio Cholerae* pathogen to bind to the cholera toxin (*ctx*) gene promoter only in dimerized form. Therefore, we fused the RET-TM (amino acids R635 to F657) to ToxR and the maltose-binding protein (MBP) for precise delivery to the *Escherichia coli* inner membrane. The fusion construct ToxR-RET-TM-MBP will then form dimers in the cytosol proportional to the oligomerization ability of the RET-TM domain. Following the binding of dimerized ToxR-RET-TM-MBP to the *ctx* DNA-binding element, the chloramphenicol transferase (*cat*) gene reporter was activated and consequent production of the CAT protein was quantified by ELISA. Indeed, the RET-TM showed a strong propensity to self-associate as the levels were comparable to other reported dimeric transmembrane receptors such as the ErbB family of receptors (Mendrola 2002). We then used a mutagenesis approach to find the self-association determinants of the RET-TM. By focusing our analysis on conserved serine and arginine residues in the RET-TM, we found the RET S649A and A641R mutants to have respectively a 50% and 80% loss of self-association measured by CAT-ELISA compared to the wild-type.

Interestingly, a rare case of the double mutation A639G/A641R in exon 11 was found in a tumor from a patient with sporadic MTC (Kalinin 2002). This double missense mutation involves three nucleotide substitutions in the first and last residues of a triad of alanines. Yet the mechanism of how these residue substitutions disrupt RET-TM activity remained poorly understood. Thus, we included the A639G mutation into the wild-type fusion construct and into our A641R mutant. The A639G mutation marginally reduced RET-TM self-association. However when A639G was inserted together with A641R, the double mutant was not different from A641R alone as it displayed the same 80% reduction of relative self-association compared to the wild-type. To determine if the double mutations impaired RET function, we generated two clonal fibroblast cell lines stably expressing either the wild-type or the mutant construct. The double mutant did not display any phenotypic change when compared to the RET wild-type following GDNF stimulation. Both constructs were expressed at similar levels and the mutant did not display any impairment in expression of the mature form of RET. However, low basal RET activity was found in the double mutant through Western blot analysis using a phospho-specific antibody. Even though the A639G/A641R mutations showed detrimental effects on RET-TM self-association, the lack of spontaneous RET activity did not support a causative role for these mutations in MTC tumor formation.

From this, we concluded that the two arginines A639 and A641 are crucial for RET dimerizing activity but do not promote an increase in RET basal activity. Nevertheless, it remains possible that these two arginines can stabilize RET homodimerization in a constitutively active setting such as the oncogenic RET C634R mutation found in MEN2A and FMTC patients. Hence, we analyzed dimer formation mediated by the C634R mutation in fibroblasts transfected with construct carrying or not the double arginine mutation. When running RET immunoprecipitates on a non-reducing SDS-PAGE, we observed RET dimers in the C634R mutant when probing the membrane with a RET-specific antibody. However, the triple mutant C634R/A639G/A641R showed a statistically significant 47% reduction in dimer formation. Besides, the transforming activity of the triple RET mutant was reduced to background levels in foci formation of fibroblasts plated in soft agar.

Taken together, our results highlight the importance of TM-TM interactions in the self-association of RET by the MEN2A oncogenic activation. Both residues that we found to disrupt the self-association of RET are aligned on the same surface of a membrane-spanning  $\alpha$ -helix. With respect to the *Ret* gain of function mutation, our results bring a novel insight into the crucial role this transmembrane domain plays in the conformational stabilization of oncogenic homodimers.

### PAPER III

In humans, several psychiatric disorders have been correlated with cortical inhibitory interneuron malfunction including epilepsy, schizophrenia, and autism (Cossart 2005, Levitt, 2005; Rubenstein 2003). Therefore, current research has focused its attention on understanding the signaling mechanisms that control the development of cortical GABAergic interneurons. GDNF is known to promote the differentiation and migration of MGE-derived GABAergic neuronal precursors (Pozas 2005). These functions have been shown to be dependent on the GPI-anchored receptor  $GFR\alpha1$ , but are independent of the two known transmembrane receptor partners RET and NCAM. Since neither RET nor NCAM are required for the effects of GDNF on GABAergic interneuron migration and differentiation, then  $GFR\alpha1$  must somehow be able to either mediate GDNF signaling on its own or  $GFR\alpha1$  may partner with other transmembrane effectors expressed on these cells. Thus, the aim of the last paper was to elucidate the role of GDNF and  $GFR\alpha1$  signaling in GABAergic differentiation and migration of cortical interneuron precursors of the medial ganglionic eminence.

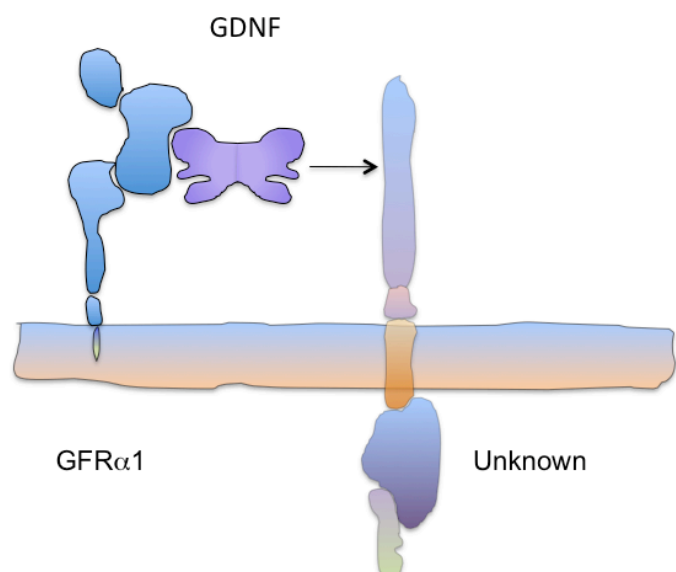
First, we evaluated if soluble  $GFR\alpha1$  would be sufficient to promote neurite outgrowth of GABAergic positive MGE neurons. To test the effect of soluble  $GFR\alpha1$ , we used a purified chimeric protein of the  $GFR\alpha1$  ectodomain (excluding the GPI-anchoring sequences) fused to the Fc domain of a human immunoglobulin ( $GFR\alpha1$ -Fc). Indeed, we found that soluble  $GFR\alpha1$ -Fc promoted the GABAergic neurite outgrowth of these cells and that GDNF further potentiated these effects. However, when both GDNF and  $GFR\alpha1$ -Fc were combined the effects were not strictly additive. This result alluded to the likelihood that the same cell population was responding to either treatment.

Since GDNF was reported to promote the tangential migration of GABAergic interneurons to the cortex, we tested both GDNF and  $GFR\alpha1$ -Fc in a Boyden chamber assay to assess cell migration. In this system, dissociated cells are seeded on the top chamber and left to migrate through the pores towards the lower compartment where substances to be tested are presented. Both GDNF and  $GFR\alpha1$ -Fc promoted cell migration measured after 48 hours. However, dissociated CGE and LGE neurons failed to migrate into the lower compartment when incubated with GDNF. This suggested that GDNF acts specifically on interneuron precursors from the MGE, which expresses  $GFR\alpha1$ , but does not act on interneuron precursors of the LGE and CGE, as no  $GFR\alpha1$  expression is observed in these eminences at E12.5 in mice.

After showing that both GDNF and soluble GFR $\alpha$ 1 induce GABAergic neurite outgrowth and migration of MGE neurons, we next verified if the biological effects of exogenous GFR $\alpha$ 1-Fc were still dependent on GDNF or were independent of its known ligand. Since both GDNF and GFR $\alpha$ 1 are expressed in the MGE at E12.5, we anticipated a loss of neurite outgrowth by GFR $\alpha$ 1-Fc if endogenous GDNF was required for mediating this effect. Although GDNF mediated both neurite outgrowth and cell migration at similar levels between both wild-type and *Gdnf*<sup>-/-</sup> neurons, soluble GFR $\alpha$ 1 indeed failed to induce any biological effect in knock-out neurons. Interestingly, these detrimental effects could be rescued when GDNF and GFR $\alpha$ 1-Fc were applied concurrently. These results demonstrate that endogenous GDNF production is required for soluble GFR $\alpha$ 1-Fc to mediate both GABAergic neurite outgrowth and migration of MGE neurons.

We further evaluated if soluble GFR $\alpha$ 1 could act in a non-cell autonomous fashion and treated *Gfra1*<sup>-/-</sup> neurons with GFR $\alpha$ 1-Fc. GDNF did not promote any differentiation or migration in primary MGE neurons from *Gfra1* knock-out embryos, confirming GFR $\alpha$ 1 as a receptor of GDNF in these cells. However, soluble GFR $\alpha$ 1 was sufficient to rescue these knock-out neurons in both GABAergic outgrowth and cell migration to similar levels observed in wild-type cells. Additionally, when both GDNF and GFR $\alpha$ 1-Fc were added together, *Gfra1*<sup>-/-</sup> neurons differentiated and migrated to a similar degree as wild-type cells. These compelling observations can only be explained by the existence of a novel transmembrane signaling partner for the GDNF/GFR $\alpha$ 1 complex in MGE GABAergic neurons. (Fig. 12).

Figure 12: Illustration depicting the probable interaction between the GDNF/GFR $\alpha$ 1 signaling complex and the novel transmembrane receptor which mediates GABAergic neurite outgrowth and cell migration in MGE neurons.





To investigate potential receptor candidates, we analyzed if ErbB4 could mediate GDNF/GFR $\alpha$ 1 signaling activities, since this Neuregulin-1 receptor is known to mediate the tangential migration of GABAergic MGE neurons (Flames 2004). To these means, we employed a biochemical approach and transiently stimulated wild-type MGE cultures with GDNF or Neuregulin-1. Although Neuregulin-1 induced a robust increase of the phosphorylation state of ErbB4 observed by Western blot, GDNF did not induce any phosphorylation of the receptor above background levels. This indicated that GDNF does not signal via ErbB4. When incubating MGE neurons with the specific pharmacological ErbB4 inhibitor AG1478, we did not observe any GABAergic differentiation induced by the inhibitor alone. Moreover, when added together with GDNF, the inhibitor failed to inhibit the differentiation mediated by GDNF. Similar results were obtained with the Boyden chamber migration assay, thereby supporting the evidence that ErbB4 is not a receptor for GDNF in MGE neurons.

We then selected another receptor candidate, the HGF receptor MET, also known to mediate the tangential of MGE neurons into the cortex (Powell 2001). With the same rationale, we transiently stimulated MGE cultures with GDNF or HGF and analyzed the phosphorylation levels of the MET receptor. Although HGF induced a rapid increase of MET phosphorylation detected by a phospho-MET antibody in a Western blot, GDNF did not phosphorylate MET above background levels. Interestingly, the downstream pathway Erk was stimulated by both ligands, which confirmed previous evidence that GDNF activities are mediated through the ERK pathway (Pozas 2005). To test if MET was involved in GDNF-induced neurite outgrowth in MGE cells, we stimulated MGE cultures with GDNF in the presence or absence of the specific pharmacological MET kinase inhibitor SU11274. To our surprise, SU11274 induced GABAergic neurite outgrowth on its own and at comparable levels to GDNF. Interestingly, at the concentration of 1 $\mu$ M SU11274 blocked the activation of the Erk pathway, indicating that GABAergic neurite outgrowth is independent of the activation of the Erk pathway. When both GDNF and SU11274 were added together, the effects on neurite outgrowth were additive. To eliminate any undesired off-target effects of this inhibitor, we tested neurite outgrowth with another MET inhibitor PHA665752. Our previous evidence was confirmed as PHA665752 did induce GABAergic neurite outgrowth by itself. Taken together, we concluded that ErbB4 and MET are not the transmembrane receptors mediating GDNF activities in MGE neurons. Instead, the inhibition of MET signaling unexpectedly promoted GABAergic neurite outgrowth in these cells.

Since the MET inhibitor SU11274 promoted neurite outgrowth by itself, we then decided to further investigate the role of MET signaling inhibition in MGE neurons using an antibody blocking HGF signaling. We chose this approach because it would eliminate the possibility of unspecific pharmacological inhibition of other kinases than MET. Although HGF did not promote any neurite outgrowth, the  $\alpha$ -HGF blocking antibody induced robust GABAergic neurite outgrowth to a higher level than GDNF. When GDNF and  $\alpha$ -HGF were added together, the effect on neurite outgrowth was additive; similar to the effect observed when GDNF and SU11274 were added together. Interestingly, HGF did not reduce the effect of GDNF when both were added together. In Boyden chambers,  $\alpha$ -HGF proved to be more potent than GDNF in promoting cell migration, even with a synergistic induction when both were added simultaneously.

These results prompted us to further analyze if the biological outputs observed in MET signaling inhibition were dependent on GDNF signaling. Therefore, we utilized a  $\alpha$ -GDNF blocking antibody to inhibit all GDNF-mediated signaling in these neurons. As control experiments, we first tested the specificity of the antibody in inhibiting GDNF activities in neurite outgrowth. We also evaluated this GDNF inhibition in conjunction with BDNF, another ligand promoting neurite outgrowth in MGE neurons (Pozas 2005), and found that  $\alpha$ -GDNF failed to inhibit the GABAergic neurite outgrowth mediated by BDNF. Interestingly, when added together with  $\alpha$ -GDNF,  $\alpha$ -HGF also failed to induce any GABAergic neurite outgrowth above background levels. These results argue that  $\alpha$ -HGF is dependent on endogenous GDNF signaling to mediate GABAergic neurite outgrowth of MGE neurons.

To verify if  $\alpha$ -HGF requires endogenous GDNF signaling to mediate neurite outgrowth, we utilized an in vitro approach and cultured knock-out neurons. Therefore, we assessed GABAergic neurite outgrowth and cell migration in cultured MGE neurons derived from *Gfra1*<sup>-/-</sup> embryos and treated them with  $\alpha$ -HGF. Both biological outputs showed an impairment of  $\alpha$ -HGF to exert its activity in *Gfra1*<sup>-/-</sup> neurons, confirming that  $\alpha$ -HGF is dependent on endogenous GDNF/GFR $\alpha$ 1 signaling. Moreover, these results imply that HGF signaling through MET may restrict GDNF activities in these neurons since MET is broadly expressed in the MGE at E12.5.

To test this hypothesis, we used MGE cultures derived from MET mutant mice carrying a double (D) point mutation of two tyrosine residues into phenylalanine in the intracellular domain the MET receptor. *MET*<sup>D/D</sup> embryos display the same phenotype as MET null mutants and are incapable of MET signaling induction both *in vitro* and *in vivo* (Maina 1996). We observed an enhanced response to GDNF in *MET*<sup>D/D</sup> neurons both in GABAergic neurite outgrowth and migration. Conclusively, these neurons exhibited a statistically significant increase in GABAergic neurite differentiation and cell migration even in untreated conditions.

In order to understand how MET signaling could negatively regulate GDNF signaling, we investigated the expression of both MET and GFR $\alpha$ 1 in the MGE. Both receptors were expressed in the postmitotic mantle zone of the MGE. While MET was more broadly expressed than GFR $\alpha$ 1, some cells did exhibit co-expression of both receptors. Next, we performed quantitative real-time PCR of RNA extracted from wild-type MGE neurons that were treated for 48 hours and measured *Gfra1* mRNA expression. Surprisingly, we found that dissociated MGE cells cultured for two days in control conditions displayed significantly reduced levels of *Gfra1* mRNA compared to freshly dissected E12.5 MGE tissue. Incubation of these cells with GDNF restored *Gfra1* mRNA expression in DIV2 MGE cultures to similar levels displayed by the freshly dissected E12.5 MGE tissue. GFR $\alpha$ 1-Fc also had an effect on *Gfra1* mRNA expression but was lower than GDNF. We further showed that the  $\alpha$ -HGF blocking antibody and the SU11274 Met inhibitor increased the level of *Gfra1* mRNA in cultured MGE cells to a similar extent as GDNF. As the trophic effects of HGF/Met inhibition were mediated by endogenous GDNF signaling, we tested if the effects observed on *Gfra1* mRNA expression were also dependent on GDNF and therefore combined  $\alpha$ -HGF and  $\alpha$ -GDNF treatments to assess the levels of *Gfra1* mRNA expression. Unlike its effect on neurite outgrowth,  $\alpha$ -GDNF did not impair the ability of  $\alpha$ -HGF to increase *Gfra1* mRNA levels in our MGE cultures, suggesting that endogenous HGF/MET represses *Gfra1* mRNA expression in MGE cells.

Together, these results conclude that endogenous HGF signaling through MET restricts the effects of GDNF/GFR $\alpha$ 1 signaling in both the differentiation and migration of GABAergic MGE neurons. This negative regulation of GDNF signaling by MET may play a crucial role in early developmental diversification of GABAergic interneuron subtypes in the MGE.

## CONCLUSION

When GDNF was discovered seventeen years ago, it was merely acknowledged for its capacity to promote the survival of embryonic midbrain dopaminergic neurons. Extensive work from recent years has now appropriately recognized the diverse biological influence of this growth factor in nervous system development and function. This thesis has contributed to our understanding of the physiological and cellular events mediated by GDNF and its receptors by offering the following new insights:

- Discovery of the first adaptor protein to bind phospho-Tyr<sup>687</sup> of RET.
- Identification of the first direct binding site for SHP2 in RET.
- Promotion of sympathetic neuron survival and neurite outgrowth mediated by SHP2 binding to RET Y687.
- Elucidation of the self-association determinants of the RET transmembrane domain.
- Description of how the RET-TM self-associates and stabilizes receptor-receptor interactions in constitutive dimers formed by the C634R mutation found in MEN2A and MTC patients.
- Localization of crucial residues for RET TM-TM interactions assuming a  $\alpha$ -helix transmembrane conformation.
- Demonstration of soluble GFR $\alpha$ 1's ability to promote GABAergic neurite outgrowth and migration in primary MGE neurons.
- Justification for the presence of a novel transmembrane receptor expressed in the MGE for the GDNF/ GFR $\alpha$ 1 signaling complex
- Validation of MET signaling restriction on GDNF activities in MGE neurons.

Our understanding of how neurons precisely integrate and interpret GDNF signaling remains unclear. Further investigation is necessary to clarify the effects these signals have on cellular function. Specifically, it would be significant to identify the function of other phosphorylated tyrosine residues in RET and in other RTKs. This information would allow for a better application of RET-mediated signaling in a clinical context. With respect to GDNF signaling, it will be important to identify the novel receptor partner of the GDNF/GFR $\alpha$ 1 complex and evaluate its interaction with MET signaling. A thorough understanding of the molecular mechanisms by which GDNF functions in different neuronal population will be essential in elucidating its contribution to nervous system development and exploiting its therapeutic potential.

## EPILOGUE

Seventeen years have passed since scientists initially embarked on the hopeful quest to make GDNF a therapeutic agent for Parkinson's disease patients. Since then our knowledge of GDNF's mechanisms of action has staggeringly increased; while the technological developments for GDNF delivery into the human brain have unfortunately faltered. A recent clinical trial by Ceregene, employing an adeno-associated virus encoding the *Neurturin* gene, failed to show any symptomatic improvement in 58 PD patients even one year after the virus injection. The first post-mortem brains of this study confirmed that only 15% of the putamen was expressing the recombinant protein. Once again, the therapeutic agent did not reach the target tissue accurately (Vastag 2010). However, GDNF demonstrated clinical improvement for PD patients in a singular clinical trial, where subsequent post-mortem brain analysis showed massive sprouting in neuronal fibers of the putamen - the site where the catheter had been surgically inserted (Gill 2003, Love 2005).

Scientifically much work remains before we will clearly understand the dynamic capabilities of GDNF and be able to apply them in a clinical setting. Nevertheless, my hopes have not faltered, as I believe there is a future for GDNF as therapeutic agent, even beyond Parkinson's disease. GDNF has yielded both neuroprotective and regenerative results in animal models of spinal cord injury, nerve trauma, ALS, and stroke. These prospective hopes now rely on the design of newly engineered technologies for drug delivery, as well as, on the will of scientists to bring the findings of their research endeavors from the bench to the bedside where they deservedly belong...

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