GDNF and GFRαs Signaling in Frog Cardiac Ganglion

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Preface

This study was performed at The Neuroscience Center at the University of Helsinki. I want to thank my supervisor Matti Airaksinen for all the support and encouragement. I also owe my gratitude to the whole group: Jussi, Kaija, Pavel, Tiina and Marika for all their help. It felt privileged to be able to work with you. Many thanks also to Crisse for helping me with the frogs. Great thanks to my dear friends Salla and Sari, with whom I shared the pleasure of studying, in and outside the lab. I also want to thank my family. Most of all, I want to express my gratitude to Simo for all his love and support.

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Tiivistelmä:

GDNF-perhe on ryhmä hermokasvutekijöitä, jotka toimivat hermosolujen kasvussa, selviytymisessä, migraatiossa ja erilaistumisessa. GDNF-perheeseen kuuluvat gliasolulinjaperäinen hermokasvutekijä (GDNF), neurturiini (NRTN), artemiini (ARTN) sekä persefiini (PSPN).
Gliasolulinjaperäinen hermokasvutekijä löydettiin alunperin viljeltyjä dopaminergisia hermosoluja ylläpitävänä tekijänä. Se toimii myös tehokkaana liikehermosolujen kasvutekijänä selkäytimessä, ja sillä on tärkeä rooli myös hermoston ulkopuolella munuaisten morfogeneesissä ja spermatogeneesissä. GDNF-perheen ligandit viestivät GPI-sidoksella solukalvoon kiinnittyneen GDNF-perheen apureseptorin (GFRα) ja solukalvon läpäisevän RET-tyrosiinikinaasireseptorin muodostaman kompleksin kautta. Jokaisella GDNF-perheen ligandilla on oma ensisijainen apureseptorinsa. GDNF viestii ensisijaisesti GFRα1/Ret kompleksin kautta, NRTN GFRα2/Ret kompleksin kautta, ARTN GFRα3/Ret kompleksin kautta ja PSPN GFRα4/Ret kompleksin kautta. Osa GDNF-perheen ligandeista voi viestiä myös jonkin toisen kuin ensijaisen apureseptorinsa kautta. NRTN ja ARTN voivat viestiä myös GFRα1:n kautta ja GDNF GFRα2:n kautta.

Nisäkkäiden monet parasympaattisen hermoston esisolut tarvitsevat GDNF/GFRα1/RET-kompeksin kautta tapahtuvaa viestintää lisääntymiseen ja migraatioon. Postmitoottiset hermosolut vaihtavat GDNF-välitteisen viestinnän NRTN-välitteiseen viestintään alassäätelemällä GFRα1:n ilmentymisen ja ylössäätelemällä GFRα2:n ilmentymisen. NRTN/GFRα2/RET-kompleksin kautta tapahtuva viestintä on välttämätöntä kohteen hermotukselle ja selviytymiselle. GDNF-välitteisen viestinnän vaihtamista NRTN-välitteiseen viestintään on todettu tapahtuvan ainakin hiiren parasympaattisissa aivohermoissa, mutta oletettavasti se tapahtuu myös muissa osissa nisäkkään kehittyvää parasympaattista hermostoa. Sammakon (*Xenopus*) genomista puuttuu neurturiinia ilmentävä geeni, mutta GFRα2 -reseptori samakolla kuitenkin on. Rakennemallien mukaan GDNF voisi mahdollisesti toimia ligandina molemmille reseptoreille (GFRα1 ja GFRα2) sammakossa. GDNF:n todella toimiessa ligandina molemmille reseptoreille *Xenopus*ta voitaisiin käyttää mallieläimenä tutkittaessa ristiinviestintää ligandien ja reseptorien välillä.

Tämän tutkimuksen ensisijainen tarkoitus oli selvittää, tapahtuuko sammakon sydämen hermosolmuissa muutos GFRα1-riippuvuudesta GFRα2-riippuvuuteen sammakon kehityksen aikana. Toisena tarkoituksena oli selvittää, toimiiko GDNF ligandina sekä GFRα1-reseptorille että GFRα2-reseptorille. NADPH-värjäystä käytettiin hermosolujen paikantamiseen *X.laeviksen* sydämestä. Whole mount *in situ* -hybridisaatio- ja RT-PCR-menetelmiä käytettiin määrittämään GDNF-, GFRα1-, GFRα2- sekä RET-geenien ilmenemistä aikuisessa sammakossa ja nuijapäässä.

Whole mount *in situ* –hybridisaatio-tulokset osoittavat GDNF:n ja RET:n ilmenemistä aikuisen sammakon suolessa ja sydämen hermosolmuissa. RT-PCR-tuloksissa on nähtävissä, että GFRα1 ilmentyy nuijapäässä. GFRα1:n alassäätely voidaan myös havaita RT-PCR-tuloksista, sillä sitä ei havaita aikuisen sammakon sydämessä, suolessa eikä aivoissa. GDNF ilmenee yhtä aikaa sekä GFRα1-, GFRα2- reseptorien kanssa, joten tulokset tukevat hypoteesiä, jonka mukaan GDNF toimisi ligandina molemmille reseptoreille.

Avainsanat: GDNF, *Xenopus tropicalis*, *Xenopus laevis*, whole mount *in situ* hyrbridisaatio,

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Abstract:

The GDNF family of ligands is a group of four neurotrophic factors that function in neurite outgrowth and cell survival, migration and differentiation. The members of this family of proteins are glial cell line derived neurotrophic factor (GDNF) neurturin (NRTN), artemin (ARTN) and persephine (PSPN).

The Glial cell line derived neurotrophic factor was first discovered as a factor promoting the survival of cultured dopaminergic neurons. It is also a very potent trophic factor for spinal motoneurons. GDNF also has an important role outside the nervous system in kidney morphogenesis and spermatogenesis. The GDNF family ligands signal trough a multicomponent receptor complex, consisting of a GPI- linked GDNF family receptor α (GFRα) subunit and a common transmembrane signaling component, the tyrosine kinase Ret. Each GFL has a preferred α-receptor. GDNF signals preferentially trough GFRα1/Ret, NRTN trough GFRα2/Ret, ARTN trough GFRα3/Ret and PSPN trough GFRα4/Ret complex. However some of the GFLs can bind to some other than its preferred α-receptors at least in vitro. GFRα1 can serve as coreceptor for NRTN and ARTN, and GFRα2 can be coreceptor for GDNF.

In mammals many parasympathetic neuronal precursors require GDNF/GFRα1 (RET) signaling for proliferation and migration. Postmitotic neurons switch from GDNF to NRTN dependence by downregulating GFRα1 and upregulating GFRα2 expression. This NRTN/ GFRα2 (/RET) signaling is required for target innervation and survival. This switch has been shown to occur at least in the development of mouse cranial parasympathetic ganglia but presumably it takes place also in other parts of the developing parasympathetic nervous system in mammals. In contrast, frog (*Xenopus tropicalis*) genome lacks the gene for NRTN. Structural modeling suggests that GDNF is the endogenous ligand for both GFRα1 and GFRα2 in frog. If GDNF is truly a ligand for both, *Xenopus* could serve a possible model organism for studying the cross talk between GDNF and its receptors.

The primary aim of this study was to determine if the switch from GFRα1 to GFRα2 expression happens in cardiac ganglia during development of *Xenopus*. The secondary aim was to find out whether the expression of GDNF would be consistent of it being the ligand for both of the receptors, GFRα1 and GFRα2. NADPH diaphorase staining was used to localize neurons in *X. laevis* cardiac ganglia. Whole mount *in situ*- hybridization and RT-PCR were used to determinate the expression of GDNF, GFRα1, GFRα2 and RET in *Xenopus tropicalis* embryos and adult *Xenopus laevis*.

The results from the whole mount *in situ*- hybridization experiments show both GDNF and Ret expression in cardiac ganglia and gut of adult frog. GFRα1 expression in tadpole can bee seen in the PCR results. Also the downregulation of GFRα1, in tissues of adult frog, is visible in the PCR results. So far the results support the hypothesis of GDNF being the ligand for both GFRα1 and GFRα2 in frog.

Keywords: GDNF, *Xenopus tropicalis*, *Xenopus laevis*, whole mount *in situ* hyrbridization,

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Abbreviations

ARTN	artemin
BCIP	5-Bromo-4-Chloro-3'-Indolyphosphate p-toluidine salt
cDNA	complementary DNA
CNS	central nervous system
DIG	digoxigenin-dUTP
GDNF	glial-cell-line derived neurotrophic factor
GFL	GDNF family ligand
GFRα	GDNF family receptor-α
MAB	maleic acid buffer
NBT	nitro-blue tetrazolium chloride
NRTN	neurturin
PNS	peripheral nervous system
PSPN	persephine
RET	Ret-tyrosine kinase
UPDRS	unified Parkinson’s disease rating scale

1. Introduction

1.1 The Parasympathetic Nervous System

The nervous system can be divided anatomically in to two parts: the central nervous system (CNS) and the peripheral nervous system (PNS). Functionally the peripheral nervous system can be divided to the somatic nervous system and the autonomic nervous system. The autonomic nervous system acts as a control system, maintaining homeostasis in the body. The autonomic nervous system is furthermore split into the sympathetic-, parasympathetic- and enteric nervous systems. The sympathetic and the parasympathetic nervous systems function usually in opposite manners. Activity of the sympathetic nervous system increases during stress and physical exercise. Sympathetic activity increases cardiac output and pulmonary ventilation, routes blood to the muscles, raises blood glucose and slows down digestion, kidney filtration and other functions not needed during emergencies. It also fastens the use of body's energy resources (catabolism). In addition, activation of the sympathetic nervous system leads to adrenaline and noradrenalin release to blood from the adrenal glands. In contrast to the sympathetic nervous system, the parasympathetic nervous system is evident when a person is resting and feels relaxed. Parasympathetic activity slows down heart rate, dilates blood vessels, and stimulates digestion and the genitourinary system and fastens the accumulation of the body's energy resources (anabolism). In addition, the enteric nervous system manages every aspect of digestion, from the esophagus to the stomach, small intestine and colon.

Sympathetic nerves originate from the thoracic and lumbar regions of the spinal cord. The preganglionic nerves are short and synapse in paired ganglia is adjacent to the spinal cord. Parasympathetic nerves originate from the cranial and sacral regions of the CNS. They have long preganglionic nerves which synapse at ganglia near or on the organ innervated.

1.2 Neurotrophic Factors

Neurotrophic factors are small secreted proteins that regulate the development and maintenance of neuronal populations, and can be seen as a growth factors acting on neural tissue. They have an important role in the genesis of the peripheral nervous system. They regulate the survival of various populations of neurons as well as some non neuronal tissues by preventing the neurons from initiating programmed cell death. Neurotrophic factors also influence neurite brancing and synaptogenesis. Besides functioning in neurogenesis, neurotrophic factors have important roles in the adult nervous system. They regulate the synaptic plasticity and maturation of electrophysiological properties. Most of the neurotrophic factors contain secretory signal sequences. They are synthesized in the rough endoplasmic reticulum, are posttranslational modified in the Golgi apparatus and released in vesicles. They can affect either neighboring cells (paracrine signaling), or act by binding the cell's own receptors (autocrine signaling). Neurotrophic factors include neurotrophins, neurokines and glial-cell-line derived neurotrophic factor (GDNF) family ligands (for review see Ip and Yancopoulos, 1996, Huang and Reichardt, 2001). In addition to these many other growth factors have effects on stem cells of the nervous system. For example epidermal growth factor, fibroblast growth factor 1- and -2, and insulin like growth factor-1 and -2 (for review see Ip and Yancopoulos, 1996). Each neurotrophic factor is important for a specific, usually very narrowly defined nerve cell population. The functions of growth factors have been commonly studied in cell cultures, because different treatments are easy to perform on them. *In vitro*- experiments however are not quite comparable to *in vivo*-experiments because nerve cells are grown a few days with the neurotrophic factors before removing the neurotrophic factors from the culture. In natural conditions this kind of exposure does not happen. Nerve cells are also influenced by many other factors which are not necessary present in the culture. The neurotrophic growth factors in the culture are also present at greater amounts than in nature. The effects of the neurotrophic factors *in vivo* are studied in knock-out mice, in which the gene coding factor or its receptor is deleted. Predictably the phenotype is similar in both cases (Snider et al., 1994).

1.2.1 Neurotrophins

Nerve growth factor was the first discovered neurotrophic factor and is the best known from the neurotrophic factors. It belongs to neurotrophins along with brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5 (for review see Ip and Yancopoulos, 1996). They signal via Trk receptor tyrosine kinase A, B, and C as well as p75, which belong to the family of tumor necrosis factor receptors (Glass DJ. 1993; for review see Ip and Yancopoulos, 1996).

1.2.2 Neurokines

The family of neurokines consist of the ciliary neurotrophic factor, the leukemia inhibitory factor, oncostatin M, interleukin 6, interleukin 1, cardiotrophin-1 and the cardiotrophin-like cytokine (also known as the novel neurotrophin-1/B cell stimulating factor-3) (Elson et al 2002, Forger et al.2003). Interleukines 6 and 11 signal through the glycoprotein 130 homodimer and the ciliary neurotrophic factor, the leukemia inhibitory factor, cardiotrophin-1, the cardiotrophin-like cytokine and probably oncostatin M, through a heterodimer of glycoprotein 130 (for review see Ip and Yancopoulos, 1996). The neurokine family does not have domains of intrinsic tyrosine kinase activity on their receptors like the other neurotrophic factors have. However, the activation of the receptors leads to Jak tyrosine kinase activation on the cells. The neurokines activate partly the same signaling pathways in the cells as other neurotrophic factors and affect many nerve cell populations that the GDNF family members maintain. Ciliary neurotrophic factor has an influence mainly on the nervous system, whereas other neurokines play a role, besides the nervous system, also in the genesis of hematocytes (März et al., 1999). The neurokines are best known for their capability of maintaining motoneurons (for review see Ip and Yancopoulos, 1996).

1.2.3 GDNF family ligands

The glial-cell-line derived neurotrophic factor was the first discovered member from the family of GDNF family ligands (GFLs) (Linne, 1993). The other members of the GDNF family are neurturin (NRTN) (Kotzbauer et al., 1996), artemin (ARTN)

(Baloh et al., 1998) and persephine (PSPN) (Mildbrant et al., 1998). The members of the GDNF family are distantly related members of the transforming growth factor-beta (TGF-β) superfamily (Unsicker, 1996). Members in both groups have seven conserved cysteine residues located in the same relative spacing in the mature protein and therefore belong to the "cysteine knot growth factor superfamily" (McDonald and Hendricson, 1993). The sequence homology of amino acids between the members of the GFLs is between 40 and 50% (NTN to GDNF: 43%, PSPN to GDNF: 50 % PSPN to NRTN: 40 %), and less than 20 % with other members of the TGF-β superfamily (Saarma, 2000). The GFLs are produced as a preproGFL precursor. The signal sequence is cleaved upon secretion, and activation of the proGFL most likely occurs by proteolytic cleavage. The GFLs seem to bind heparan-sulfate side chains of extracellular-matrix proteoglygans. This probably restricts their diffusion and increases their local concentration (Hamilton et al. 2001). It is not yet fully understood, how the genes of the GFLs are regulated. Also the mechanism of secretion and the activation of GFL precursors are unknown. Functions of the GFLs are discussed in detail in the following paragraphs.

1.2.4 Glial cell line Derived Neurotrophic Factor

GDNF was purified and characterized from the supernatant of a rat glial cell line and originally identified as a survival factor for midbrain dopaminergic neurons (Linne, 1993). The human GDNF gene contains three exons and two introns. The 3´-end region of the second exon possesses an alternative splicing site, leading to two isoforms (Woodbury et al., 1998). The GDNF gene is highly regulated. The gene's 5′-regulatory region is extremely rich in canonical cis-elements, with more than 12 potential transcription factor binding sites leading to members from multiple families of transcription factors binding to this promoter, thereby effecting its expression (Woodbury et al., 1998). The processed and secreted mature GDNF protein has 134 amino acids containing two N-linked glycosylation sites and it is a disulphide bridge-linked homodimer (Trupp et al., 1995).

The GDNF receptor complex is expressed very early on during development. Both GFRα1 and ret are expressed at this time (Shepherd et al., 2004). Besides

interacting on the survival of midbrain dopaminergic neurons GDNF is also very potent trophic factor for spinal motoneurons (Henderson et al., 1994). It has also important roles outside the nervous system in kidney morphogenesis and spermatogenesis (for review see Saarma and Sariola 1999). GDNF knockout mice revealed the importance of GDNF. These mice have defects in the developing kidneys and lack the enteric nervous system due to failed migration of neural crest cells (Moore et al., 1996, and Pichel et al., 1996). GDNF knockout mice die soon after birth because of a renal failure, and they do not have enteric neurons. The phenotype of these mice is comparable to both RET knockout -and GFRα1 knockout mice, suggesting that they operate on similar signaling pathways (Moore et al., 1996, and Pichel et al., 1996). GDNF signaling is dependent of TGFβ cooperation at least on ciliary ganglion neurons (Peterziel et al., 2007).

The critical role of GDNF in the development of the enteric nervous system is conserved between mammals and zebrafish. The amino acids sequence of *Xenopus laevis* GDNF has 60.8%, 58.6%, 55.9%, 55.5% and 52 % similarity with chick, human, mouse, rat and zebrafish GDNF, respectively. The *X. laevis* protein instead has 75.0%, 69.7%, 66.7%, 65.9% and 62.9 % similarity with chick, human, mouse, rat and zebrafish GDNF, respectively. (Kyono and Jones, 2006)

GDNF has received much attention since the discovery because of its potential therapeutic value in neurological diseases such as Parkinson's disease (Airaksinen and Saarma 2002). Parkinson's disease is characterized by degeneration of substantia nigra dopaminergic (DA) neurons in the midbrain. GDNF prevents the degeneration of these cells and therefore could potentially function as a therapeutic agent in treatment of Parkinson's disease. GDNF treatment has been tested in several animal models of Parkinson's disease, with promising results (Grondin and Gash 1998; Björklund et al., 2002). In the first clinical trial, the growth factor was delivered into the lateral ventricles of patients. GDNF appeared to be ineffective probably because GDNF did not reach the target, the putamen, and caused severe side-effects (Grondin and Gash, 1998). A recent clinical trial in which GDNF was infused unilaterally directly into the putamen of the patients with Parkinson's disease, showed more promising findings. Unified Parkinson's Disease Rating Scale (UPDRS) total scores after one year of therapy were improved by ~40% but these improvements were lost within 9 months of

drug withdrawal (Slevin et al., 2007). However, in another trial intraputaminal GDNF infusion was ineffective, probably because of limited distribution within the putamen (Peterson and Nutt, 2008).

1.2.5 Neurturin

NRTN was found due to the fact that conditioned medium of Chinese hamster ovary cells could support the long term survival of superior cervical ganglion sympathetic neurons in culture (Kotzbauer et al., 1996). Mature NRTN protein shares 42% sequence similarity with mature GDNF protein (Kotzbauer et al., 1996). NRTN gene has two exons, containing 594 base pairs. It promotes the survival of several neuronal populations including peripheral parasympathetic, nodose and dorsal root ganglion sensory neurons, as well as central midbrain dopaminergic neurons. (Heuckeroth et al. 1997)

1.2.6 Persephin and Artemin

PSPN was identified as a member of the GFL family by using degenerate PCR and homology cloning. PSPN has approximately 40% sequence similarity with GDNF and NRTN and it also shares the similarity of biological activity of NRTN and GDNF on central neuronal populations, but not on peripheral neurons (Mildbrant et al., 1998). The PSPN gene contains one intron and it was revealed to have two isoforms (a long and a short one) by RT-PCR (Jaszai et al., 1998).

ARTN is the most newly discovered GDNF family member. Just as GDNF and NRTN, it is a survival factor for sensory and sympathetic neurons in culture. Its expression pattern suggests that it also has effect on these neurons *in vivo*. (Baloh et al., 1996)

1.3. GFL receptor complex

The GDNF family ligands bind a receptor complex, which consist of a cell membrane crossing RET tyrosine kinase receptor and a ligand binding component, the GDNF family receptor-α (GFRα) (Figure1.). Each GFL has a preferred α-receptor:

GDNF signals preferentially trough GFRα1/Ret, NRTN trough GFRα2/Ret, ARTN trough GFRα3/Ret and PSPN trough GFRα4/Ret complex. However some of the GFLs can bind to some other than its preferred α-receptor at least *in vitro* (Airaksinen et al., 1999). GFRα1 can serve as coreceptor for NRTN and ARTN, and GFRα2 can be coreceptor for GDNF (Figure 2.). PSPN has not been shown to bind any other receptors than the GFRα4 coreceptor (Lindahl et al., 2001). The GFRα-coreceptors are consequently responsible for binding specific GFLs, whereas RET mediates the signal to the cell, and is the same for all GFLs. The GFLs signal *in vivo* mainly trough their preferred α-receptor, because the deletion of a neurotrophic factor or its preferred α-receptor leads to a similar phenotype at least in mice. Alternative interactions are avoidable probably because different neurotrophic factors and their receptors are expressed at different times or in different places and also because the amounts of neurotrophic factors are very small. (Airaksinen et al., 1999)

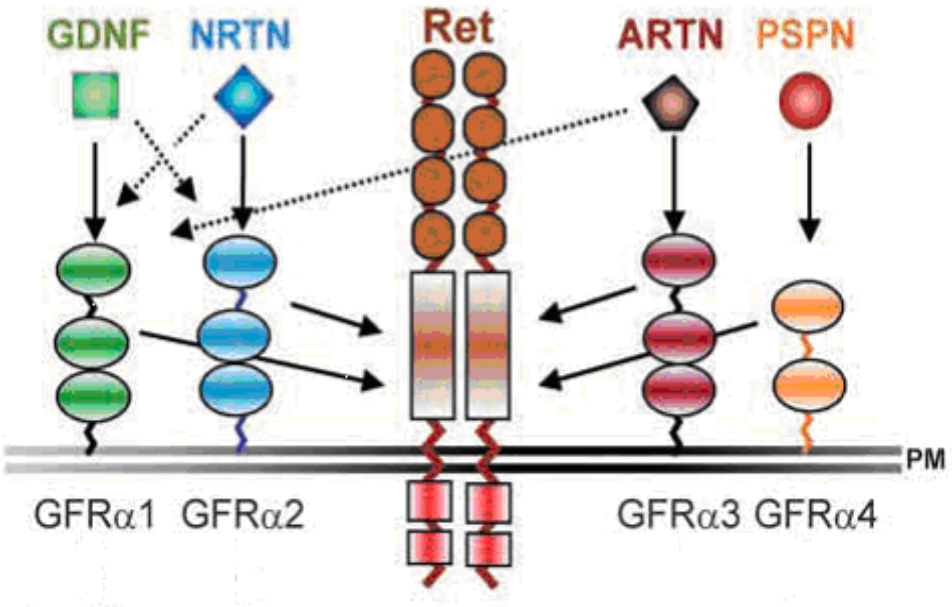

Figure 1. GDNF family ligand and their receptor interactions. All GFLs activate the transmembrane RET tyrosine kinase trough their preferred GFRα receptors. GDNF signals preferentially trough GFRα1, NRTN trough GFRα2, ARTN trough GFRα3 and PSPN trough GFRα4 (solid arrows), also alternative interactions occur at least in vitro: GDNF can bind also coreceptor GFRα2 and NRTN and ARTN coreceptor GFRα1 (dashed arrows) (Airaksinen et al. 1999). Mammalian GFRα-4 lacks the first cysteine rich domain which is likely not involved to ligand binding (Lindahl et al., 2001). From Sariola and Saarma, 2003.

1.3.1 RET- tyrosine kinase

The members of the TGF-β superfamily signal usually trough a serine-threonine kinase. However, the GDNF family members signal trough the Ret-tyrosine kinase (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996; Saarma, 2001). Ret is a cell surface glycoprotein which has three known isoforms produced by alternative splicing: a long (RET-51) an intermediate (RET-43) and a short (RET-9) one differing in their C-terminal end of the protein (Myers et al., 1995). The human RET oncogene was mapped to chromosome 10q11.2 (Ishizaka et al., 1989) and it contains 21 coding exons (Myers et al., 1995). The product of the proto-oncogene is a cell surface and transmembrane receptor with an intracellular tyrosine kinase domain (Takahashi and Cooper, 1987). The intracellular domain contains 14 Tyr residues, RET -9 however lacking two Tyr at the C-terminus (Myers et al., 1995). The extracellular region of the protein contains four domains, each of about 110 residues in length containing the consensus motif of the cadherin sequence and a Ca^{2+} binding site located between two domains (Anders et al., 2001). Ca^{2+} binding is required for the activation of RET (Airaksinen and Saarma, 2002). Ret is expressed in a smaller scale than GDNF family receptors-α. The cells that express Ret typically express also one of the four GFRα receptors (Yu et al., 1998, Golden et al., 1998, Golden et. al., 1999).

RET was originally identified as an oncogene activated by DNA rearrangement (Takahashi et al., 1985) and later on it has been discovered to be related on many diseases. A mutation that causes continuous activation of RET in humans generates multiple endocrine neoplasia type 2, which is a group of medical disorders associated with tumors of the endocrine system. Inactivation of the kinase part of the RET causing mutations can lead to Hirschsprung´s disease. It is a congenital disorder of the colon in which certain nerve cells, known as ganglion cells, are absent, causing chronic constipation. (Airaksinen et al., 1996)

1.3.2 GDNF family receptors-α

The GDNF family ligands activate the transmembrane Ret tyrosine kinase via GDNF family receptors-α 1-4 (GFRα1-4). The GFRαs have three globular cysteine-rich

domains (D1, D2, D3), except for GFRα-4, which has only two (Lindhal et al., 2000). The second domain is essential for Ret binding, in addition to GFLs binding to it (Scott and Ibanez, 2001). All the GFRα genes have a similar shape and organization of exons. They have a 30-45 % amino acid similarity and have a similar arrangement of conserved Cys residues. The GFRα proteins have a glycosyl phophatidylinositol (GPI) anchor, through which they are linked to the plasmamembrane (Jing et al., 1996). Proteins, having a GPI anchor, are usually located in lipid rafts on the cell membrane (Muniz and Reizman, 2000). Lipid rafts, containing a large number of sphingomyelin and cholesterol, have great impact on GDNF family signaling (saarma 2001, Tsui-Pierchala et al., 2002). The GFRα proteins can also be produced in a soluble form by phosphatidylinositol specific phospholipase C treatment, which cuts the GPI anchor and releases GFRα receptors from the cell membrane. The GFRαs may also be alternatively spliced producing soluble isoforms, as demonstrated in the GFRα4 gene (Lindahl et al., 2000).

1.3.3 GFL receptor complex formation and signaling

GFL signaling can be either RET-dependent or RET-independent. RET dependent signaling can be *in cis* or *in trans*. In the original model, a dimeric neurotrophic growth factor binds first to its coreceptor, which is located on the cell membrane. Growth factors bring the coreseptors closer to each other forming a GFL/GFRα complex, which promotes the dimerization of transmembrane RET. This leads to autophosphorylation of intracellular tyrosines (Jing et al., 1996, Airaksinen et al., 1999, Airaksinen and Saarma, 2000). Phosphorylated RET is able to activate many signaling cascades, which are typical for tyrosine kinase signaling. These include the Ras–MAPK (Santoro et al., 1994;Worby et al., 1996) and phosphoinositol-3-kinase (van Weering et al., 1997) as well as Jun N-terminal kinase (Chiariello et al., 1998; Xing et al., 1998) and phopholipase-Cγ (Borrello et al., 1996) dependent pathways. Also neurotrophins activate partly the same pathways, even though lipid rafts are not so important for their signaling as they are for the GFLs (Airaksinen and Saarma 2000; Kaplan and Miller, 2000). In this preceding signaling model both, the GFRα and Ret are located on the same cell and that is why the interaction is called *in cis*. GFRα receptors

can also be in soluble form in the cytosol, in which case it is called an interaction *in trans*. (Paratcha et al., 2001; Airaksinen and Saarma, 2002; Tsui-Pierchala et al., 2002). In this case the GFRα bind the growth factor outside the cell and presents it to Ret, which is then activated outside the lipid rafts and only thereafter moved to the raft. The activation of Ret *in cis* and *in trans* probably leads to the activation of different signal molecules and thereby to different responses (Paratcha et al., 2001; Saarma, 2001; Tsui-Pierchala et al., 2002). The significance of *in trans* signaling is still unclear and animal tests have not shown any notable effects, at least, not in organogenesis or nerve regeneration (Enomoto et al., 2004).

It was unclear for long time, why the GFRα receptors are expressed more abundantly in the nervous system and especially in the cerebellum, than Ret. It was partly explained by *in trans* signaling, but it also suggests that GFRα receptors can signal independently from Ret (Airaksinen and Saarma, 2002; Sariola and Saarma, 2003). This was proven to be a fact. GDNF activates Src family kinases independently of Ret (Poteryaev et al., 1999; Trupp et al., 1999). This leads to the phosphorylation of phopholipase-Cγ, ERK1/ERK2, MAPK and cAMP response element binding protein. Ret-independent signaling has been recently shown to be mediated by the phophorylation of receptor tyrosine kinase Met via Src family kinases (Popsueva et al., 2003). Altogether, quite a few possible interactions within and between receptor systems are emerging, which may have effect on both normal development and on pathological states.

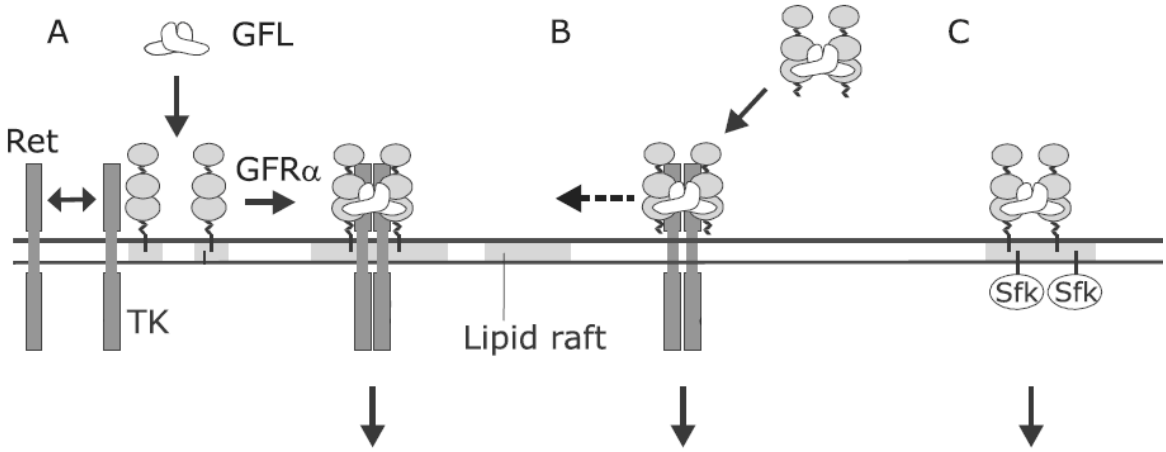

Figure 2. The Interactions of GDNF-family ligands and their receptors. (A) GPI-anchored GFRα proteins are located mainly in the rafts in the absence of ligands. A dimeric GFL binds to GFRα receptor, which recruits RET to rafts and promotes its dimerization and autophophorylation in cis. (B) Soluble or an adjacent cell's membrane anchored GFRα binds GFL, recruits Ret to a lipid raft and induces a raft specific signaling in trans. (C) A conceivable RET-independent signaling mechanism where a GFL/GFRα complex induces the phophorylation of Src-family kinases. From Airaksinen and Saarma, 2002.

1.4. Use of Xenopus in a genetic research

Amphibian embryos have for a long time been the favored systems in studying the mechanisms of development. The two main reasons for this are the external development of embryos and their relatively large size. It enables microsurgery and manipulation of the embryos in ways that are very difficult to perform on other vertebrate embryos. Embryos of *Rana* (common frog), *Triturus* (European newt) and *Ambystoma* (American salamander) were used for most of the early experiments. Use of the African clawed frog, *Xenopus laevis* became general after 1950 (Amaya et al., 1998). *X. laevis* offers many advantages for gene and protein expression and knockdown studies. Individuals of this species can live and sustain fertility for over 10 years in a laboratory environment (Tinsley and Kobel, 1996). Ovulation of a female frog can be induced every 1 and 2 months by injection of human gonadotropin. Embryos can be produced anytime of the year and hundreds of embryos can be generated per ovulation. The development can be followed continuously because of the external development of the embryos. *X. laevis* embryos are very large, are easy to culture and use in experiments. RNA from other organisms can easily be injected into the large oocytes and after manipulation the embryos can be analyzed by localizing mRNA transcripts by whole mount *in situ* hybridization or by localizing proteins with immunohistochemistry (Sive et al., 2000). A detailed developmental stating system is available, which allows for comparison of developmental stages between embryos based on external morphology (Nieuwkoop and Faber, 1994). Experimentation is greatly facilitated by the fact that the developmental rate is predictable and can be manipulated with temperature (Khokha et al., 2002).

Despite all the advantages *X. laevis* has to offer for research it has one remarkable drawback. Its genome is allotteraploid, which complicates the genetic studies substantially. The genus *Xenopus* contains nearly 20 separate species, which are all, except for one, polyploid. *Xenopus* (*Silurana*) *Tropicalis* is the only species in the *Xenopus* genus that has a diploid genome (Bisbee et al., 1977). *X. tropicalis* has recently been adopted for research in developmental genetics and functional genomics.

It has a highly similar morphology to *X. laevis, X. tropicalis* being nonetheless significantly smaller. It shares all the advantages with *X. laevis* besides having a few more, the diploid genome being the most significant advantage (Amaya et al., 1998). *X. tropicalis* has a relatively small genome size (only 10 chromosome pairs) and also has a short generation length ranging from 4 to 6 months. In comparison, the generation length of *X. laevis* ranges from one to two years (table 1.). *X. tropicalis* can produce more eggs per ovulation than *X. laevis* and the embryos can be easily microinjected even though being smaller than the embryos of *X. laevis*. (Khokha et al., 2002)

The research use of frogs was greatly eased by the creation of a database (www.xenbase.org), found on the internet. It is a model organism database with biological and genomic data on the frogs, *Xenopus laevis* and *Xenopus tropicalis*. There is a lot of information about gene function, full-length cDNA sequences and *in situ* databases on *X. laevis*, but no genome sequence or genetics. *X. tropicalis* conversely has a sequenced genome, genetics and strong EST support but little literature or gene expression data. Integration of data from both organisms allows these different strengths to complement each other. *X. laevis* having larger embryos is better suited to microsurgery experiments and *X. tropicalis* to mutant and experimental gene knockdown experiments, having the advantages of a diploid animal. (Bowes et al., 2008)

Kokha et al., (2002) have established that many of the reagents available for X. laevis can be successfully transferred to *X. tropicalis*. They have also shown that whole mount *in situ* hybridization protocols of X. laevis can be effectively used for *X. tropicalis* without modification and that *X. laevis* probes often work in *X. tropicalis*. *X. tropicalis* embryos develop at the same rate as *X. laevis*, only tolerating a narrower range of temperature (Khokha et al. 2002).

Table 1. Comparison of differences between *X. laevis* and *X. tropicalis*.

Species	X. laevis	X. tropicalis
Ploidy	allotetraploid	diploid
N	18 chromosomes	10 chromosomes
Genome size	3.1 x 10^9 bp	1.7 x 10^9 bp
Adult size	10 cm	4-5 cm
Egg size	1-1.3 mm	0.7-0.8 mm
Eggs/spawn	300-1000	1000-3000
Temp. optima	16-22^{o}C	25-30^{o}C
Generation time	1-2 years	<5 months

1.4.1 Cardiac ganglia of frog

Xenopus heart development is well known from the heart patch stage to chamber formation. The cardiac ganglion is the parasympathetic ganglion that innervates the heart. The ganglion consist a single type of neurons, a pool of motoneurons. The neuronal cell bodies form several small clusters, which are all closely located on the atrium of heart.

All peripheral neurons in vertebrates are derived from cells that migrate from the the neural crest. In mammals, the proliferating neural crest-derived precursor cells become postmitotic neurons soon after they arrive to the correct tissue region and cluster to form peripheral ganglia during embryogenesis (around E12 in mouse). In contrast, the crest-derived cells in frog heart become postmitotic over a long period of time and accumulation is continued in the ganglion even after metamorphosis. (Heathcote and Sargent, 1984, 1987a). The first neurons are present in the *Xenopus* cardiac ganglion at stage 42 (day 3) and number of neurons increases at the constant rate for some time (Heathcote and Sargent, 1984). Cell death is common in the developing nervous system; however there is little or no cell death during frog cardiac ganglion morphogenesis (Heathcote and Sargent 1987b). Adult *Xenopus* and its heart can grow continuously for at least 1-2 years, therefore making the effectively innervation of target challenging. The cardiac ganglion increases by size in tree separate

stages. First, cells migrate to the ganglion from neural crest. Second new neurons are added to the ganglion continuously. The time of proliferation is the main determinant of neuronal number and it's quite long, continuing even after metamorphosis. Third, after proliferation, the cells start to increase by size (Heathcote and Sargent, 1987a).

1.4.2 Evolution of GDNF family signaling and role in frog parasympathetic nervous system development

The general order and development of the parasympathetic nervous system is thought to be rather comparable between frog and mammals. In mice, the postmitotic parasympathetic neurons switch from GDNF to NRTN dependence by downregulating GFRα1 in the neurons and upregulating NRTN expression in the target tissue (Enomoto et al., 2000, Rossi et al., 2000). NRTN signaling via GFRα2 (and RET) is required for target innervation and survival of the parasympathetic neurons (Heuckeroth et al., 1999, Rossi et al., 1999). This switch has been shown to occur during the development of mouse cranial and pancreatic parasympathetic ganglia but presumably it takes place also in other parts of the developing parasympathetic nervous system in mammals (Rossi et al., 2000, Lähteenmäki et al., 2007).

All vertebrate classes, from teleost fishes to mammals, have four GFRα-receptors (Hätinen et al., 2007). Consistently, genes orthologous for all the four GFLs are found in mammalian and fish genomes. In contrast, there are only three GFLs in the genomes of *X. tropicalis*. Synteny analysis shows that the *Xenopus* genome lacks the ortholog of NRTN (Hätinen et al., 2006). Lacking the NRTN gene, frog has to have some other ligand for the GFRα2-receptor. Protein models suggest that GDNF is the ligand for both GFRα1 and also for GFRα2 in frogs (Figures 3 and 4) (Hätinen et al., 2007).

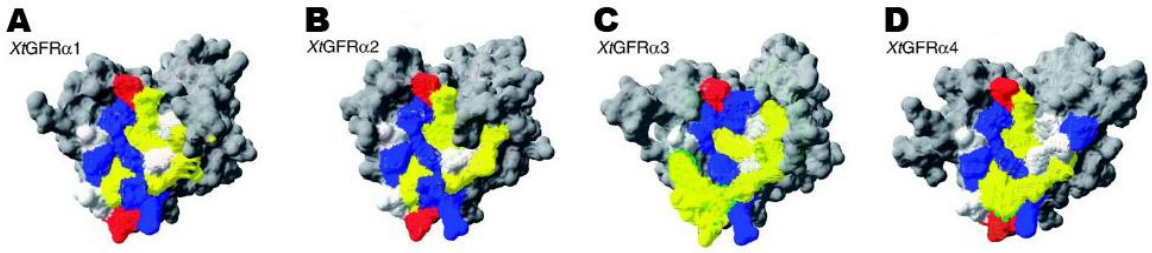

Figure 3. Homology modeling of the ligand-binding surfaces on frog GFRα receptors. Residues corresponding to the ARTN-binding surface of human GFRα3 crystal structure (Wang et al., 2006) are colored according to type (blue = basic, positive, red = acidic, negative, yellow = polar, white = hydrophobic). There is notable similarity of binding sites between GFRα1 and GFRα2, GFRα3 and GFRα4 being significantly different. (Adapted from Hätinen et al., 2006)

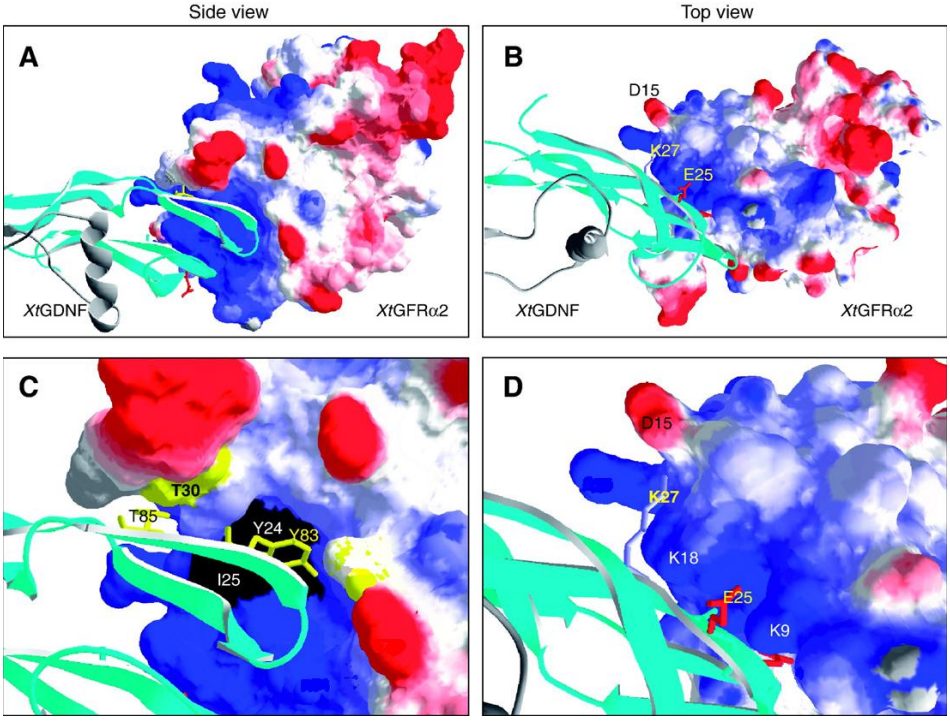

Figure 4. Structural modeling of GDNF in complex with GFRα2 (domains D2 and D3) in the Xenopus tropicalis. Frog GDNF is shown as ribbons with individual chains in light blue and gray. The molecular surface of frog GFRα2 is colored by electrostatic potential. Side chains are shown for selected residues at the binding interface. (A, C) Side view of the complex, showing that one of the evolutionary trace residues in GFRα2, the buried hydrophobic residue T30 (yellow) is close to a patch of hydrophobic residues (including T85) in GDNF. (B, D) Top view of the complex, demonstrating that the positive "trace" residue in GDNF, K27, may interact with the negative residue D15 on GFRα2. Shown are also key residues in frog GDNF/GFRα2 on two binding epitopes that are proposed to constitute conserved "anchor" points for GFLs interactions with their GFRα receptors (Wang et al., 2006): Y83 in GDNF is predicted to form contact with Y24 and I25 in GFRα2 (B), and E24 in GDNF with K9 in GFRα2 (D). Shown is also another proposed specificity determining residue in frog GDNF, E25, matching residues K9 and K18 in GFRα2. (Adapted from Hätinen et al., 2006)

The critical role of GDNF in development of enteric nervous system is conserved between mammals and zebrafish (Kyono and Jones, 2006). The *X. laevis* GDNF has ~60-70%, % similarity with GDNF from other vertebrates (Kyono and Jones, 2006). *Xenopus* GDNF is detectable for the first time at stage 12 at low level, gradually increasing up to stage 22. Expression of GDNF sharply increases and continues at similar level after stage 24 (Kyuno et al., 2006).

2. Aims of the Study

The primary aim of this study was to determine whether the parasympathetic neurons in frog switch from GFRα1 dependence to GFRα2 dependence during the development of *Xenopus*. This was done by determining which GFRα receptors are expressed in the cardiac ganglia of *Xenopus* larvae and adult frogs. Secondary aim was to find out whether GDNF expression in the cardiac tissue is consistent of it being the ligand for both GFRα1 and GFRα2 receptors. These questions were studied by using whole mount *in situ* hybridization and RT-PCR.

3. Materials and methods

3.1 Animals

Adult *Xenopus laevis* and tadpoles of *Xenopus laevis* and *Xenopus tropicalis* were used in this study. *X. laevis* were obtained from the breeding colony of the University of Helsinki and *X. tropicalis* were obtained from the breeding colony of Université de Rennes, France.

3.2 NADPH- diaphorase histochemistry

Frogs were anesthetized with a 0,2% solution of MS-222 (3-aminobenzoic acid ethyl ester, Sigma), the heart was removed and the ventricles were detached from the atria. The atria were pinned to a dish and fixed in 4% PFA for 30 minutes at room temperature and washed twice in 1 x PBS for ten minutes. The samples were then put into 1x PBS, which contained 1 mg of β-NADPH, reduced form, 5 mg of nitroblue tetrazolium and 0,2 % of TX-100. Samples were incubated for one hour at 37 ºC. The samples were then rinsed with 1 x PBS, mounted (2% propyl gallate, 1% PBS, 90% glycerol and 0,001% Hoechst) air dried and microscopied (Carl Zeiss, Axioplan 2, Göttingen, Germany). Pictures were taken with Zeiss Axiocam digital camera and the Axiovision 4.3 software.

3.3 Probes

The EST clones for making complementary RNA probes for *Xenopus* GFRα1, GFRα2 and Ret were obtained from ImaGene. Clone IDs are presented in table 2. The plasmid clone for making the GDNF probe was kindly provided by Dr. Jones from Warwick University. GFRα1 and Ret clones were inserted in to a pCMV-SPORT6 plasmid and GFRα2 into a pCS107 plasmid. The clones were received in agar and plated on LB-ampicillin plates and grown overnight at 37ºC. The next day a single colony was picked and inoculated in 3ml of LB-medium with ampicillin (100 µg/ml)

for eight hours at 37 ºC with vigorous shaking. A starter culture was diluted 1/1000 into LB-medium with ampicillin (100µg/ml) and grown at 37 ºC for at least 12 hours with vigorous shaking. Plasmids were purified by using a QIAGEN Plasmid Midi kit.

3.4 Transformation

The GDNF clone was received in a pCMV-SPORT6 plasmid. It was transformed into *E. coli* (DH5α) cells by a heat shock method. Cells were first thawed on ice and were then transferred to Eppendorf tubes, 100 µl/tube. One µl of plasmid was added to each tube, not including the negative control and kept on ice for 30 minutes. The tubes were then transferred to 42 ºC for 45 s. and then back to ice for two minutes. 450 µl of 42 ºC SOC was added to each tube and the tubes were then incubated at 37 ºC for an hour. After that, 200 µl and 100 µl were plated on LB-AMP plates and incubated overnight at 37 ºC. The next day a single colony was picked and inoculated in 3 ml of LB-medium with ampicillin (100 µg/ml) for eight hours at 37 ºC with vigorous shaking. The purification of plasmids was done similarly as for the GFRα1, GFRα2 and Ret clones.

3.5 DIG-labeling of the RNA probe

Purified plasmids were first linearized. The following components were added to an Eppendorf tube: 15 µg of the purified plasmid, 10 µl 10 X enzyme buffer, 5 µl of enzyme and sterile distilled water up to 100 µl. The solution was then incubated for three hours at 37 ºC. The vectors and restriction enzymes used for the clones can be seen in table 2. Linearized plasmids were purified by adding 100 µl of phenol: chloroform: isoamyl alcohol (25:24:1) solution into the tube and mixed well. The mixture was then centrifuged at full speed for five minutes to separate the phases. The upper phase was transferred to a clean tube, 100 µl of chloroform was added and the solution was mixed and centrifuged again at full speed for five minutes. The upper phase was transferred again to a clean tube and 10 µl of 3 M NaCl and 275 µl of cold 100% EtOH were added. The plasmids were precipitated for one hour at -80 ºC and centrifuged at full speed for 25 minutes at +4 ºC. The pellet was then washed with cold

70% EtOH and centrifuged at full speed for five minutes at +4ºC. The pellet was then air-dried and dissolved in 20µl of sterile RNase free water. The linearization was checked by running 500ng of the linearization product in a gel together with a nonlinearized plasmid.

The linearized plasmid was used as a template for preparing digoxigenin-dUTP (DIG) -labeled probes. 1,5 µg of the linearized plasmid was added to a eppendorf tube along with two µl of 10 X transcription buffer, two µl of 10 X DIG labeling mix, 0,5 µl of RNase inhibitor (Promega), two µl of Polymerase (T3, T7 or SP6) and RNase free water up to 20µl. The transcription of the anti-sense probes was performed with T7 RNA polymerase following linearization with *Sal*I, and for the sense probes SP6 RNA polymerase and *Not*I were used (Table 2.). The mixture was incubated at 37ºC for two hours and after that 0.8 µl of 0,5M EDTA was added to stop the reaction. 2.5 µl of 4M LiCl and 75µl of cold 100% EtOH were added to the tubes and the solution was incubated at -80ºC for an hour. The tubes were then centrifuged at full speed for 25 minutes at +4ºC. The pellet was washed once with 70% EtOH and centrifuged at full speed for five minutes at +4ºC, followed by air-drying and dissolving in 100µl sterile RNase free water. The product was checked by running five µl of it in a gel together with 500ng of the linearized plasmid. The labeling of the probe was checked by dot blot analyses. The labeled probes were stored at -80ºC.

Table 2. Vectors, restriction enzymes and ImaGene IDs for clones.

Clone	GFRα-1	GFRα-2	RET	GDNF
ImaGene ID	IMAGp998A1316915Q1	IMAGp998M0816037Q1	IMAGp998C0116901Q1	-
Vector	pCMV-SPORT6	pCS107	pCMV-SPORT6	pCMV-SPORT6
Restriction Enzyme for Antisense Probe	SalI	EcoRI	SalI	SalI
Restriction Enzyme for Sense Probe	NotI	XhoI	NotI	NotI
Polymerase for Antisense Probe	T7	T3	T7	T7
Polymerase for Sense Probe	SP6	SP6	SP6	SP6

3.6 Whole Mount *in situ* Hybridization

3.6.1 Fixation

All the procedures were done at room temperature unless noted otherwise. The animals were anesthetized with 0,2 % of MS-222. The heart was removed and ventricles were detached from the atria. The atria were pinned to a dish and fixed for two hours in MEMFA. Also gut were pinned to a dish and fixed for two hours in MEMFA. The fixed samples were dehydrated with methanol-PTW (PBS with 0.1 % Tween 20) series; 25%, 50%, 75%, 5 min in each solution and twice in 100% MetOH-PTW. The samples were stored in 100% MetOH-PTW in -20ºC.

3.6.2 Prehybridization and hybridization

The samples were rehydrated in 75 %, 50% and 25 % MetOH-PTW; for 5 min in each solution and twice in 100% PTW. The samples were permeabilized with a Proteinase K-treatment for five minutes and then rinsed twice in 0,1 M triethanolamine for five minutes. Acetic anhydride was added (12,5 µ/ 5 ml triethanolamine)and the solution incubated for five minutes after which the step was repeated. The samples were

then washed twice in PTw for five minutes. Refixation was done in 4 % PFA in PTw for 20 minutes. After that the samples were washed five times in PTw for five minutes. *X. tropicalis* were prehybridized for six hours and *X. laevis* for one hour at 60 º C. The solution was replaced with 0,5 ml probe solution and hybridized overnight at 60 º C. The probe was removed and kept for reuse. The probes were used twice at the maximum. The samples were washed twice with the hybridization buffer from the previous step and incubated in hybridization buffer for three times three minutes at 60 º C and then three times twenty minutes at 60 º C.

3.6.3 Blocking and color reaction

The samples were washed twice in maleic acid buffer (MAB) for ten minutes. The buffer was replaced with MAB + 2 % BMB Blocking reagent and incubated for at least 1 hour. Antibody binding was performed by using a 1/3000 dilution of the antidioxygenin AP antibody in MAB + 2 % BMB Blocking reagent either overnight at 4 º C or for four hours at room temperature. Next the samples were rinsed several times with MAB and washed in MAB several times for 15 minutes and then overnight.

The samples were washed twice, 5 minutes each time, with a Alkaline phosphatase buffer. The color reaction was performed using 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP) and Nitro-Blue Tetrazolium Chloride (NBT). Together they yield an intense, insoluble black-purple precipitate when reacted with a Alkaline phosphatase, which is a part of the antibody. Staining was monitored every fifteen minutes until the stains had developed. It took 15 minutes to two hours depending on the probe. The chromogenic reaction was stopped with a quick wash with MAB.

3.6.4 Bleaching

Xenopus tissues have lots of pigmentation which disturbs the analyses of results. To reduce the pigmentation, the samples were bleached. The samples were incubated overnight at Boiun´s to fix the stain. Then the yellow Bouin´s was removed by multiple washes with 70 % EtOH. The samples were rehydrated with buffered EtOH- SSC series; 75 %, 50% and 25 %; 5 min in each solution and twice in 100% SSC. The

samples were bleached under direct light on top of a aluminium foil for two hours and then washed twice in 1 X SSC for five minutes. The samples were dehydrated by washing in MeOH for multiple times and stored in methanol at – 20 º C.

3.6.5 Microscopy

amples were placed on microscopic slides which had a well in the center. The wells were made by covering the slides with SYLGARD (Dow Corning Corp., Midland, USA) and cutting the center away. The samples were covered with benzyl benzoate/benzyl alcohol and microscopied with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Pictures were taken with a Axiocam digital camera (Zeiss) mounted on the microscope. Axiovision 4.3 software (Zeiss) was used to process the pictures. Some of the samples were embedded in to paraffin and cut on to microscopy slides. This was done by a research technician Erja Huttu. These slides were also microscopied with the same equipment.

3.7 RNA isolation and RT-PCR

3.7.1 RNA isolation

RNA isolation from adult frogs and tadpoles was performed using the QIAzol reagent. Brain, gut and atria of heart, were dissected from adult *X. laevis*. Triplicate samples were taken. One milliliter of GIAzol was added and each sample was then homogenized for twenty seconds with homogenizator (Heidolph Diax 900; Heidolph Elektro GmbH, Kelhaim, Germany)

The samples were then centrifuged for ten minutes (12.000g) at + 4 º C. The supernatant was transferred to a new eppendorf tube, incubated for five minutes at room temperature after which 0,2 ml of chloroform was added to the samples. The samples were incubated for two minutes at room temperature and centrifuged for 15 minutes (12.000g) at + 4 º C. The cleared supernatant was transferred to a new tube and 0,5 ml of isopropanol was added to each sample. The samples were centrifuged for ten minutes (12.000g) at + 4 º C and the pellet was washed with 1 ml of 75 % EtOH and centrifuged

again for five minutes (7.500g) at + 4 º C. The RNA pellet was then air dried and dissolved to 50 µl of RNase free water.

3.7.2 cDNA synthesis

Complementary DNA (cDNA) was synthesized using the SuperScript III Reverse Transcriptase (Invitrogen). The following components were added to a nuclease-free microcentrifuge tube; random primers (200ng), total RNA (2µg), 1µl of dNTP Mix (10mM) and sterile, distilled water up to 13µl. The mixture was heated to 65ºC for five minutes, incubated on ice for one minute and then briefly centrifuged. Four µl of 5X First strand buffer, 1µl of 0,1M DTT, 1µl of RNase OUT and 1µl of SuperScript III RT were added to each tube and mixed by pipeting gently up and down. The tubes were incubated for 60 minutes at 50 ºC and the reaction was then inactivated by heating for 15 minutes at 70ºC. The cDNA was stored at -20ºC.

3.7.3 PCR

The cDNA was used as a template for amplification in polymerase chain reaction (PCR). The primers for the PCR reaction are presented in table 2. and the PCR mix is presented in table 1. The PCR program was as follows; two minutes starting denaturation at 94ºC, 30 seconds denaturation at 95ºC, 30 seconds annealing at 60ºC or 56 ºC, one minute extension at 72 ºC and five minutes final extension at 72 ºC.

Table 3. PCR mix

Component	Quantity
10X DyNAzyme EXT buffer	2,5µl
dNTPs	0,6µl
DyNAzyme EXT	0,6µl
Forward primer	1,26µl
Reverse primer	1,26µl
Sample	0,4µl
Sterile, distilled water	16,8µl
Total	25µl

Table 4. Primers (Proligo, USA) used for PCR.

Gene	Forward (F) and Reverse (R) Primers 5´→ 3´	Product size (pb)	Annealing Tm (ºC)
GFRα-1	F:GACCCACAGACTCCTACAATCCC R:CATTGCTTCACTTCATTTAC	306	56
GFRα-2	F:AATGAGCTGTGTGCCGCAGAC R:CGGAGTCGCTTACATGCATCATTC	401	60
GFRα-3	F:AGCATCGCATGAGAAGGGAAG R:GCACAGTAACTCTCTTCACACGGAC	411	56
GFRα-4	F:GAATGGCGCTAACAAACTGGG R:CACTTGGAGCGGTTGCACTG	402	60
GDNF	F:TTATGGGCTATTCTGGCTGTCTG R:AGGGAGGCTGCTTGTTGGAC	310	56
RET	F:TGACTGTGTGGGATGCCGACTC R:CATAGCGGTCTGCATTTCGGTTG	378	56
ARTN	F:TTATTGTGTCATTTCTGCTGCTGTC R:TTCTACGTCCCTCAGGCTTCTTC	234	56
PSPN	F:CCCTGTTCTTCACGCTCCTTG R:TGCTCCACCGTATGCCACTG	457	60

4. Results

4.1 NADPH- diaphorase histochemistry

NADPH-diaphorase staining showed the location of parasympathetic neurons in the cardiac ganglia of frog. Atria were detached from the ventricle and pinned to the slide. An example of the staining is shown in figure 5. It can be seen that neurons are located at clusters on the sinus venous portion of atria.

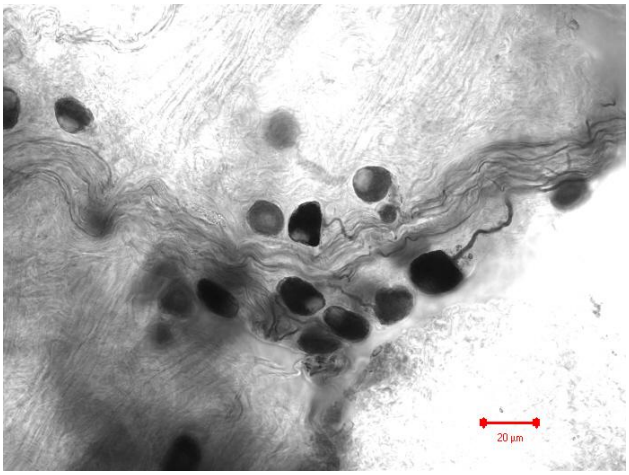

Figure 5. Parasympathetic neurons are located on the atria of *X. laevis* NADPH-diaphorase whole-mount staining. Scale bar is 20 µm and magnification is 400 fold.

4.2 Whole Mount *in situ* Hybridization

Results from the whole mount *in situ* hybridization were limited for reasons discussed in chapter 7. Figure 6. shows an example of GFRα2 mRNA expression in gut of adult *X. laevis*. GFRα2 mRNA is expressed along the gut, presumably in the enteric neurons.

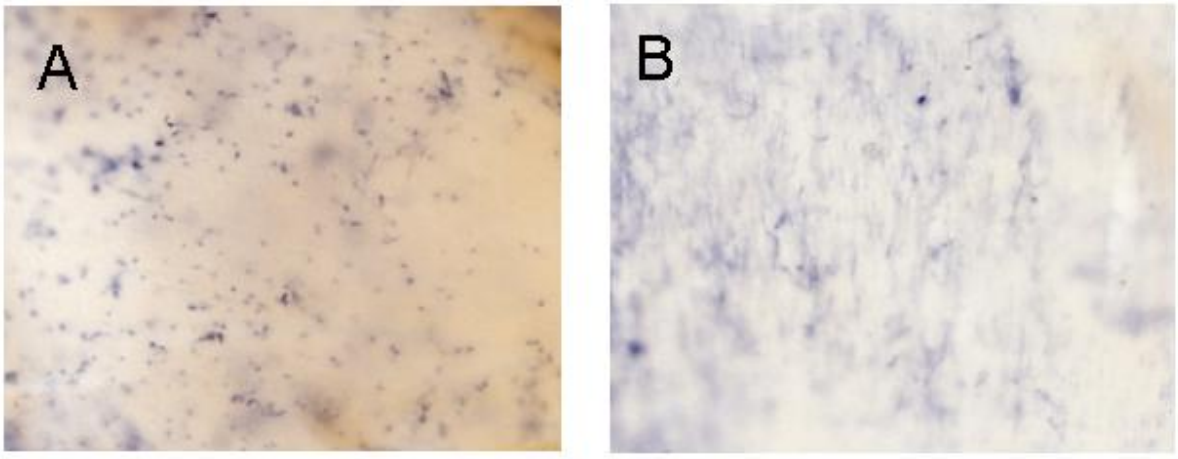

Figure 6. (A) Adult *X. laevis* gut hybridized with GFRα2 antisense-probe and (B) with GFRα2 sense-probe. Magnification is 200 fold.

GDNF expression in a whole-mount preparation the adult frog heart can be seen on the middle of atria (Figure 7), where the cardiac ganglion neurons are located. Also RET expression can be seen on the same location in the whole mount in situ hybridization pictures (figure 8).

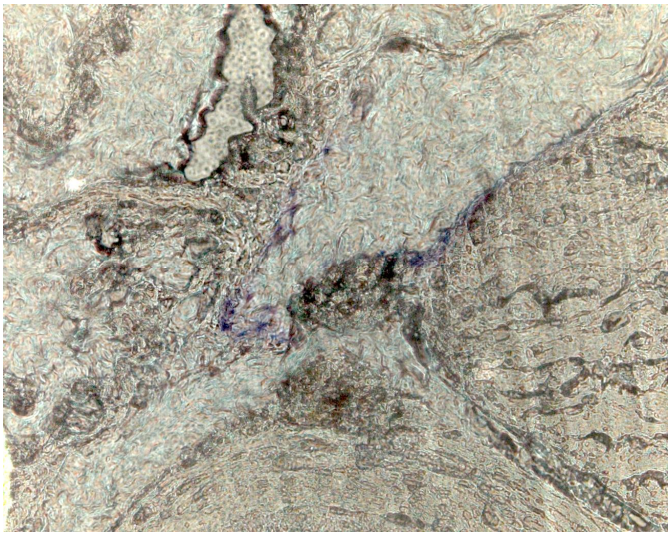

Figure 7. GDNF expression in atria of the adult *X. Tropicalis.* (Antisense probe). Magnification is 200 fold.

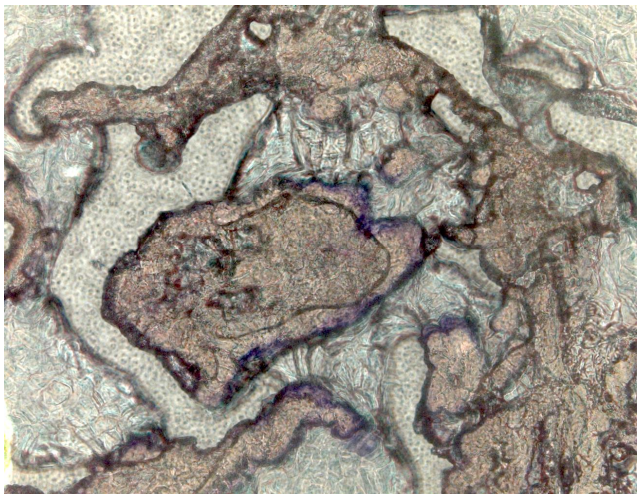

Figure 8. RET expression in *X. Laevis* atria with antisense probe. Magnification is 200 fold.

GDNF expression in gut of adult *X. laevis* is shown in figure 9. Sense probe gives also a signal although it is weaker than the signal with antisense probe.

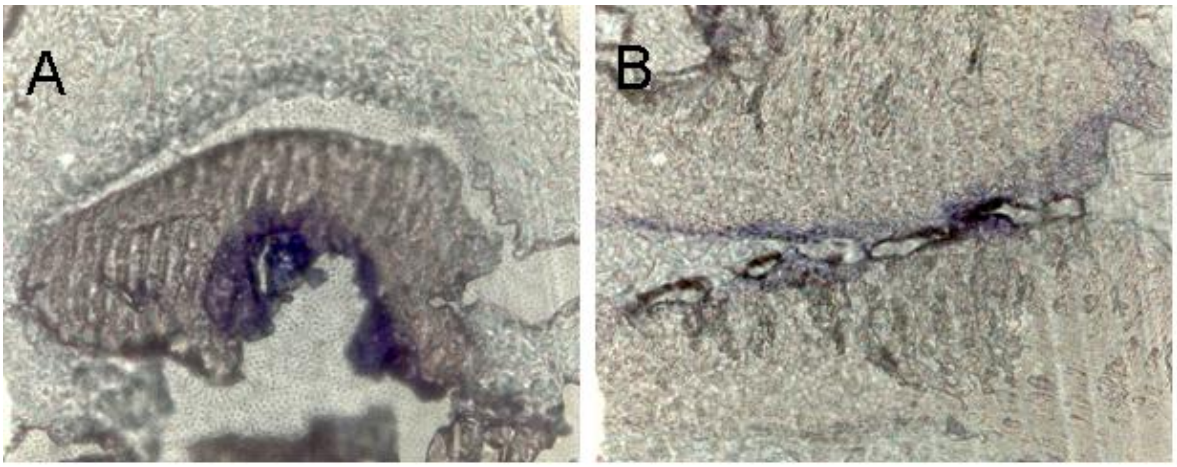

Figure 9. GDNF expression in *X. laevis* gut with (A) antisense probe and with (B) sense probe. . Magnification is 200 fold.

4.3 PCR

The data from RT-PCR experiments are summarized in table 5. GDNF, Ret, GFRα1, GFRα2, GFRα3 and GFRα4 are all expressed in *X. tropicalis* tadpole. GDNF,

Ret, GFRα2, GFRα3 and GFRα4 are expressed also in adult *Xenopus*. GFRα1 expression was not detected in any of the tissues on adult *Xenopus*.

Table 5. Summary of RT-PCR results from *X. tropicalis* tadpole and *X. laevis* heart, brain and gut.

		Adult Xenopus laevis		
	Tadpole	Heart	Brain	Gut
GDNF	+	+	+	+
Ret	+	+	+	+
GFRα1	+	-	-	-
GFRα2	+	+	+	+
GFRα3	+	+	+	+
GFRα4	+	+	+	+

5. Discussion

5.1 Whole mount *in situ* hybridization

In this study, the parasympathetic nerve cells in the Xenopus heart were first located using whole-mount NADPH staining. Cholinergic neurons are clustered in the sinus venous portion of the atrium early in development (Heathcote and Chen, 1991). The location of nerve cells at the postmetamorphic heart has been shown previously by Heathcote and Sargent (1987a). Visualization the nerve cells in the whole mount preparation helped the analysis and localization of the whole mount *in situ* hybridization signal because both methods the heart was prepared in the same way.

The main focus of this study was to establish the whole mount *in situ* hybridization method in *Xenopus* to determine whether the switch from GFRα1 to GFRα2 dependence happens in the frog cardiac ganglia. Although radioactive *in situ* hybridization has been successfully used in the laboratory for sections and whole-mount *in situ* hybridization is used routinely in the campus for mouse embryos, the non-radioactive whole-mount *in situ* hybridization method was introduced for frogs for the first time in the group. The results from the experiments were not entirely as expected. There were unexpectedly many difficulties so that in most of the experiments the techniques worked poorly and signals were not seen in the supposed places. There are numerous possible reasons for these unsuccessful or unclear results as discussed below.

The adult frog samples were prepared from *Xenopus laevis* animals that were not albinos and therefore had a lot of pigmentation. Even though the samples were kept in a bleaching solution for several hours, some of the pigmentation remained on the samples and possibly masked the detection of a weak signal. The bleaching step was done under a direct light on top of an aluminium foil. A proper light box might have improved the bleaching.

One potential cause of these major drawbacks is the possible degradation of RNA during the hybridization procedures. This is however unlikely, because the tissues were well-fixed and all the steps were done in sterile conditions and under great care. In order to exclude the possible RNA digestion during hybridization procedures all the solutions

were freshly prepared and all the replaceable equipments were changed. The possible degradation of RNA at the time of tissue preparation is also unlikely because all the equipments were sterile. Finally, the whole-mount *in situ* hybridization method worked succesfully using a positive control probe (gift from Dr Juha Partanen) on mouse embryos prepared in the same room (data not shown).

Another thing to be considered as a cause of the unexpected results was the possibility that the mRNA could not be reached with the probe. Fixation of the tissue effectively secures the mRNA within the tissue from RNAse digestion. Overfixation of the tissue however blocks the probe from reaching the target. To help the probe in reaching the intended location, in the tissue or the embryo, the time for the proteinase-K treatment was slightly lengthened and a small cut was done to the tail of the embryos.

Yet another reason for the unsuccessful results could be the inactivity of the probe. However, the GFRα2-, RET and GDNF-probes functioned a couple of times. The GFRα1 probe did not give any signal in adult tissues and in embryos, but this was expected based on the RT-PCR results from adult tissues and in embryos the expression might have been relatively low. In any case, an increase in probe concentration could be tried. There should be clear difference between the patterns of the antisense probe signal and sense probe signal as seen in figure 6. This was not always the case as in some cases the sense probe gave a quite a similar pattern as the antisense probe (Figure 9). This might have been caused by nonspecific binding or *X. laevis* pseudogenes could possibly cause some unexpected results.

As a reference, we used the published data by Kyuno et al. (2007) about *in situ* hybridization in *Xenopus laevis* embryos with a GDNF probe. The protocol used here was the same, although the probe used was different. Additional positive controls (such as a housekeeping, pan-specific actin or beta-tubulin probe or a *Xenopus*-specific probe against a more abundant target than *gdnf*) and negative “nonsense” probes (with similar CG content and length than the antisense) would be helpful to address these possibilities. It would also be useful to do the whole mount *Xenopus in situ* hybridization in a laboratory where it has been done earlier under the surveillance of somebody who has experience with the technique.

In sum, the whole mount *in situ* hybridization study and its results remained incomplete and inconclusive, because of the many unsuccessful or inconsistent experiments. The minor results that were achieved from the whole mount *in situ* hybridization show clear expression of GDNF in embryos and adult frog. Several things could be done in trying to achieve better results from whole mount *in situ* hybridization. A different probe concentration could be tried and also stringency of washes should be increased to decrease the nonspecific binding. One very important thing is to include of a positive control. Probes against house keeping sequences can be used for this purpose.

5.2 RT-PCR

In mammals, GDNF is thought to function in the early devolvement of parasympathetic neurons (Enomoto et al., 2000, Rossi et al 2000), whereas NRTN signaling (Heuckeroth et al., 1999; Rossi et al., 1999) is needed for the later development and maintenance of the parasympathetic neurons. During *Xenopus* development GDNF is first detectable at stage 12, and gradually increased up to stage 22. From stage 24, the expression sharply increased and continued at a similar level as development progressed (Kyuno et al., 2006). Consistent with that study, the RT-PCR results (Table 5) show that GDNF is expressed in frog embryos but also in the cardiac tissue of an adult *Xenopus*. In contrast, Golden et al. (1999) did not detect any GDNF expression in the cardiac tissue of an adult mouse. They did not either detect GFRα1, GFRα2 expression in the heart of an adult mouse, whereas GFRα2 (but not GFRα1) expression was detected by RT-PCR on cardiac of an adult *Xenopus*.

Downregulation of GFRα1/GDNF (but not GFRα2) and upregulation of NRTN happens in the development of mouse parasympathetic ganglia (Enomoto et al 2000, Rossi et al 2000). The RT-PCR results suggest that a similar downregulation of GFRα1 but not GFRα2 may happen also during the development of frog parasympathetic system. As expected, Ret is expressed simultaneously with GFRα's and GDNF in *Xenopus* as has been reviewed earlier in vertebrates by Takahashi (2001).

Neurons of frog cardiac are formed over a prolonged period of time and accumulate at a constant rate (Heathcote and Sargent, 1984). Thus, at any particular time during the early phases of development, neurons at all stages of their differentiation are

present in the heart. The *Xenopus* genome lacks the gene for NRTN and Hätinen and colleagues (2006) suggested that GDNF is the ligand for both receptors GFRα1 and GFRα2. The RT-PCR results are consistent with this possibility by indicating the simultaneous expression of GDNF with both GFRα1 and GFRα2 receptors in the developing but only with GFRα2 in adult frog heart.

If GDNF truly is the ligand for both receptors GFRα1 and GFRα2 in *Xenopus tropicalis*, this species could serve a possible model organism to study the cross talk of GDNF and its receptors. Protein binding experiments should be done to ensure that GDNF is indeed the ligand for both GFRα1 and GFRα2 in frog.

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