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Helsinki 2015 ISSN 2342-3161 ISBN 978-951-51-1405-1

JUSSI KUPARI Studies on Peripheral Nervous System Development and Function in Mice Lacking the Neurturin Receptor GFRα2 61/2015

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

61/2015

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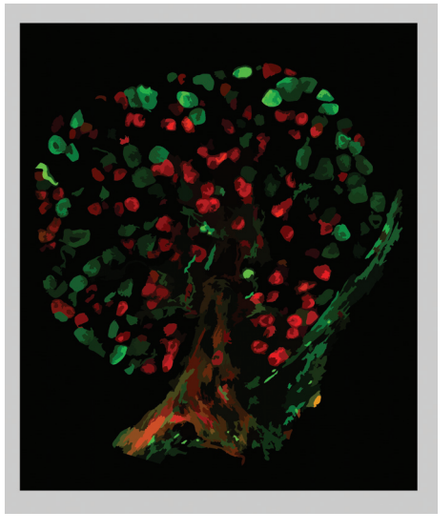

DEPARTMENT OF ANATOMY
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN BRAIN & MIND
UNIVERSITY OF HELSINKI

Studies on peripheral nervous system development and function in mice lacking the neurturin receptor GFRα2

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ACADEMIC DISSERTATION

To be presented for public examination, by due permission of the Faculty of Medicine of the University of Helsinki, in Lecture Hall 2, Biomedicum Helsinki (Haartmaninkatu 8), on the 28th of August 2015, at 10 o'clock

Helsinki 2015

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Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

ISBN 978-951-51-1405-1 (paperback)
ISBN 978-951-51-1406-8 (PDF)
ISSN 2342-3161 (print)
ISSN 2342-317X (online)
http://ethesis.helsinki.fi
Hansaprint, Vantaa

Cover image by the author.
Cover layout by Anita Tienhaara.

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ABSTRACT

The Glial cell line-derived neurotrophic factor (GDNF) family ligands, which include GDNF, neurturin (NRTN), persephin (PSPN) and artemin (ARTN), signal through a glycosyl phosphatidyl inositol (GPI)-linked cognate-receptor (GFRα1-4) and the transmembrane receptor tyrosine kinase receptor RET. The members of the GDNF family play a particularly important role in the development of the peripheral nervous system (PNS). In the autonomic nervous system, GDNF and NRTN regulate important steps in the development of the enteric and parasympathetic nervous systems from migration and proliferation to soma size and target innervation, whereas ARTN takes part in the early phases of sympathetic nervous system development. In the sensory system, GFRα2 –the co-receptor of NRTN–has been shown to mediate trophic signaling for nonpeptidergic nociceptive neurons, and is also required for their innervation of the glabrous epidermis. However, several aspects of the role of GFRα2-signaling in normal PNS development and function remain poorly understood. Therefore, the aims of this study were to elucidate (1) the role of GFRα2-signaling in the development of parasympathetic neurons; (2) the role of GFRα2-signaling in two classes of somatosensory mechanoreceptor neurons and their target innervation; and (3) the role of GFRα2-signaling in the cholinergic innervation of the gastric mucosa and the role of this innervation in gastric secretion.

We discovered that programmed cell death (PCD) is a normal part of parasympathetic neuron development in mice. GFRα2-signaling was found to regulate parasympathetic neuron survival in pancreatic and submandibular ganglia during late embryonic development; lack of GFRα2-mediated signaling resulted in the loss of intrapancreatic neurons through PCD. In argreement with previous studies, apoptosis in the ENS was found to be rare, and was not increased in the absence of GFRα2, implying that the normal number of enteric neurons is not determined by PCD. In the dorsal root ganglia (DRGs), we found that GFRα2 regulates the cell size, but not the peripheral innervation of hair follicles in both the large early-RET RA Aβ-class low threshold mechanoreceptors (LTMRs) and in the small C-LTMRs. In contrast, GFRα2 was found to regulate both the cell size and the epidermal innervation in the small Mrgprd+ C-nociceptors. We also found evidence that the RA Aβ-LTMRs downregulate GFRα2-expression at some point after birth, suggesting a possible switch in neurotrophic signaling pathways. In the enteric nervous system, we demonstrated that GFRα2-signaling via NRTN is required for cholinergic innervation of the gastric mucosa. Interestingly, this innervation was found to be unnecessary for maintaining the gastric mucosa and for gastrin secretion and basal acid secretion. Even though vagally-stimulated secretion is lost in the GFRα2-KO mice, their ability to secrete acid in response to direct parietal cell stimulation remains in the absence of gastric mucosal innervation.

SELECTED ABBREVIATIONS

ANS	autonomic nervous system
ARTN	artemin
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic protein
CGRP	calcitonin gene-related peptide
CLD	cadherin-like domain
CNS	central nervous system
CRD	cystein-rich domain
DRG	dorsal root ganglion
E	embryonic day
ECL	enterocromaffin-like
EGFP	enhanced green fluorescent protein
ENCC	enteric neural crest cell
ENS	enteric nervous system
GDNF	glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFRα	GDNF family receptor alpha
GPI	glycosyl phosphatidyl inositol
GRP	gastrin-releasing peptide
HA	histamine
HF	hair follicle
IB4	isolectin B4
KO	knockout
LLE	longitudinal lanceolate ending
LTMR	low-threshold mechanoreceptor
Mrgprd	Mas-related G-protein-coupled receptor d
NC	neural crest
NCAM	neural cell adhesion molecule
NCC	neural crest cell
NFH	neurofilament-heavy
NGF	nerve growth factor
NRTN	neurturin
NT	neurotrophin
NTF	neurotrophic factor
P	postnatal day
PACAP	pituitary adenylate cyclase activating protein
PCD	programmed cell death
PGP9.5	protein gene product 9.5
PNS	peripheral nervous system
PSPN	persephin
RA	rapidly-adapting
RET	RET tyrosine kinase (*re*arranged during *t*ransfection)
SA	slowly-adapting
SCG	superior cervical ganglion
SST	somatostatin
TGF-β	transforming growth factor beta
TH	tyrosine hydroxylase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TTX	tetrodotoxin
VAChT	vesicular acetylcholine transporter
VIP	vasoactive intestinal peptide
WT	wild-type

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text with their assigned roman numerals I-III:

I Lähteenmäki M, Kupari J, Airaksinen MS. Increased apoptosis of parasympathetic but not enteric neurons in mice lacking GFRα2. Dev Biol. 2007 May 1;305(1):325–32.

II Kupari J, Airaksinen MS. Different requirements for GFRα2-signaling in three populations of cutaneous sensory neurons. PLoS One. 2014 Aug 11;9(8):e104764.

III Kupari* J, Rossi* J, Herzig KH, Airaksinen MS. Lack of cholinergic innervation in gastric mucosa does not affect gastrin secretion or basal acid output in neurturin receptor GFRα2 deficient mice. J Physiol. 2013 Apr 15;591(Pt 8):2175–88.

* Denotes equal contribution

INTRODUCTION

The Peripheral Nervous System and Neurotrophic Factors

The peripheral nervous system (PNS) consists of the neurons and glia that reside outside the central nervous system (CNS). Whereas the CNS organizes information and regulates behavior, the role of the PNS is to relay afferent information from inside and outside the body into the CNS, and to serve as an effector system that allows the regulation of vital body functions. For these purposes, the PNS is divided into several motor (efferent) and sensory (afferent) divisions. The sensory divisions communicate stimuli from the outside world through the skin, oral cavities, nasal cavities, eyes and ears. The sensory systems also relay proprioceptive body-awareness from within the musculo-skeletal system and visceral signals from the internal organs (interoception). The motoric section of the PNS is called the autonomic nervous system (ANS). Its subdivisions (sympathetic, parasympathetic, and enteric) are responsible for maintaining body homeostasis together with the endocrine system. The axons from the somatic motoneurons that control the skeletal muscles are often included in the PNS. However, their cell bodies lie inside the CNS and therefore do not fit the definition of PNS used in this thesis.

The origin of peripheral neurons is in the neural crest, which is a transient population of migratory cells that is only found in the vertebrates. At the start of neurulation, the developing neural crest cells form a band-like neural plate border that runs bilaterally between the neuroectoderm of the neural plate and the outer non-neuronal ectoderm. As the neural tube folds and closes, the neural crest cells begin to proliferate and migrate extensively to generate a multitude of different cell types, including autonomic and sensory neurons and peripheral glial cells (Figure 1A) [1]. The generation of such a substantial diversity of peripheral neuronal cell types requires precise control over cellular migration, cell numbers, and the innervation of target tissues. Neuronal numbers in the mature PNS are regulated to fit the need of the innervated tissue, partly by controlling the rate of precursor cell proliferation. However, a hallmark of the PNS is the vast occurrence of programmed cell death (PCD): a tightly regulated method of culling excessive numbers of neurons produced during development. The controlled cell death observed in PNS neurons and other aspects of their development and maintenance are largely under the control of specific proteins secreted mainly by their target tissues. These proteins are commonly known as neurotrophic factors.

The early work of Viktor Hamburger in the 1930's demonstrated that the removal of a wing bud in a developing chicken resulted in the death of differentiated motor and sensory neurons projecting to the missing area; similarly, increasing the size of the target zone lead to the hypertrophy of the corresponding motor nuclei and sensory ganglia [2]. This allowed Hamburger to postulate that peripheral tissues control the proliferation, differentiation, and migration of corresponding neurons via secreted substances transported retrogradely to the somas. Later, it was discovered that this retrograde signaling actually works mostly in controlling the number of neurons that survive the programmed cell death process.

Work by Rita Levi-Montalcini and her colleagues beginning in the late 1940's led to the discovery of the first neurotrophic factor, Nerve growth factor (NGF), and the postulation of the neurotrophic factor hypothesis [3]. According to this theory (Figure 1B), the number of neurons and their peripheral projections in the adult organism are determined by a process where, to escape elimination by cell death, the neurons actively compete for a limited supply of a specific trophic factor released from the target organ [4].

Since the discovery of NGF, three other factors belonging to the same protein family have been discovered: Brain-derived neurotrophic factor (BDNF) and Neurotrophin-3 and -4 (NT-3, NT-4). Together, these factors are known as the *neurotrophins*. The neurotrophins signal mostly via their tyrosine kinase receptors TrkA, TrkB, and TrkC expressed on specific subsets of neurons; NGF signals via TrkA, BDNF and NT-4 via TrkB, and NT-3 via TrkC.

In addition to the neurotrophins, several other groups of growth factors with strong neurotrophic properties have been discovered, such as the glial cell line-derived neurotrophic factor and its related ligands (GDNF-family, GFLs) [5], the neuropoietic cytokines (CNTF and LIF) [6], fibroblast growth factors (FGFs) [7] and the CDNF/MANF-family [8]. These factors influence many aspects in nervous system function and development, while many of them have important functions also outside the nervous system. In the PNS, the neurotrophins and GDNF-family factors have the most important roles. Different factors from these families often control development or provide trophic support in the same neuronal populations, sometimes functioning at distinct developmental stages or influencing different aspects of development, such as proliferation, migration, survival, or target innervation [9].

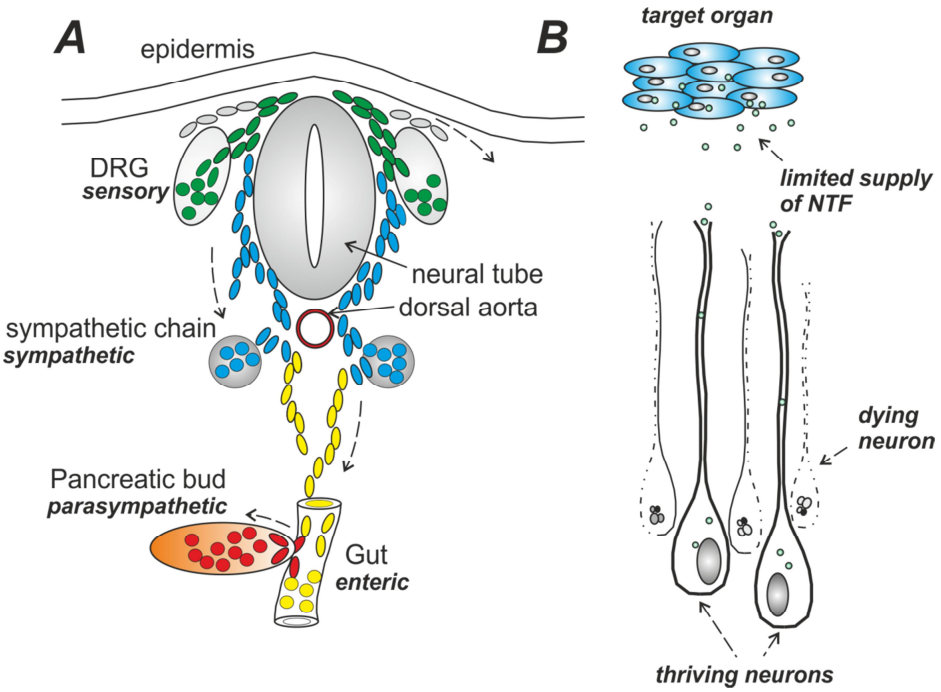

Figure 1. The neural crest and the neurotrophic factor theory. ***(A)*** Neural crest cells (NCCs) proliferate and migrate extensively around the developing embryo to create neurons and glial cells of the peripheral nervous system as well as several other cell types. Cells of the different divisions of the PNS are indicated with specific colors. ***(B)*** Peripheral neurons compete for a limited supply of a neurotrophic factor (NTF) expressed and secreted by a target organ. The neurons which are able to secure the factor thrive and proceed to innervate the target, whereas cells left without the factor die via programmed cell death.

REVIEW OF THE LITERATURE

The GDNF Family Ligands and Receptors

GDNF Family Ligands

The GDNF family of neurotrophic factors consists of four members: GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN). They form a subgroup of the transforming growth factor beta (TGF-β) superfamily [10] based on a conserved cysteine residue arrangement found on all the ligands and other members of the superfamily [11–14]. All TGF-β related proteins, including the GFLs, are first synthesized as premature preproproteins with an N-terminal signal sequence and a variable prodomain that is cleaved upon secretion [10]. The prodomain is cleaved by proprotein convertases at specific RXXR cleavage sites to produce mature proteins, which are biologically functional as homodimers [5,10]. The general structure of a GFL monomer includes a "cysteine knot" motif formed by the conserved cysteine residues, two antiparallel β-sheet "fingers", and a connecting α-helix section in the middle [15–17]. In the dimer, the subunits are connected by a disulfide bond in a face-to-face orientation, where the β-sheet fingers of the two monomers cross in the middle and extend to the opposing lateral sides [15–17].

The founding member of the group, GDNF, was purified from a rat glial cell line on the basis of its ability to promote survival, differentiation, and dopamine uptake in cultured embryonic rat midbrain neurons [11]. These findings were followed by a number of studies showing the ability of GDNF to promote the survival and target reinnervation of dopaminergic neurons *in vivo* in several injury paradigms [18–24]. In addition to dopaminergic neurons, GDNF was also effective in supporting the survival of *locus coeruleus* noradrenergic neurons [25] and basal forebrain cholinergic neurons after injury [26].

Outside the brain, GDNF was shown to act as a trophic and survival factor for central motoneurons [27–30] and peripheral sensory, autonomic, and enteric neurons [31–36]. Although GDNF is expressed in multiple areas of the rodent central nervous system [37–39], it is found most abundantly in the developing peripheral tissues, with embryonic whisker pads, gastrointestinal tract, and kidney showing some of the highest transcript levels [34,39]. In the developing gut and kidney, GDNF is expressed, respectively, in the muscular wall and the surrounding mesenchyme[39–41]. Consistent with this, GDNF is essential for the development of the enteric nervous system and kidney morphogenesis [42–44]. In the testis, GDNF is secreted by the sertoli cells and regulates differentiation and self-renewal in spermatogonia [45].

NRTN was discovered and isolated following the observation that the conditioned medium of Chinese hamster ovary cells was able to support the survival of superior cervical ganglion (SCG) sympathetic neurons in culture [13]. In addition to sympathetic neurons, purified NRTN also promoted the survival of visceral sensory neurons from the nodose ganglion and of a small subset of dorsal root ganglion (DRG) neurons. Like GDNF, NRTN protects adult dopaminergic neurons *in vivo* [46,47] and also supports survival in spinal motoneurons and basal forebrain cholinergic neurons *in vitro* [48,49]. Peripherally, NRTN promotes proliferation and survival in enteric neuron progenitors [33] and supports survival in embryonic chicken autonomic and sensory neurons to varying degrees [32,35]. In the CNS, NRTN transcripts are found in the postnatal striatum, brainstem, and pineal gland [50]; however, similarly to GNDF, it is most strongly expressed in peripheral tissues.

NRTN transcripts are found (among other locations) in the circular muscle layer and mucosa of the intestine, salivary gland epithelium and sweat glands, tubular epithelium of the developing kidney, and developing whisker follicles [39,50–53].

The last two members of the group, PSPN and ARTN, were discovered using polymerase chain reaction (PCR) and bioinformatics [12,14]. PSPN is expressed in the ventral midbrain and striatum during late embryonic to early postnatal development [54]. It is able to protect nigral dopamine neurons *in vivo* [12,54,55] and also supports motoneurons and embryonic basal forebrain cholinergic neurons [12,49]. PSPN, however, does not support neurons from peripheral ganglia [12]. ARTN [56,57], on the other hand, is a powerful survival factor for sensory and sympathetic neurons and is highly expressed in the DRG nerve roots and along the surface of the superior mesenteric artery in developing mouse embryos [14]. Outside the nervous system, ARTN has been shown to be involved in secondary lymphoid formation in the gut [58].

GDNF Family Receptors

The receptor complex for GDNF was discovered in 1996, when several research groups obseved that GDNF was able to phosphorylate the orphan receptor tyrosine kinase RET (short for Rearranged during transfection) [59–61] and that this activation was mediated via an additional ligand-binding co-receptor [62,63]. This co-receptor was later named GFRα1. In a short period of time, three more of its kind were characterized (Figure 2): GFRα2 is the preferred receptor for NRTN [48,64–70], GFRα3 for ARTN [14,66,71–76], and GFRα4 for PSPN [77–80].

RET was first identified 30 years ago as a part of a fusion gene that formed in a lymphoma transfection study [81]. The C-terminal of the formed fusion protein was later found to belong to the tyrosine kinase superfamily [82]. The full-length RET receptor is a dimer composed of two RET monomers with a ligand-binding domain in the extracellular space, a transmembrane helix-region, and a cytoplasmic tyrosine kinase domain [83]. The extracellular N-terminal is composed of four cadherin-like domains (CLD1-4) with a calcium-binding site between CLD2 and CLD3, and a cysteine rich domain close to the cell membrane [84–86]. The intracellular part has a juxtamembrane section and a two-part tyrosine kinase domain, as well as a C-terminal tail [87].

The four GFRα co-receptors share a similar basic structure containing a hydrophobic N-terminal secretion signal, several N-glycosylation sites, a C-terminal hydrophobic region for the addition of a glycosylphoshadityl-inositol (GPI) plasma membrane anchor [88,89], and three conserved cysteine rich domains (D1-D3) [17,78,90,91]. In contrast to GFRα1-3, the mouse GFRα4 is smaller and lacks D1 [78]. Although each of the different GFRα co-receptors shows preference for a specific ligand, a certain amount of cross-talk has been documented. GDNF can phosphorylate RET through GFRα2 and NRTN through GFRα1. Also, both ARTN and PSPN can activate RET via GFRα1 [14,92].

GFRα receptors are often expressed in a complementary pattern with GDNF family ligands. For example, in the kidney and gastrointestinal tract GFRα1 is expressed, respectively, by the wolffian bud epithelium and enteric plexus neurons. These patterns are matched precisely by GDNF expression in the kidney mesenchyme and gut muscle layers [93]. GFRα1-3 are strongly expressed in the peripheral nervous system in subsets of developing and adult autonomic, enteric, and sensory neurons and Schwann cells [39,51,52,71,75,94–99]. GFRα1 and GFRα2 can also be found abundantly in neurons throughout the rodent CNS [37–39,70,100–102]. In addition, all GFRα's are also expressed in many non-neuronal tissues. GFRα4 is produced in multiple splice forms in the mouse, of which functional GPI-linked forms are found apparently only in some endocrine cells. [78,103].

The GFRα receptors are expressed in many tissues that lack RET, but RET has never been detected without the company of at least one of its co-receptors [37–39,93,98]. During embryogenesis, RET is expressed by migrating neural crest cells that form the peripheral nervous ganglia, the developing excretory system, and parts of the rodent CNS [39,93,104–106]. Multiple splice isoforms of RET exist, the best characterized being RET9, RET51, and RET43 [107,108]. The RET9 and RET51 isoforms are the prevailing isoforms with distinct expression patterns [109] and separate signaling complexes [110]. RET9 isoform appears to be crucial for kidney and enteric nervous system development, whereas RET51 is required by mature sympathetic neurons [111,112]. RET is also required for male fetal germ cell survival [113]. Loss-of-function mutations of RET in humans result in Hirschprung's disease with chronically contracted and obstructed aganglionic segments of the bowel, leading to the dilation of the proximal part of the gut [114]. In contrast, gain-of-function mutations of RET can lead to multiple types of cancer [115]. Together with ARTN and GFRα3, RET is required for the secondary lymphoid structure formation in the gut [58].

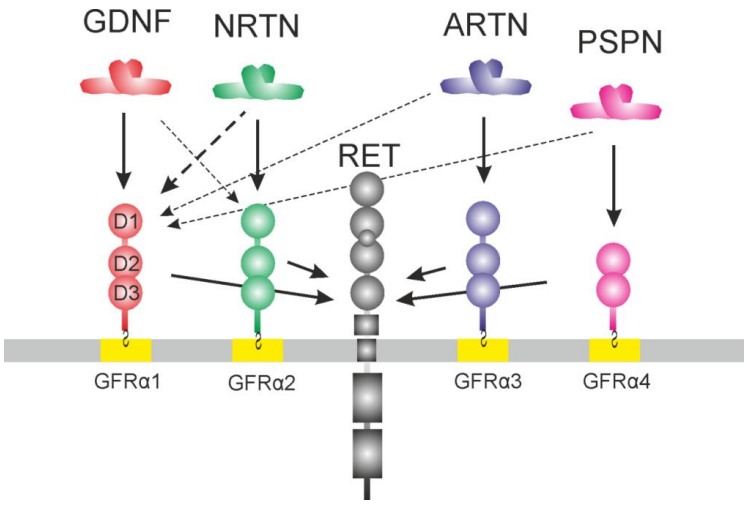

Figure 2. The GDNF family ligand-receptor complex and interactions. All of the four GFLs form homodimers and activate the transmembrane receptor tyrosine kinase receptor RET via their preferred GFRα co-receptors (indicated by solid arrows). GFRα4 lacks the first cysteine-rich domain D1 in mammals. Modified from [5].

GDNF Family Signaling

Receptor complex formation

According to the original model of GDNF mediated RET phosphorylation, a dimer of GDNF first binds two GFRα1 co-receptors (Figure 3) and this complex then recruits two molecules of RET, leading to the autophosphorylation of the catalytic tyrosine kinase domains [62]. However, RET and GFRα1 have been shown to weakly associate even without the ligand being present [67,74]. Also, some GDNF mutants unable to bind GFRα1 can still induce GFRα1-dependent RET activation [116]. These findings suggest that at least some of the receptors could be present as preformed GFRα1-RET complexes before GDNF binding; however, exact details of the complex formation remain unknown [5,116].

Based on mutagenesis studies, the most critical sites for GFRα1 and GFRα2 binding have been localized to two adjacent locations on finger 2 of the GDNF molecule [117]. Homologous sites on NRTN and ARTN molecules can also mediate binding to GFRα1 and RET activation, but additional regions are required for binding with GFRα2 and GFRα3 [117]. In addition, GDNF binding to GFRα1 receptors in the absence of Ret has been shown to require additional residues in finger 1 and in the N-terminus of GDNF [116].

The crystal structures of ARTN and GDNF, alone as well as complexed with GFRα3 and GFRα1,respectively, have been characterized [16,17,118,119]. According to these configurations, GFL homodimers bind to D2 of the GFRα monomers and D3 stabilizes this connection [17,119]. D1 is not required for ARTN/GFRα3 binding [17]. Similarly, the N-terminal region in GFRα1 has been shown to be dispensable for complex formation and RET activation [120]; however, D1 appears to contribute by stabilizing the ineraction between GFRα1 and GDNF [121]. The most recent model of the GDNF/GFRα1/RET complex suggests a flower-like shape in which the extra-cellular part of RET wraps around th GFRα1-GDNF –complex [122] (Figure 3). In the model, RET contacts GFRα1 at five positions along the CLD1, 2, 3 and CRD of RET. These interactions drive homotypic association between the membrane-proximal regions of RET CRD. There appears to be very little actual contact between GDNF and RET, which could explain the ability of RET to accommodate any of the four GFLs [122].

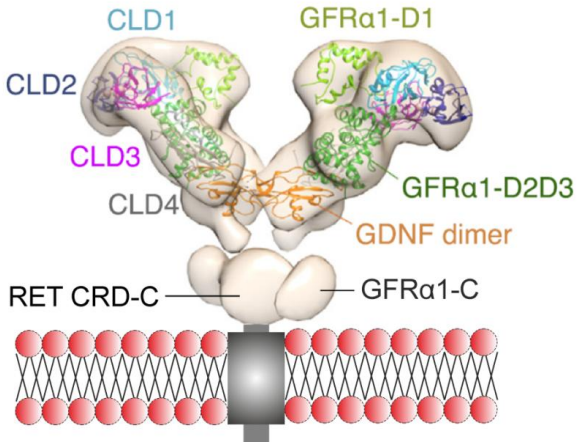

Figure 3. The most recent model of the GDNF/GFRα1/RET- signaling complex. Adjacent to the plasma membrane are the C-terminal parts of RET CRD-regions and the flanking C-terminal tails of the GFRα1 co-receptors. Modified from [122].

Signaling in cis

As GPI-anchored proteins, the GFRα co-receptors are bound to the plasma membrane on lipid rafts. These rafts are scattered sphingolipid and cholesterol enriched microdomains in the membrane outer leaf. They are associated with specific signaling complexes inside the cell, such as Src kinases [123,124]. RET, on the other hand, normally resides outside the lipid rafts (or possibly in a weak interaction with GFRα's) but is readily recruited to the rafts when the GFL-GFRα-RET complex is formed [125]. This type of *cis* signaling (cis meaning *on this side*; Figure 4A) is the best characterized way of RET activation and was proposed in the original model of GDNF mediated signaling [62]. The translocation of

RET to lipid rafts is not required for RET phosphorylation, but it may improve the activation of important downstream signaling pathways, such as Akt and MAP kinases [125]; nevertheless, RET has been shown to mediate both cell survival and neurite outgrowth via PSPN/GFRα4 outside lipid rafts [126].

Signaling in trans

The expression of GFRα receptors is more widespread than that of RET, suggesting another form of signaling [37,98]. In this *trans* mode of RET activation (trans meaning *across*; Figure 4B), the tyrosine kinase is located alone on the target cell membrane, while the coreceptors are on adjacent cells, in the extracellular matrix, or as a soluble form in the extracellular fluid. In this signaling mode, a preformed complex of the ligand and a GFRα coreceptor binds RET, resulting in the autophosphorylation of the kinase domains. During *trans* signaling, the full receptor complex is also eventually recruited to a lipid raft but in a manner that is dependent on phosphorylated RET kinase activity [127]. Inside the lipid rafts, activated RET interacts with the lipid-anchored adaptor protein FRS2; outside the rafts, RET associates with the soluble Shc, adding to the diversity of signaling possibilities [5,127].

Trans type of signaling shows a more prolonged type of activation of RET and is able to potentiate the effects of *cis* activation on neuron survival, differentiation, and growth cone development *in vitro* [127,128]. Functional GFRα's can be released from cultured Schwann cells [127]. Moreover, it has been suggested that *trans* expressed GFRα molecules from Schwann cells could function as aggregators of GFL molecules to form concentration gradients along which nerves could grow, for example in situations of nerve injury [37]. Enteric neuron precursors have also been shown to release soluble GFRα1 in culture, promoting survival [129]; however, the effects on enteric neuron axonal growth appear to be minimal [130]. Some support for a non-neuronal form of GFL signaling *in trans* was recently demonstrated in the embryonic mouse gut, where GDNF, NRTN, and ARTN together with soluble GFRα1, α2, and α3 (respectively) could initiate the aggregation of hematopoietic cells to lymphoid organs [130]. In cancer biology, soluble GFRα1 released by nerves has recently been suggested to enhance cancer cell perineural invasion through GDNF-RET signaling [131]. Concerning the PNS, a new study suggested that GFRα1 produced by neighboring DRG neurons could activate RET in adjacent large DRG neurons *in vitro* and that these neurons could also signal *in trans* via GFRα1 *in vivo* [132]. Still, mice lacking GFRα1 expressed *in trans* are normal in terms of organogenesis and nerve regeneration, suggesting only a minor physiological role for this type of signaling [133].

Downstream signaling pathways

RET dimerization leads to the autophosphorylation of several tyrosine residues in the intracellular tyrosine kinase domains. The active tyrosine residues can interact directly with signaling molecules, such as phospholipase Cγ (PLCγ) and SRC, or with a multitude of adaptor proteins, which facilitate the activation of downstream signaling pathways [115]. GDNF, NRTN, and ARTN have been shown to activate four key tyrosine residues (Tyr905, Tyr1015, Tyr1062, and Tyr1096) with similar kinetics, indicating that the downstream signaling pathways are mostly similar between GDNF family members [128,134]. From these four residues, Tyr1062 appears to be the most important signaling hub and can bind an array of adaptor proteins [134,135]. The phosphorylation of Tyr1062 is required for the full activation of RAS-MAPK and PI3K-AKT pathways [128,136–138]. It is critical for the development of the enteric nervous system and kidneys, the maintenance of spermatogonial stem cells, and the survival of embryonic sensory neurons [128,139–142]. Moreover, the phosphorylation of Tyr1062 is also required for recruiting RET to the lipid

rafts [127]. The PI3K-AKT pathway can be activated also through tyr981 via Src-family [143] or through Tyr1096, a residue that is found only on the RET51 isoform [134]. The phosphorylation of residue Tyr1015 activates PLCγ/Ca2+; mutations here cause impairments in the neuronal migration of the neocortex and renal abnormalities [144]. In addition to tyrosines, cAMP dependent RET phosphorylation on serine 696 by PKA also has a role in neural crest cell migration [145]. However, the function of the majority of activated tyrosine residues in RET remains poorly known [146].

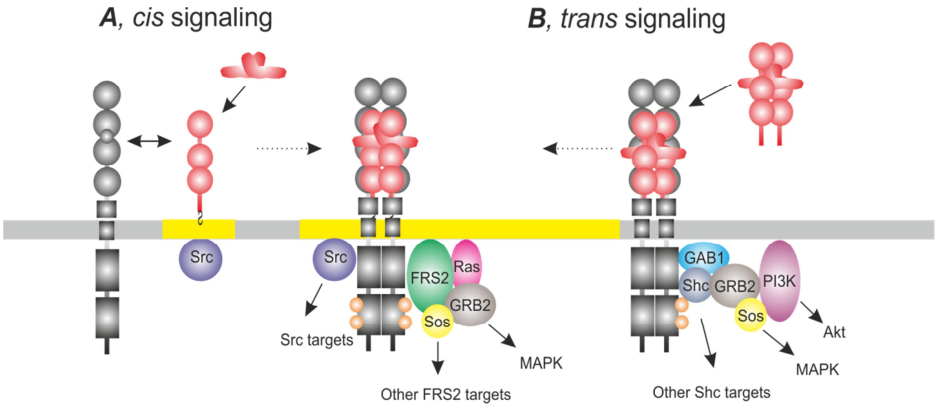

Figure 4. Putative pathways for GFL signaling inside (cis) and outside (trans) of lipid rafts. ***(A)*** The GFRα receptors are mostly attached to the plasma membrane inside the lipid rafts, whereas RET localizes outside the rafts. The binding of a GFL dimer to GFRα recruits RET to the rafts. This promotes interaction with adaptors such as FRS2 and the activation of Src. ***(B)*** The binding of a soluble GFL-GFRα complex to RET outside the rafts leads to the translocation of the full complex to a lipid raft, and initially activates signaling pathways mediated by soluble adaptors such as Shc. Modified from [5].

Factors involved in GFL-signaling

The survival promoting effects of GDNF both *in vitro* and *in vivo* (albeit not for motoneurons) require TGF-β –signaling [147]. This co-operation involves the recruitment of GFRα1 to the plasma membrane and the protection of the GPI-anchors by TGF-β; consequently, GDNF can signal without TGF-β if soluble GFRα1 is present [148]. In contrast, NRTN/GFRα2-signaling does not require TGF-β [35]

Similarly to many other growth factors, GDNF, ARTN, and NRTN bind heparin and heparan sulfate proteoglycans (HSPGs) [149,150]. In a previous study, it has been suggested that surface-bound heparan sulfate is required for GDNF mediated RET activation and the downstream effects on neurons [151]. Certainly, mice with a dysfunctional gene for the enzyme heparin sulfate 2-sulfotransferase exhibit renal agenesis, resembling RET- GFRα1- and GDNF-KO mice in that sense [152]. Still, the heparin-binding sequence of GDNF has been shown to be dispensable both for GFRα1 binding and *in vitro* neurite outgrowth [149]. Also, exogenously added heparin sulfate and heparin have been shown to inhibit GDNF signaling [151,153] and the release of GDNF from heparin sulfate to facilitate GFRα/RET signaling *in vivo* [154]. Therefore, the full role of HSPGs in GFL signaling remains undisclosed.

RET-independent GFL-signaling

GDNF has been shown to be a potent survival promoting factor for spiral ganglion neurons both *in vitro* and *in vivo*, although these neurons only express GFRα1 and not RET [155]. GDNF is, indeed, capable of signaling through GFRα1 in the absence of RET, by inducing Src-kinase activity that leads to downstream phosphorylation of MAPK and CREB *in vitro* [156,157]. The neural cell adhesion molecule NCAM has been shown to function as an alternative receptor for GDNF/GFRα1, inducing rapid phosphorylation of the Src-family kinase Fyn in neuronal and glial cells [158]. GDNF stimulation via NCAM can induce Schwann cell migration and axonal growth in hippocampal and cortical neurons in the absence of RET [158]. However, mice lacking NCAM mediated RET-signaling are normal, which puts the physiological relevance of this alternate signaling mode to question [133]. In addition, GDNF also stimulates axonal growth and functions as a chemoattractor in a subpopulation of developing GABAergic neurons that lack both RET and NCAM but express GFRα1, suggesting further modes of RET-independent GDNF signaling [159]. Recently, it was shown that syndecan-3, a transmembrane heparan sulfate proteoglycan, binds GDNF, NRTN, and ARTN and mediates cell spreading and neurite outgrowth on immobilized GFLs with the involvement of Src-kinase activation [150].

Programmed Cell Death in PNS development

PCD is an important part of the development of the nervous system; around 50% of newly formed neurons have been estimated to perish before the final maturation of the PNS [160]. In contrast to necrosis, where cells burst after irreversible injury or pathology, PCD is a controlled, active phenomenon that requires energy and leads to a clean disposal of the cell remnants through a condensation of the nucleus and cytoplasm, cell fragmentation, and finally phagocytosis [161]. This type of PCD is known as *apoptosis*. In comparison to the other non-apoptotic types, it is the most common PCD type in the nervous system [162].

PCD in the nervous system can be divided to early-PCD, involving young neural progenitors and postmitotic neuroblasts, and late-PCD, which depends on target derived neurotrophic factors. The early bout of neuronal death is more poorly understood, but is likely to be related to the removal of erroneous, unwanted, or extra cells. It is regulated by many of the factors that are involved in progenitor proliferation, migration, and differentiation, such as BMPs, Sonic hedgehog, Wnts, IGFs, FGFs, and NT-3 [163,164]. In the PNS, neuronal apoptosis occurs already in the initial stages of DRG neuron development, before target innervation has started, and is increased in the absence of NT-3 [165–167].

As the neurotrophic theory states, the target derived NTF-dependent PCD is responsible for adjusting the number of neurons suitable for the size of the innervated target. The NTF-dependent PCD can occur only during an "apoptotic window", which opens around the time when the axons reach the target tissue [168,169]. After the apoptotic stage of the neuron population comes to an end, the cells eventually become less dependent of neurotrophic support for survival and resistant to apoptosis [168,170]. The timing of this apoptotic window also appears to be genetically regulated [169]. Moreover, although neurotrophic factor theory holds true for sympathetic and many DRG neurons, which require target derived NGF support for survival via the TrkA receptor [171], it is important to note that the occurrence of normal PCD varies between different peripheral neuron types, and its role in the development of some subdivisions of the PNS remains unclear (as discussed later).

The mechanisms of neuronal apoptotic pathways are best known in sympathetic neurons. They involve the Bcl-2 family of proteins, the adaptor protein Apaf-1, and members of the cysteine protease caspase family [172] (Figure 5). When deprived of TrkA activation by NGF, apoptosis in sympathetic neurons commences via the intrinsic mitochondrial pathway [170]. The Phosphorylation of the c-jun transcription factor leads to the transcription of the pro-apoptotic members of the Bcl-2 family, which in turn mediate the activation of yet another member of Blc-2 family, Bax. The insertion of oligomerized Bax to the outer mitochondrial membrane causes the release of cytocrome-c from mitochondria. Together with the initiator-type caspase-9 and Apaf-1, cytochrome-c forms a complex, which leads to the activation of caspase-9. Caspase-9 then activates the executioner-type caspase-3, and death soon follows.

A different type of apoptosis pathway is activated, via an extrinsic route, when a ligand binds a specific "death receptor". This pathway also leads to the activation of executor-caspases, but not by cytohcrome-c. The nonselective neurotrophin receptor p75 is one such death receptor: when bound by neurotrophins, it can induce apoptosis [173].

The activation of the neurotrophic factor tyrosine kinase receptors by their ligands leads to the induction of survival promoting pathways. Especially the PI3-K/Akt pathway has been related to the survival promoting effects of NTFs [172]. However, in the absence of their ligands, the neurotrophin receptors TrkA and TrkC actively cause the death of their host neurons, further explaining the dependence of certain peripheral neurons on neurotrophic support [174]. RET has also been suggested to induce apoptosis in the absence of GDNF via caspase-3 cleavage in certain cultured cell lines and primary cultures of pituitary somatotrophes [175,176]. Interestingly, depriving sympathetic neurons of GDNF support has been reported to cause apoptosis via a novel non-mitochondrial pathway that does not require caspase-3, but activates the executor caspases 2 and 7 [177].

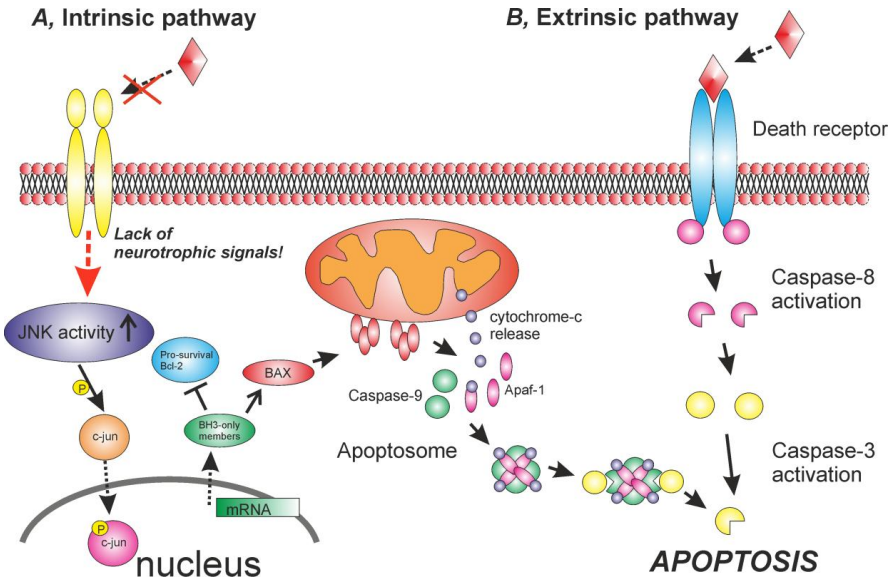

Figure 5. Apoptosis pathways. ***(A)*** In the intrinsic pathway, apoptotic signals (e.g. the loss of neurotrophic signaling) activate the c-jun N-terminal kinase (red arrow to JNK). JNK then induces the phosphorylation of c-jun, which is subsequently translocated to the nucleus. This leads to the production of pro-apoptotic BH-3 proteins followed by the attachment of oligomerized BAX proteins to the outer mitochondrial membrane. Cytochrome-c is then released from the mitochondrial intermembrane space and forms a complex (apoptosome) with caspase-9 and Afap-1. Activated Caspase-9 cleaves caspase-3, which ulti-

mately leads to cell death. ***(B)*** In the extrinsic pathway, the binding of a ligand to a death receptor (e.g. NGF to p75) activates caspase-8 which then cleaves caspase-3 and commences apoptosis.

The Somatosensory system

The somatosensory system serves the body to perceive stimuli originating from both inside and outside the body. Signals from the inside, or proprioceptive signals, originate from the muscles and tendons and relay information concerning the position and movement of the trunk and limbs. Outside signals are communicated via the skin and convey modalities of mechanical, thermal, and chemical stimuli (Figure 6A). The peripheral neurons that communicate somatosensory information from the trunk and limbs are located in the DRGs adjacent to the spinal cord. These primary sensory neurons are pseudounipolar in nature: they send one axon branch to a peripheral target and another through the dorsal root into the spinal cord to synapse with second order neurons (Figure 6B).

Cutaneous sensory neurons

Cutaneous DRG neurons can be classified in a number of ways (see Table 1). The neurons that respond to innocuous low level mechanical and thermal stimulation are called low-threshold mechanoreceptors (LTMRs), whereas neurons that respond to high intensity and painful stimuli are called nociceptors [178]. LTRMs can be further divided into slowly adapting (SA), which maintain activity during sustained stimuli, and rapidly adapting (RA), which fire only at the beginning and end of a given stimulus [179]. In terms of size and conduction velocity, the cutaneous fibers can be grouped into three major classes: Aβ-, Aδ-, and C-fibers. Aβ-fibers consist of large myelinated axons with the fastest conduction velocities; they are low threshold mechanoreceptors that respond to innocuous sensations, although some are able to encode nocious stimuli as well [178,179]. In comparison to Aβ-fibers, Aδ-fibers are smaller, lightly myelinated, and conduct at an intermediate velocity. The C-fibers, for their part, are the smallest but the most common of the cutaneous fibers.. They have unmyelinated axons and, therefore, the lowest conduction velocities. The majority of both Aδ- and C-fibers belong to nociceptors based on their responses to nocious mechanical, heat, or cold stimuli [179].

Cutaneous end organs

The Mammalian skin is divided into non-hairy (glabrous) and hairy skin. Nocious signals originating from the skin are mostly detected through free nerve endings that innervate both glabrous and hairy epidermis. Innocuous somatic sensations, on the other hand, are detected by specialized end organs. The glabrous skin hosts four types of sense organs: Merkel cells, Ruffini endings, and Pacinian and Meissner corpuscles. In the hairy skin, special palisades of longitudinal nerve endings and circumferential endings are associated with individual hair follicles. Merkel cells are innervated by SA afferents and respond with very low thresholds to skin intendation caused by edges and contours, allowing the sensing of textures and shapes [179]. Merkel cells are also found in the hairy skin adjacent to the largest hair follicles. Ruffini endings can be found in the glabrous dermis and possibly also in the hairy skin [179]. Like Merkel cells, they are innervated by SA afferents, but specialize in detecting low resolution changes in tissue shape, such as the compression of the hand and fingers when holding an object. Meissner and Pacinian corpuscles are associated with RA fibers and detect rapid changes in skin movements and vibration, for example when losing a grip on an object [179].

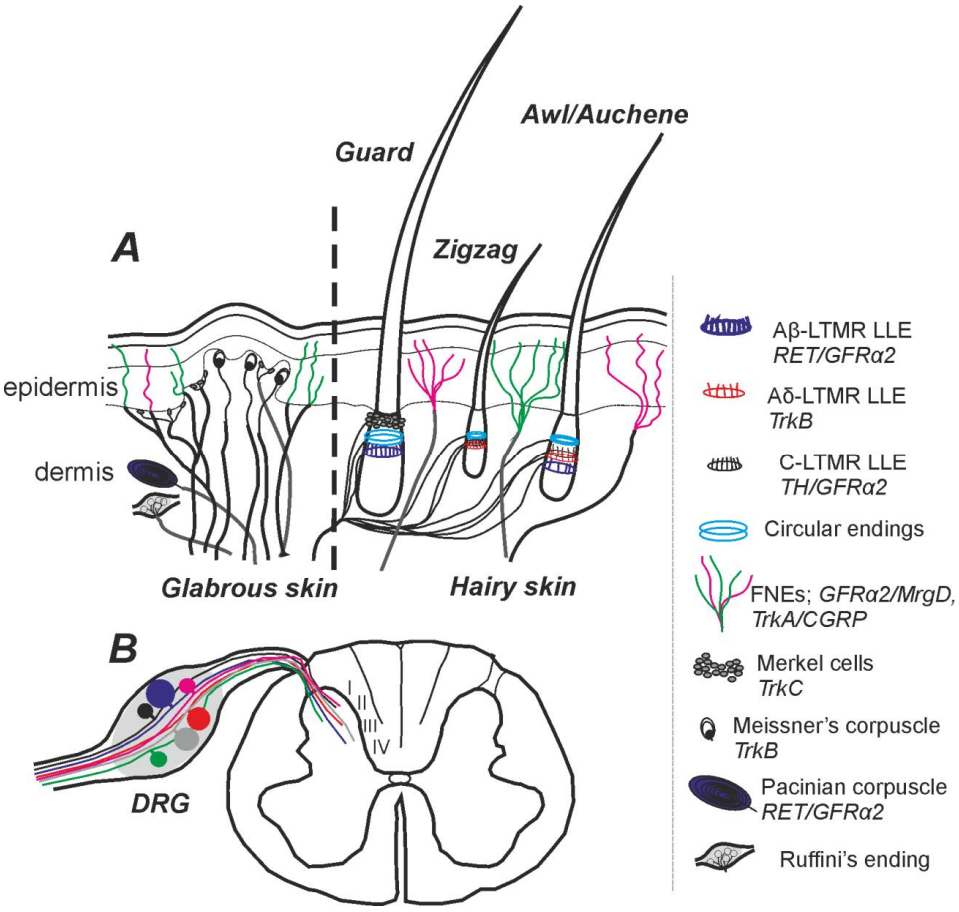

Figure 6. The organization of cutaneous innervation. ***(A)*** In the glabrous skin, innocuous touch is mediated by four types of mechanoreceptors: the Merkel cell-neurite complex, Meissner corpuscles, Pacinian corpuscles, and Ruffini's endings. In the hairy skin, tactile stimuli are communicated through three types of hair follicles and their associated LTMR longitudinal lanceolate endings (LLEs): guard follicles are associated with Aβ-LTMRs, awl/auchene hairs are innervated by all three (C, Aδ, and Aβ) LTMRs, and zigzag hair follicles by both C- and Aδ-LTMRs. All hair follicle types are also innervated by circumferential endings. Painful stimuli are detected through free nerve endings (FNEs) in the epidermis, which are characterized by both Aδ- and C-responses. ***(On the right)*** Molecular markers and neurotrophic factor receptors associated with different types of cutaneous endings and end organs. ***(B)*** The cell bodies of primary somatosensory neurons are located in the dorsal root ganglia (DRG) and form synapses centrally in cell type specific laminae of the spinal cord. Modified from [179].

Hair follicle types and innervation

The mouse back skin is covered by three major types of hair follicles: zigzag, awl/auchene, and guard. These categories can be distinguished on the basis of hair shaft length, the number of medulla cells in the shaft, and the presence and number of kinks in the shaft [180]. Zigzag hairs are the most abundant type, adding up to three fourths of all the hair follicles [181]. They are the shortest and thinnest type with three to four alternating bends in the shaft [182]. Awl/auchene hairs are almost identical with each other and are usually grouped together [180]. They have three to four medullary cell rows, are longer and thicker than zigzags, and represent about one fifth of mouse coat hair [181,182]. The last group,

guard hair follicles, are by far the largest and also the rarest [180]. This type of hair follicle amounts to only about one percent of all hair follicles and can be identified by its exceptional size and two associated sebaceous glands [180–182]. The hair follicle types on mouse trunk skin are organized in a 1:23:76 iterative pattern, where one guard follicle is surrounded by 23 awl/auchene follicles in a regular arrangement. 76 zigzag hairs evenly populate areas surrounding the two larger hair follicle types [181].

Three kinds of nerve endings are associated with pelage hair follicles: longitudinal lanceolate endings (LLEs), which are vertical finger-like projections surrounding the hair follicle outer sheath partly ensheated from the outside by projections from terminal schwann cells; circumferential endings forming loops around the hair follicle outer root sheath above the longitudinal endings; and Merkel cell complexes, which are only found together with guard follicles [179,183,184]. All the hair follicle types have been shown to associate with a unique combination of longitudinal lanceolate endings deriving from different types of sensory neurons [181,184]. According to these findings, the smallest zigzag fibers are innervated by both C-LTMRs and Aδ-LTMRs, the awl/auchene follicles are innervated by C-LTMRs, Aδ-LTMRs, and RA Aβ-LTRMs, and the guard follicles only by the large RA Aβ-LTMRs. Circumferential endings, which remain poorly characterized in existing research, are found around each follicle type [179,185]. In addition to lanceolate endings, a cluster of Merkel cells and their associated nerve fibers forms a "touch dome" structure associated with guard follicles [181].

Table I. Cutaneous sensory neuron subtypes (Modified from [179]).

NEURON TYPE	CONDUCTION VELOCITY	END ORGAN OR ENDING	LOCATION OF ENDINGS	STIMULUS	RESPONSE
SA1-LTMR	Aβ(16-96m/s)	Merkel cells/ touch dome	Basal epidermis/ around Guard HFs	Intendation	
SA2-LTMR	Aβ (20-100ms)	Ruffini endings	Dermis	Stretch	
RA1-LTMR	Aδ (26-91m/s)	Meissner corp & LLEs	Dermal papillae/ Guard & Awl-Auch HFs	Skin & Hair movement	
RA2-LTMR	Aβ (30-90m/s)	Pacinian corpuscles	Deep dermis	Vibration	
Aδ -LTMR	Aδ (5-30m/s)	LLEs	Awl-Auch & Zigzag HFs	Hair movement	
C-LTMR	C(0.2-2m/s)	LLEs	Awl-Auch & Zigzag HFs	Hair movement	
Nociceptors	Aβ, Aδ,C (0.5-100m/s)	Free nerve endings	Epidermis & Dermis	Nocious mechanical	

The origin of DRG neurons

In mice, the trunk NCCs destined to become sensory neurons start delaminating from the dorsal neural tube at around E8.5, migrate ventrally between the somite and the neural tube, and begin to coalesce into dorsal root ganglia around E9.5 [186,187]. The development of DRGs occurs mostly in two successive waves [188–190]. These waves correspond with the sequential expression of the helix-loop-helix transcription factors Ngn2 and Ngn1

[189]. In the first wave (Figure 7A), the developing neurons express Ngn2, proliferate in a limited fashion, and form myelinated, medium to large sized proprioceptive neurons and cutaneous mechanoreceptors [189–192]. Neurons in the second wave (Figure 7B) express Ngn1 [189]. These cells develop mostly into numerous small and medium diameter nociceptive neurons, but also, to a minor extent, into the early born population of large neurons [189,191]. In addition, a small third wave of neurogenesis, from the NC derived boundary gap cells at the nerve roots, contributes a minor population to the small unmyelinated DRG neurons [193,194].

Following the initiation of neurogenesis, sensory neurons begin to express the homeodomain transcription factors Brn3a and Islet1 [195,196]. These factors are crucial for ending the neurogenic phase and initiating most aspects of the following sensory neuron diversification, such as the expression of neurotrophic factor receptors and the transcription factors Runx1 and Runx3 in different sensory neuron populations [197–199]. At least five different types of developing sensory neurons – expressing TrkA, TrkB, TrkC, RET, and several other markers – can be identified in mouse DRGs during early sensory neuron specification [199]. The sensory neuron lineages go through a dynamic diversification process during development and early postnatal life, with many transient and final subpopulations in both myelinated and unmylinated lineages [192,199,200].

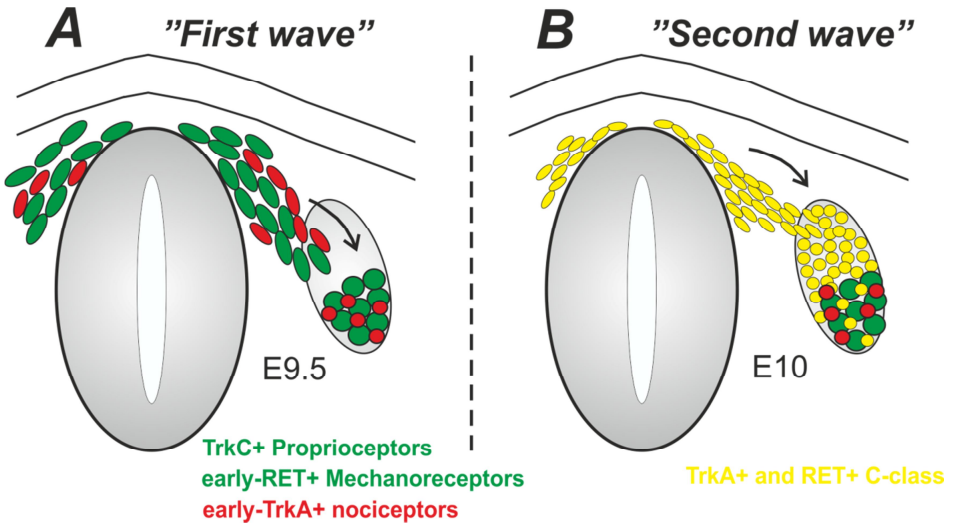

Figure 7. DRG neurons arise in two major waves of neurogenesis. ***(A)*** During the first wave, starting around E9.5 in the mouse, the large, myelinated TrkC+ proprioceptors, early-RET+ mechanoreceptors, and early-TrkA+ -δ-nociceptors are born. ***(B)*** In the second wave, abundant numbers of small, unmyelinated TrkA+ C-class neurons emerge. A subgroup of these neurons gives rise to the nonpeptidergic RET+ neurons during late prenatal to early postnatal development.

Myelinated DRG neurons

During the initial wave of neurogenesis, large TrkC expression appears first, followed shortly by TrkB and RET [201,202]. In these early stages, there is a strong colocalization of TrkC and TrkB expression, which eventually disappears during the first two weeks of gestation as the two populations segregate [166,201]. This segregation is driven partly by the transcription factor Runx3 in the TrkC+ neurons, which downregulates TrkB expression [201]. The proportion of TrkC+ neurons in the mouse DRG rapidly declines from about

70% to less than 10% in the adult animal [166,203]. A great majority of the remaining TrkC+ DRG neurons represent type Ia proprioceptor neurons, which project to the ventral spinal cord and innervate muscle spindles in the periphery [204]. In addition, TrkC emerges in a group of neurons that send projections to cutaneous targets [205,206]. Accordingly, a recent study showed that cutaneous Merkel cells are innervated by TrkC expressing myelinated fibers [207]; in support of these findings, significant deficits in Merkel cell development and innervation have been found in mice lacking TrkC and its ligand NT-3 [208,209].

TrkB-positive neurons arise from the initial population of TrkB+/TrkC+ double-positive neurons at around E14.5 in mice [201]. In this double-positive population, the transcription factor Shox2 promotes the expression of TrkB, which in turn downregulates TrkC+ from the same neurons [210,211]. In the adult mouse thoracic DRGs, most TrkB+ neurons have been shown to represent a group of medium-sized, lightly myelinated Aδ-LTRMs, which form longitudinal lanceolate endings associated with hair follicles [181,212]. The survival of these Aδ-LTRMs requires NT-3 during early postnatal life, and NT-4 later in adult animals [213,214]. NT-3 signaling via TrkB in Aδ-LTRMs could explain why significantly more DRG neurons are lost in NT-3- than in TrkC-deficient mice (60% vs. 20-30%) [215]. Further findings suggest that, in addition to the hair follicle innervating Aδ-fibers, TrkB is required for other neuron populations innervating cutaneous targets. In mice in which the segregation of TrkB+ DRG neurons fails due to loss of Shox2, the innervation of Merkel cells and Meissner corpuscles is significantly reduced [210]. Moreover, in mice lacking TrkB altogether, Meissner corpuscles are lost and Merkel cell innervation is disrupted, along with a loss of ~30% of DRG neurons [216]. A reduction in the number of DRG neurons (about 30%) has also been reported in BDNF-KO mice [217,218]. This requirement for BDNF appears postnatally and has been suggested to involve an autocrine or paracrine way to promote the early postnatal survival of small NGF and GDNF responsive neurons [219].

In addition to the TrkB-expressing LTRMs, the Aδ-class of DRG neurons includes nociceptors. A significant number of Aδ-fibers express the NGF receptor TrkA and the peptidergic marker CGRP; in the adult rat lumbar DRGs, about 30% of myelinated neurons fall into this class [220,221]. These neurons are dependent on TrkA-mediated signaling for survival and are mostly lost in TrkA-KO mice [213,222]. A recent study showed that TrkA+ Aδ-nociceptors are present in mouse DRGs already at E10.5, suggesting that they are born in the first wave of neurogenesis [223].

GFL-signaling in myelinated sensory neurons

RET is present in DRG neurons already at E10.5 in the mouse [104]. These early-RET neurons are a distinct population from the small RET+ neurons that appear later during development [187,224]. The early-RET neurons represent around one fourth of NFH+ DRG neurons, express MafA and GFRα2 during development, and some sub-populations have also been reported to express TrkB or TrkC at P0 [201,225]. Early-RET neurons are Aβ-class low-threshold mechanoreceptors. They innervate Meissner and Pacinian corpuscles and form longitudinal lanceolate endings around large hair follicles in the skin; centrally they project to laminas III-V and the dorsal column of the spinal cord [225,226].

The role of RET-signaling in the early-RET neurons remains incompletely understood. From two studies using two different RET-cKO models, one showed no decrease in DRG neuron numbers and no loss of size in the large NFH+ neurons, whereas the authors of the other study observed a 15% loss of DRG neurons at P15.5 [187,225]. In a third study that used a $RET^{GFP/GFP}$-KO mouse line, a greater than 30% loss of GFP+ sensory neurons was observed after two weeks of gestation [227]. The most recent findings showed that 50% of early-RETneurons are lost in RET-cKO mice at E18.5 [132] In NRTN and

GFRα2-deficient mice, however, normal early-RET neuron numbers have been reported at birth [226]. This discrepancy in early-RET neuron survival between RET- and GFRα2-/NRTN-KO mice was recently suggested to be the result of *in trans* GDNF/GFRα1-signaling compensating for GFRα2/NRTN [132]. In the periphery, the development of Pacinian corpuscles appears to be critically dependent on RET- and NRTN/GFRα2-signaling, as only rudimentary corpuscles and associated nerve branches are found in the KO animals [226]. NFH+ LLEs, on the other hand, have been reported to be either completely missing or only slightly affected in the absence of RET-signaling [225,226]. All in all, additional research is needed to clarify the role of RET- and GFL-signaling in RA Aβ-LTMR development.

Unmyelinated neurons

Most, if not all, unmyelinated DRG neurons are generated in the second wave of neurogenesis and start expressing the neurotrophin receptor TrkA [189]. These neurons, like the Aδ-neurons which express TrkA, depend on NGF for survival during development [228]. During embryogenesis, the unmyelinated neurons begin segregating into two distinct lineages as TrkA expression is downregulated in a subgroup of small DRG neurons [229,230]. The neurons that lose TrkA expression switch to expressing RET and one of its GFRα1 or GFRα2 co-receptors in a process that begins in mice at around E15 and is complete two weeks after birth [187,224]. The transcription factor Runx1 and the receptor tyrosine kinase Met, which binds the hepatocyte growth factor (HGF), play key roles in the segregation of the two lineages. Runx1 expression - induced by FGF molecules secreted from the large myelinated DRG neurons [231] - emerges in the small neurons at around E12.5 and eventually extinguishes TrkA expression while enabling RET expression in the developing nonpeptidergic neurons [187,232]. Met, on the other hand, is expressed in the TrkA+ lineage and promotes the extinction of Runx1 and the expression of CGRP [233]. The neurons that continue expressing TrkA develop into the peptidergic C-nociceptors, marked by their expression of classic neuropeptides such as CGRP, Substance P, and TRPV1 [192]. They synapse centrally in the lamina I and the outer region of lamina II of the spinal cord and project to the *stratum spinosum* of the epidermis [234,235]. A small subgroup of TrkA+ neurons remains RET+ and expresses the ARTN co-receptor GFRα3 [203,236]. Furthermore, TrkA/GFRα3 neurons express the capsaicin/heat activated channel TRPV1, while many of them can also express the nocious cold/pain channel TRPA1. These findings together implicate a role in cold and pain sensation for these neurons [237,238].

GFL-signaling in unmyelinated sensory neurons

Most of the small RET+ neurons, however, are nonpeptidergic, bind the *Griffonia simplicifolia* isolectin B4 (IB4), and express GFRα2 or GFRα1 [224,239,240]. The discovery of new molecular markers has made it possible to divide the nonpeptidergic neurons to more precise subclasses [192,200]. The great majority of RET+/IB4+ neurons express GFRα2, the Mas-related G-protein-coupled receptor d (Mrgprd), and the purinoceptor P2X3. These neurons belong to a group of polymodal nociceptors that form free nerve endings in the *substantia gelatinosa* of the epidermis and project centrally to the inner lamina II of the spinal cord [234,235,240]. RET/Mrgprb4+/IB4+ neurons, on the other hand, form large arborizing endings around hair follicle roots and have been implicated in sensing gentle, pleasurable touch [241,242]. A group of RET+/GFRα2+/IB4- nonpeptidergic neurons defines the C-LTRMs that form longitudinal lanceolate endings around hair follicles on murine skin [181]. These neurons express tyrosine hydroxylase (TH), while many of them also express Vglut3 and TAFA4 [181,243,244]. In addition to innocuous mechanical sensations, the C-LTMR neurons are also sensitive to cooling and can contribute to mechanical pain hyper-

sensitivity [181,245]. In contrast to GFRα2+ populations, little is known about the nonpeptidergic populations that express GFRα1.

RET-signaling is generally considered dispensable for the survival of nonpeptidergic DRG neurons [187,227,246], although conflicting observations have been reported [247]. RET-signaling is required for normal soma size in nonpeptidergic neurons, as indicated by the atrophy of small unmyelinated DRG neurons in RET-cKO mice [187]. A similar phenotype is seen in GFRα2-KO mice, in which IB4+ neurons remain, but are markedly smaller than in WT littermates [240]. Also in NRTN-KO mice the loss of trophic support leads to the downregulation of GFRα2-expression in DRG neurons and the reduction of soma size [248].

NRTN is expressed in the developing and adult mouse skin epidermis [39], and GFRα2-KO mice show a severe reduction in the density of nonpeptidergic free nerve endings of the glabrous skin epidermis, indicating that NRTN/GFRα2-signaling is a major factor influencing target innervation in nonpeptidergic neurons. In line with this, NRTN or GDNF overexpression in the skin leads to nonpeptidergic hyperinnervation of the glabrous epidermis and hypertrophy of the relevant neuronal somas [249,250]. In the developing mouse skin, NRTN is expressed at high levels also in hair follicles [39]; however, it is not known whether NRTN/GFRα2-signaling is required for hair follicle innervation by C-LTMRs (or RA Aβ-LTMRs).

The Autonomic Nervous System

According to John Langley's original definition, the autonomic nervous system "consists of the nerve cells and nerve fibers, by means of which efferent impulses pass to tissues other than multi-nuclear striated muscle" [251]. Langley further divided the ANS into the sympathetic, parasympathetic, and enteric divisions, and this organization still holds today [251,252]. The sympathetic and parasympathetic divisions consist of two sets of neurons each, one central (preganglionic) and one peripheral (postganglionic). The sympathetic preganglionic neurons are located between the first thoracic and early lumbar levels of the spinal cord, whereas the parasympathetic preganglionic fibers originate from specific brainstem nuclei and from the S2-S4 sacral levels of the spinal cord. Acetylcholine functions as the preganglionic neurotransmitter via nicotinic receptors in both the sympathetic and parasympathetic systems. In the target tissues, most sympathetic signaling is mediated via noradrenalin, whereas acetylcholine (via muscarinic receptors) is found in the parasympathetic endings. Traditionally, the sympathetic and parasympathetic divisions have been regarded as mostly antagonistic in action: where sympathetic activity relates to intense activity, catabolic functions, and energy expenditure ("fight or flight"), parasympathetic activity functions to reduce energy expenditure and increase anabolic processes and energy stores ("rest and digest") [253]. The third division, the enteric nervous system (ENS), encompasses the largest, most autonomous and complex part of the ANS and regulates the functions of the gastrointestinal tract. In contrast to the sympathetic and parasympathetic divisions, the enteric nervous system lacks preganglionic neurons. However, it exchanges afferent and efferent signals with the sympathetic and parasympathetic divisions.

Sympathetic nervous system

Peripheral sympathetic neurons reside in two types of ganglia. The paravertebral ganglia form bilateral interconnected chains (sympathetic trunks) adjacent to the vertebral column and project axons to somatic tissues in all parts of the body [252]. Major paravertebral ganglia are the superior cervical ganglion (SCG), which projects along the internal carotid artery to targets in the head and upper cervical regions, and the stellate ganglion, which in-

nervates the upper extremity and thoracic targets. The prevertebral ganglia, a class of sympathetic ganglia, are unpaired ganglia further away from the vertebral columns and are mostly located close to the main branches of the abdominal aorta [254]. The prevertebral ganglia innervate organs in the abdominal and pelvic cavities. The adrenal medulla cells, which release adrenalin and noradrenalin to the blood stream, are homologous to postganglionic sympathetic neurons. They are innervated by sympathetic preganglionic fibers.

Sympathetic neurons

The future sympathetic neurons from the trunk neural crest cells migrate through the same ventromedial pathway as the developing sensory neurons [186]. After passing through the somites, the NCCs continue migrating ventrally and begin coalescing into the developing sympathetic trunks near the dorsal aorta. Some of the NCCs continue their migration to the foregut and some develop into the prevertebral ganglia and the chromaffin cells of the adrenal medulla [255–257]. The migration of sympathoadrenal NCCs and the development of sympathetic trunks is dependent on many both attracting and repulsing factors, such as Neuregulin-1, Semaphorins 3 and 3F, and SFD-1 [258–262]. The primordial ganglia first appear in the thoracic region, followed by the development of the cervical region where the SCG is formed after additional migratory steps [263,264]. In the SCG, many of the contributing NCCs appear to derive from the vagal neural crest [265]. Bone morphogenetic proteins (BMPs) expressed by the dorsal aorta induce the differentiation of sympathetic neurons from the coalescing NCCs [262,266–268]. In addition, BMPs initiate the expression of a network of transcription factors (Mash1, Phox2a, Phox2b, Gata3, and Hand2), which regulates the expression of pan-neuronal genes and genes regulating the differentiation of NCCs into cholinergic, noradrenergic, and other lineages [269].

The early sympathetic neuroblasts appear to be independent of neurotrophic factor support for survival [270], although HGF/Met signaling has been shown to promote the survival of sympathetic neuroblasts [271]. Sympathetic neurons soon become dependent on NGF signaling via TrkA for survival [228,272]. Early sympathetic neurons also express TrkC [215], and about 50% of sympathetic neurons are lost in NT-3-KO mice [273,274]. NT-3 promotes early sympathetic axon growth [274,275], whereas distal target innervation is dependent mostly on NGF [276]. Only NGF promotes survival through retrograde signaling, suggesting that the reduced sympathetic neuron numbers in NT-3-KO mice could result from an innervation failure and the subsequent loss of target-derived NGF for survival [274]. Moreover, a recent study has shown that both TrkC and TrkA initiate cell death in the absence of a ligand; this could also explain the loss of sympathetic neurons in mice lacking these receptors [174].

GFL-signaling in sympathetic neurons

RET is expressed in the developing mouse sympathetic chain neurons starting from E9.5 [104]. In the mouse SCG, RET mRNA is abundant at E12.5, transiently downregulated around E14.5 and then upregulated after E16.5 until post natal stages [39,264]. A thorough analysis of RET-KO embryos has indicated abnormalities in sympathetic neuron migration and axonal outgrowths leading to the displacement and hypotrophy of sympathetic ganglia, including the SCG [277]. Most SCG cells express GFRα3 during development [264]; consequently, ARTN expressed adjacent to developing sympathetic ganglia and on the surface of blood vessels is required for the migration and initial outgrowth of sympathetic neurons [277–279]. GDNF-KO mice have a 35% reduction in SCG neurons at P0 [42], and GDNF has recently been shown to support a similar sized population of embryonic sympathetic neurons *in vitro* [36]. Together, these findings suggest that GDNF could have a role in sympathetic neuron development. GFRα1, however, is expressed only transiently in the

developing SCG [39,264], and GFRα1-KO mice have no abnormalities in the sympathetic neurons [280,281]. RET also controls the maturation of the cholinergic phenotype in this population of sympathetic neurons [282]. Accordingly, GFRα2 is expressed in the cholinergic sympathetic neurons of the mouse stellate ganglion and is required for normal soma size and target innervation in these neurons [51].

Parasympathetic nervous system

The cranial preganglionic parasympathetic neurons are located in several nuclei of the brainstem. These include the dorsal motor nucleus of vagus (DMV), ambiguous nucleus, Edinger-Westphal nucleus (E-W), and salivatory nuclei. Axons from the E-W and salivatory nuclei project through cranial nerves to four parasympathetic ganglia found in the head. From the ambiguous nucleus and DMV, preganglionic fibers project through Vagus nerve to ganglia associated with the airways, heart, pharynx, and the gastrointestinal organs up to the last third of the transverse colon. The remainder of the gastrointestinal tract and the pelvic organs receive preganglionic parasympathetic fibers from neuronal clusters in the sacral spinal cord through pelvic splanchnic nerves [254].

In the head, neurons from the ciliary ganglion innervate the pupillary sphincter and ciliary muscles in the eye; pterygopalatine ganglion innervates lacrimal, nasal, and palatal glands; and submandibular and otic ganglia innervate the salivary glands. Parasympathetic ganglia in the trunk and abdomen are generally smaller than the cranial ganglia. They are located close to or inside their target organs, forming network like plexuses [253]. In the gastrointestinal tract, preganglionic parasympathetic fibers from the vagus nerve and sacral spinal cord synapse with enteric neurons in the gut wall. Postganglionic neurons innervating the pelvic organs, including the genitals, are located in the pelvic splanchic ganglia [254].

Parasympathetic neurons

Relatively little is known about the development of parasympathetic neurons, and the bulk of information on the subject comes from studies using the chick ciliary ganglion as a model [283]. Similarly to sympathetic neurons, the pan-autonomic network of transcription factors Mash1, Phox2a, and Phox2b is required for parasympathetic neuron development. All parasympathetic neurons fail to develop in mice lacking Mash1 or Phox2b [284–286]. Phox2a, on the other hand, is required by parasympathetic neurons to varying degrees: in Phox2a-KO mice the otic and sphenopalatine ganglia fail to develop, submandibular ganglion is only partially affected, and cardiac ganglia are normal, suggesting a rostral to caudal dependency gradient on Phox2a in parasympathetic neurons [287]. According to studies on the chick ciliary ganglion, BMPs induce the expression of pan-autonomic transcription factors in parasympathetic neurons, as in the sympathetic neurons [288].

Recently, the origin and migration of parasympathetic neurons in mammals have been further elucidated by two studies [289,290]. The studies showed that streams of NCCs expressing Schwann cell markers migrate along cranial, truncal, and splanchic nerves and develop into parasympathetic neurons and glial cells. During the migration, many of the Schwann cell precursors (SCP) begin expressing the autonomic neuron markers Mash1 and Phox2b and downregulate the expression of glial markers [289]. Mash1 expressing cells were found at sites closest to the developing ganglia; deleting this factor from the SCPs resulted in the loss of parasympathetic neurons [289]. In these studies, Phox2b was first expressed by all nerve associated SCPs, but later exclusively by cells in the developing parasympathetic ganglia [290]. As BMPs have been implicated in the development of ciliary ganglia in chicks [288], it was hypothesized that rising levels of BMPs near the developing parasympathetic ganglia could serve to induce the expression of Mash1 and begin the deri-

vation of parasympathetic neurons from the progenitor cells [289]. Another important discovery in these studies was that, in the most economical way, the SPCs use the preganglionic nerve outgrowths as guidelines to migrate to the sometimes distant locations of the future parasympathetic ganglia [289,290].

Another recent study shed light on the mechanisms of parasympathetic gangliogenesis by showing that it is regulated by Wnt- and FGF-signaling [291]. This work showed that Wnt molecules secreted by epithelial precursor cells in the developing submandibular gland promote parasympathetic neuron proliferation, survival, and the subsequent innervation from the submandibular ganglion. FGF-signaling on the other hand acts to inhibit Wnt expression, and is itself inhibited by Sprouty1 and Sprouty2 to allow Wnt expression [291].

Organs that form as outgrowths of the embryonic gut (like the lungs and pancreas) receive their parasympathetic neurons from the pool of vagal NCCs that migrate along the developing alimentary tract [292,293]. In the rat, the NCCs colonize the foregut by E11 and from E12 onwards a subset of the enteric NC progenitors starts a secondary migration into the developing pancreas [293]. In the mouse, vagal NCCs enter into the foregut at around E9.5 [294] and begin colonizing the developing pancreatic buds by E10 [295]. The enteric NCCs express DCC (deleted in colorectal cancer) and use the DCC ligand netrin-1 as a guidance cue for their migration to the pancreas [296].

The regulation of parasympathetic neuron numbers during development appears to differ depending on the studied animal species. On the one hand, about 50% of ciliary ganglion neurons in the chicken die via apoptosis during the time of target innervation [297]. On the other hand, neuron numbers in the parasympathetic cardiac ganglia of the developing frog are regulated through proliferation of progenitor cells, apoptosis being either nonexistent or playing only a minor role [298,299]. Very little is known about the mechanisms of cell number regulation in mammalian parasympathetic ganglia [283].

GFL-signaling in parasympathetic neurons

RET, GFRα1, and GFRα2 are expressed in developing mouse cranial parasympathetic ganglia [39,300,301]. Here GFRα1 expression is downregulated at E12, whereas GFRα2 expression remains high throughout development into adulthood [301]. In embryonic and early postnatal rats, RET and GFRα2 are expressed by the cardiac ganglion neurons and GFRα1 by the cardiac glial cells [302]. In the pelvic ganglia of rats, GFRα1, GFRα2, and RET transcripts have been observed in the majority of neuronal bodies [303], whereas in mice, GFRα2 protein mostly localizes to the abundant cholinergic pelvic ganglia neurons and rarely to the noradrenergic neurons [97]. Low levels of GFRα1 and GFRα3 expression have also been reported in subpopulations of developing pelvic ganglia neurons of mice [304,305]. In the pancreas, most parasympathetic neurons of newborn and adult mice also express GFRα2 along with glial cells that occupy the periphery of the islets of Langerhans [52]. Both GDNF and NRTN are expressed in tissues associated with the parasympathetic nervous system; however, whereas NRTN expression is located to the target tissues and persists to adulthood, GDNF expression is found along the migratory routes of the neurons and is downregulated during prenatal development [39,300,306].

RET-signaling via GDNF/GFRα1 is absolutely required for the proper migration and proliferation of otic and sphenopalatine neurons, as well as for many submandibular neurons [300,301]. In GFRα2-deficient mice, the otic, submandibular, and sphenopalatine ganglia are present, but the former two are smaller in size [301,307]. This atrophy is the result of both reduced neuron numbers and the loss of cell size, and is amplified postnatally [301]. A similar loss of submandibular neurons has been observed in NRTN-KO mice [248]. NRTN is expressed in the lacrimal and salivary glands, and the parasympathetic innervation of these tissues is greatly reduced in both NRTN- and GFRα2-KO mice [248,301,307].

The requirement for NRTN/GFRα2 signaling in parasympathetic innervation is evident outside the cranial ganglia as well. Cholinergic innervation of the cardiac ventricles is reduced in GFRα2-KO mice [302]. Also, the soma size of sacral parasympathetic neurons is reduced and their target innervation to the reproductive organs is deficient [97,303].

In the pancreas, GDNF mediated signaling is required for the parasympathetic neural colonization of the tissue, reminiscent of the enteric colonization of the bowel (as discussed later) [308]. Interestingly, GFRα2-KO mice also show a roughly 85% reduction in the number of intrapancreatic neurons and a loss of innervation of both the endocrine and exocrine pancreatic tissue [96]. Thus, it appears that parasympathetic neurons generally use GDNF/GFRα1 signaling for early development and switch to NRTN/GFRα2 signaling during later stages [5]. However, whether the parasympathetic neuron loss in GFRα2-KO pancreas and cranial ganglia results from reduced progenitor proliferation, poor cell migration, increased cell death (possibly due to loss of target innervation), defunct differentiation, or from the compounding effects of several factors, remains unknown.

Enteric nervous system

The third division of the ANS, the enteric nervous system (ENS), is housed in the wall of the alimentary tract. It is by far the most abundant and complex part of the ANS. In humans, it contains around 500 million neurons – roughly equaling the number of neurons found in the spinal cord [309]. Although the ENS has a remarkable ability to function nearly independently of the CNS through reflex circuits, it is nevertheless abundantly innervated by extrinsic autonomic and sensory nerve fibers and modulated by the brain [310].

The ENS is structurally divided into two major networks of interconnected ganglia and fibers: the myenteric plexus and the submucosal plexus. The myenteric (or Auerbach's) plexus forms a continuous meshwork of neural tissue between the longitudinal and circular muscle layers of the gut wall and extends from the early esophagus all the way trough the GI tract. The myenteric plexus can be divided to three levels of organization [310]. The primary plexus is formed from numerous ganglia and the interganglial fiber bundles connecting them. The secondary level of fibers is constituted from thinner strands of the ganglia and interganglial fibers. It runs parallel to the circular muscle fibers between the primary plexus and the circular muscle layer; many secondary fibers innervate the circular and longitudinal muscle layers and form the deep and longitudinal muscular plexuses. Finally, the tertiary plexus is formed by the thinnest fibers that originate from the ganglia, interganglial fibers, and secondary fibers.

A fully developed submucosal (or Meissner's) plexus is located in the wall of the small and large intestines but not in the esophagus or the stomach. This continuous plexus has generally smaller ganglia and a finer network of interconnecting nerve bundles compared to the myenteric plexus. Larger animals, including humans, have two distinct submucosal plexuses, while smaller mammals such as mice and guinea-pigs have only one [310].

Seventeen types of neurons have been characterized in the ENS [311]. These include excitatory and inhibitory motoneurons, secretomotor neurons, interneurons, intrinsic primary afferent neurons, and intestinofugal (innervating prevertebral ganglia) neurons. The most common transmitters in excitatory motoneurons and interneurons are acetylcholine and tachykinins. Inhibitory motoneurons, on the other hand, are nitrinergic, but often use GABA and several neuropeptides such as VIP, PACAP, NPY, and Enkephalins [311].

Development of the ENS and the role of GDNF/GFRα1/RET

The dual neural crest origin of the ENS was first demonstrated using chick-quail embryonic chimeras [312]. By grafting fragments of the quail neural primordium at various levels of the chick neural axis, it was shown that enteric neurons throughout the alimentary

tract originate from the vagal neural crest (somite levels 1-7), with caudal sacral levels (beyond level 28) contributing to the post umbilical gut. Since then, a similar dual origin of the ENS has also been demonstrated in the mouse [265,313–315].

The vagal NCCs destined to become enteric neurons begin migrating at around E8.5 in the mouse, and reach the foregut about 24 hours later [265,313]. The enteric progenitors migrate in streams along the gut mesenchyme in a process that takes five days in the mouse (E9.5-14.5) and three weeks in the human (weeks 4-7) [294,316]. The sacral level NCCs emigrate between E9-9.5 in the mouse [315], but enter the hindgut only after it has first been colonized by the vagal NCCs [314].

Acting via GFRα1/RET, GNDF controls the proliferation and survival of the ENCCs, also functioning as an activator of cell migration [317–319]. In the gut, GNDF expression advances in front of the migrating NCC front as a spatio-temporal wave, starting from the splanchnic mesenchyme of the stomach and proceeding to the posterior caecum [320]. In a GDNF dependent manner, a major subpopulation of ENCCs exits the gut mesenchyme and shortcuts the caecum to colonize the hindgut via the mesentery [321]. Beyond the caecum, the colonic mesenchyme expresses endothelin-3 (ET-3); its receptor Endothelin receptor B (EDNRB) is found in the enteric neuroblasts [322]. ET-3/EDNRB-signaling is crucial for enteric gangliogenesis in the distal colon [323,324]. In the process, it directly interacts with GDNF/RET signaling by enhancing the proliferative and inhibiting the chemo-attractive effects of GDNF, possibly thus enabling the ENCCs migration to proceed beyond the high concentration peak of GDNF in the caecum [325,326]. The expression of GFRα1 diminishes in the enteric ganglia postnatally and is lost altogether from the gut mesenchyme already before birth [317]. Similarly, GDNF expression is downregulated in the gut, and is practically undetectable in the adult [39,327]. Several other factors also influence the neuronal colonization of the alimentary tract. For example, Netrin/DCC-signaling contributes and is indispensable to the perpendicular migration of enteric NCs to form the submucosal plexus, although this process still requires GNDF [296,326,328]. Moreover, a multitude of transcription factors have also been implicated in the neural colonization of the gut. These include the familiar autonomic transcription factors Phox2b, Mash1, and Hand2, but also many other factors such as SOX10, PAX3, and Foxd3 [329].

RET-, GFRα1-, and GDNF-KO mice all share a phenotype of complete intestinal aganglionosis [42–44,265,280,281,330]. In RET-KO mice, a large subpopulation of enteric NCCs undergoes apoptotic cell death shortly after entering the foregut [265,318,331]. In contrast to full KOs, heterozygous RET- and GFRα1-mice have normal numbers of enteric neurons, whereas GNDF+/- mice are hypoganglionic, likely due to reduced ENCC proliferation [317]. In mice where RET or GFRα1 has been conditionally knocked out, but only after gut colonization has finished, non-apoptotic neuronal cell death follows in the distal gut. This suggests that especially colonic ENCCs require GFRα1/RET signaling for survival [331,332]. The expression level of RET can be reduced to less than half, after which ENS development in the colon becomes severely impaired, due to both affected trans-mesenteric migration and non-apoptotic death of the enteric NCCs in the colon [321,331].

In contrast to other populations of the peripheral nervous system, programmed cell death has been regarded as a rare (and relatively insignificant) phenomenon in the normal development of the enteric nervous system; caspase-3 activity has not been observed in the developing ENS, and deleting Bax or Bid has no visible effects on enteric neuron numbers [317]. However, in the embryonic chicken, many vagal NCCs on the way to the developing alimentary tract die via apparently conventional apoptotic death, and inhibition of this cell death leads to hyperganglionosis in the foregut [333]. Whether this phenomenon is also found in mammals remains to be investigated [334].

GFRα2/NRTN in enteric neurons

NRTN expression begins in the gastrointestinal tract at around E12 in the mouse, and high levels of NRTN can be found especially in the embryonic and adult stomach mucosa [39]. The mucosa and muscular layers of the intestine also express NRTN, with high levels in the circular muscle layer, although the expression decreases later on [52,70]. GFRα2 expression, on the other hand, can be observed in the newborn myenteric and submucosal ganglia and remains high also in adulthood [52,317].

NRTN-KO mice have normal numbers of myenteric neurons, but the cell bodies are atrophic [248,317]. Moderate reductions in neuron numbers have been reported in the myenteric plexus of GFRα2-KO mice and in the submucosal plexus of NRTN-KO mice, suggesting that NRTN/GFRα2 plays only a minor role in the survival of enteric neurons [52,307,317]. However, the density of the myenteric plexus cholinergic nerve fiber network is severely reduced in both NRTN-KO and GFRα2-KO mice (especially in the small intestine), accompanied by impairments in the motility of the gut [52,248,307,317]. These findings suggest that, whereas GDNF/GFRα1-signaling is required for the neuronal colonization of the gut, NRTN/GFRα2-signaling functions mostly in the innervation and soma maintenance of enteric neurons.

Due to the severe loss of parasympathetic innervation of target tissues, GFRα2-KO mice show impaired secretory capability to vagal stimulation in both the exocrine and endocrine pancreas [52,96]. The stomach mucosa shows high levels of NRTN expression and is heavily innervated by cholinergic enteric neurons (as discussed later), suggesting a major involvement of NRTN/GFRα2-signaling. Nevertheless, possible deficits in the innervation of gastric mucosa and the implications on the gastric physiology of GFRα2-KO mice have not been studied previously.

PNS regulation of gastric physiology

Gastric mucosal anatomy

The stomach can be divided into four anatomical regions: the cardia, which is a small area around the esophageal opening; the fundus, expanding laterally to the left of the cardia; the corpus, comprising the main region of the stomach; and the pyloric antrum, the most caudal part of the stomach. Two glandular regions line the stomach lumen and define the functional areas: the oxyntic and pyloric mucosa. The oxyntic mucosa covers the fundus/corpus and is responsible for gastric acid secretion, whereas the pyloric mucosa covers the antrum. The mucosa houses gastric pits into which tubular gastric glands open. These oxyntic glands have three regions: the pit, the most apical part that contains mucus secreting cells; the neck, housing stem cells, mucous neck cells, and acid secreting parietal cells; and the body/base, covering most of the gland. Parietal cells are abundant in the upper body and neck of the gland. They secrete hydrochlorid acid, intrinsic factor, and several other factors. Pepsinogen, a propeptide of pepsin, is secreted by chief cells near the base of the gland. In addition, several endocrine/paracrine cells are also found in the oxyntic glands [335]. From these, ECL-cells are the most abundant and secrete histamine, an important stimulator of acid secretion. The other endocrine cell types in the oxyntic glands are: D-cells, which secrete somatostatin; EC-cells, which secrete atrial natriuretic peptide; and A-like cells, which secrete ghrelin. Unlike in the oxyntic mucosa, pyloric glands house only rare parietal cells. Instead, they contain abundant mucus cells and endocrine cells, of which the gastrin producing G-cells are the most important.

In the mammalian stomach wall, most myenteric neurons are cholinergic [336–338]. In addition, most of the myenteric neurons express a combination of neuropeptides including vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP), and pituitary adenylate cyclase-activating peptide (PACAP) [339]. In both rodents and humans, gastric myenteric ganglia also contain non-cholinergic neurons, including nitrergic inhibitory interneurons [336–338,340]. Extrinsic CGRP+ afferent fibers also innervate the gastric mucosa and provide sensory feedback on factors such as gastric wall distension and luminal pH.

Regulation of gastric mucosal secretion and homeostasis

The process of gastric acid secretion is regulated by a complex system of hormonal, paracrine and neural factors [341] (Figure 8). Gastrin, released by the antral G-cells in response to feeding, circulates through the bloodstream and increases acid secretion mainly by increasing histamine release from ECL-cells via the CCK2-receptor. It also functions as an important trophic factor for the gastric mucosa. Histamine is a paracrine factor in the stomach mucosa and a powerful stimulator of acid secretion from the parietal cells through H2-receptors. Another paracrine factor is somatostatin, an inhibitory secretagogue produced by D-cells. Somatostatin binds SST2-receptors and inhibits acid secretion directly from parietal cells as well as via lowering gastrin and histamine secretion. The low basal level of acid secretion is maintained through a tonic secretion of somatostatin.

The neural effector component of gastric acid secretion is communicated via cholinergic neurons of the myenteric plexus. These neurons are the primary regulators of acid secretion [341]. When a meal is anticipated and initiated, the myenteric neurons receive central activating signals via preganglionic parasympathetic innervation through the vagus nerve. Acetylcholine released from the myenteric neurons induces acid secretion both by activating muscarinic M3-receptors on parietal cells and G-cells and by inhibiting somatostatin release from D-cells. The overall effect of cholinergic activation is the release of somatostatin mediated inhibition and the enhancement of stimulatory influences on acid secretion [341]. The myenteric cholinergic neurons also express neuropeptides, of which GRP and PACAP are able to stimulate acid secretion. VIP, on the other hand, can stimulate somatostatin release, although the physiological importance of this pathway is not clear [342,343]. An intact vagus nerve and muscarinic M3 receptors are indispensable for basal and stimulated acid secretion [342,344–346]. After vagotomy, the loss of secretory capacity is almost immediate in the rat [347]. The vagus also appears to provide trophic support for the gastric mucosa independently of the effects of gastrin [348]. Importantly, the mechanisms through which the vagus regulates mucosal proliferation and acid secretory responses to gastrin and histamine remain poorly understood [349].

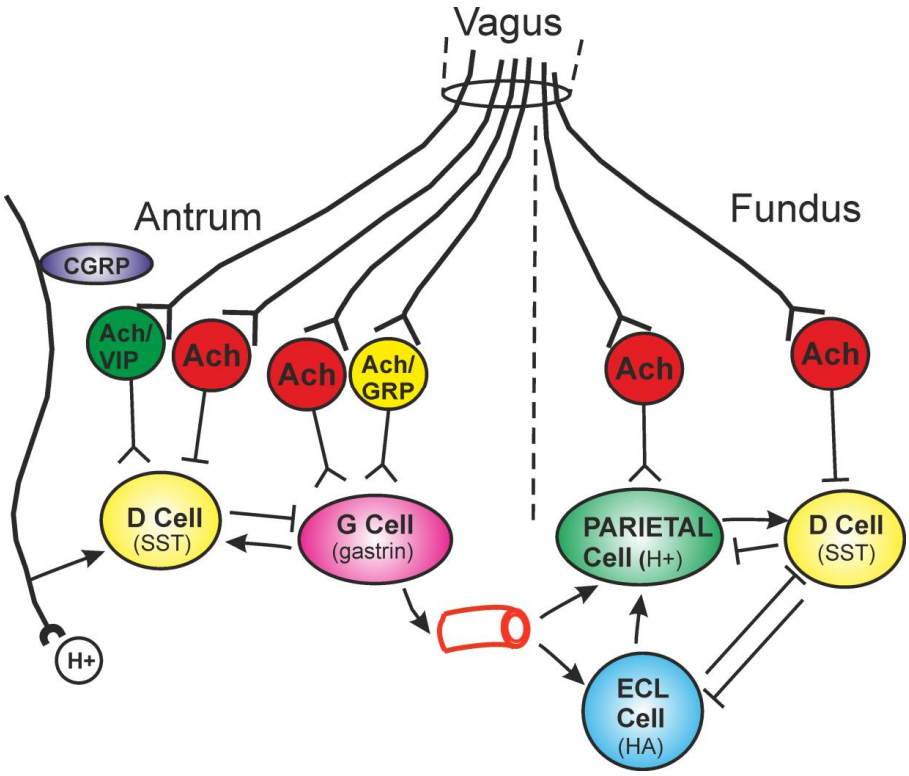

Figure 8. A current view of how gastric acid secretion is regulated by neural, paracrine, and hormonal factors. Efferent fibers from the vagus nerve synapse with cholinergic (ACh) and cholinergic/peptidergic (ACh/GRP; ACh/VIP) neurons in the myenteric plexus. In the fundic and antral (oxyntic) mucosa, ACh neurons stimulate acid secretion from parietal cells, while simulatenously inhibiting somatostatin (SST) secretion from D-cells, thus eliminating the SST brake on parietal cells and histamine releasing ECL-cells. ACh neurons in the antrum stimulate gastrin secretion and inhibit SST secretion, removing the SST brake effect also on G-cells. VIP neurons stimulate D-cells and can inhibit gastrin secretion. Histamine from ECL cells stimulates acid secretion from parietal cells and may also augment acid secretion by inhibiting SST secretion. On the antral side, paracrine pathways link D- and G-cells. Acid release into the stomach lumen increases SST secretion; this effect is mediated via CGRP+ extrinsic sensory neurons in the antrum. Modified from [335].

AIMS

The specific aims of the studies presented in this thesis were to investigate:

(i) The occurence of developmental programmed cell death in parasympathetic versus enteric and sympathetic neurons;

(ii) The role of GFRα2-signaling in parasympathetic neuron development and the mechanisms behind the postnatal loss of parasympathetic neurons in GFRα2-KO mice;

(iii) The role of GFRα2-signaling in cutaneous low-threshold mechanosensory neurons;

(iv) The role of GFRα2-signaling in the innervation of gastric mucosa; and

(v) The role of the intrinsic cholinergic innervation of the gastric mucosa in the regulation of gastric secretion and mucosal homeostasis.

MATERIALS AND METHODS

The experimental methods used in this work are listed in Table II with a reference to the appropriate original publication where detailed descriptions can be found. Tables III-V list some of the most salient materials used in the experiments.

Table II. Methods used and described in the original articles

Method	Used in
Animals and tissue processing	I,II,III
Immunohistochemistry, microscopy, imaging	I,II,III
Cell profile nuclear area estimation, marker colocalization	I,III
Cell counting	I,II,III
In situ hybridization	II
Gastric juice pH analysis	II
Gastric acid secretion analysis	II
Plasma gastrin analysis	II
Real-Time quantitative PCR	II
Statistics	I,II,III
Quantifying cell size distributions	III
Quantifying epidermal and hair follicle innervation	III

Table III. Probes for *in situ* hybridization

Probe	Host	Vector	Size	Nucleotides	Acc. No	Used in
*Nrtn**	Ms	pcDNA	587	349-936	U78109	II
*Gdnf***	Ms	PGEM-T	616	52-668	L15305	II

*Kotzbauer et al., 1996; **Suvanto et al., 1996
Ms (mouse)

Table IV. Mouse strains

Strain	Reference	Used in
GFRα2-KO	Rossi et al., 1999	I,II,III
MrgprdΔ^{EGFPf}	Zylka et al., 2005	III

Table V. Primary antibodies for immunohistochemistry

Against	Host	Supplier	Reference	Used in
BrdU	Ms	Amersham		I
Caspase-3	Rb	Cell Signaling Tech.		I
Galanin	Rb	Millipore		II
GFP	Rb	Molecular Probes		III
GFRα2	G	R&D Systems		I,II,III
Ghrelin	G	Santa Cruz Biotech.		II
GRP	Rb	Dr Panula	Panula et al., 1982	II
H/K-ATPase	Rb	Thermo Fisher		II
Histamine	Rb	Dr Panula	Panula et al., 1988	II
Insulin	Gp	Abcam		I
NFH	Rb	Millipore		III
PGP9.5	Rb	Millipore		I,II,III
PGP9.5	Sh	Serotec		I
Phox2b	Rb	Dr. Pattyn	Pattyn et al., 1999	I
Somatostatin	G	Santa-Cruz		II
RET	G	Neuromics		III
TH	Rb	Millipore		II,III
TH	Sh	Millipore		III
TrkB	G	R&D Systems		III
VAchT	Rb	Phoenix Pharm.		II
VIP	Rb	Progen		II
100β	Rb	Swant		II

Ms (mouse), Rb (rabbit), G (goat), Sh (sheep), Gp (Guinea pig)

RESULTS AND DISCUSSION

GFR**α**2 in trophic support

*GFR**α**2 promotes parasympathetic but not enteric neuron survival (I)*

To elucidate the mechanisms behind the lack of parasympathetic neurons in the GFRα2-KO pancreas, we first investigated the time-line of the development of this phenotype. No significant differences in the numbers of intrapancreatic neurons were found at E16.5 or earlier. However, at E17.5 and later, the number of neurons in GFRα2-KO pancreata declined rapidly, and a reduction of over 70% compared to WT was observed at birth. Since the loss of intrapancreatic neurons is complete soon after birth [52], our results indicate that the neuron loss happens mostly during the later stages of embryonic development. The late emergence of the neuronal loss also suggests that neuronal precursor migration to the pancreas is normal in the GFRα2-KO mice, as this process occurs for the most part between E13 and E15 in the mouse [296,308]. As has previously been predicted [52], and as recent work has also confirmed [308], the migration of neural precursors to the pancreas depends on GDNF and GFRα1. This is consistent with the fact that these neurons represent a subpopulation of enteric neural crest cells known to rely on GDNF/GFRα1/RET signaling during migration.

We used antibodies against the pan-neuronal marker PGP9.5 to demonstrate intrapancreatic neurons. It is noteworthy that, although PGP9.5 expression has been reported in the endocrine cells of the embryonic rodent pancreas [350], in our study this signal was found to be weak and clearly distinguishable from the intensely stained neurons. In addition to mature neurons, PGP9.5 is also expressed in neuron precursors in the PNS [351,352]; therefore, the possibility remains that some of the observed PGP9.5+ cells were in fact premature neurons. Some myenteric neurons do not show immunoreactivity against PGP9.5 antibodies [353], which is why, using this marker could have led to a slight underestimation of total enteric neuron numbers. However, as the main objective in our study was to quantify relative differences between the genotypes, a small bias in absolute numbers should play only a minor role.

To investigate the possibility that reduced proliferation of neural precursors underlies the loss of pancreatic neurons in GFRα2-KO–mice, we assessed the relative proportion of BrdU+/Phox2b+ positive cells out of all Phox2b+ cells in pancreatic ganglia. The number of proliferating Phox2b+ cells decreased by 50-60% between E15.5 and E17.5, but to a similar degree in both WT and GFRα2-KO mice, implying that intrapancreatic neural precursors proliferate normally in the mutant mice. Because Phox2b is expressed by NCCs, which develop into both autonomic neurons and the surrounding glial cells [308], our quantifications are likely to include a subset of glial precursors. However, the intrapancreatic S100β+ glial cells appear to be normal in adult GFRα2-KO mice. Therefore, it is doubtful that they have skewed the analysis of neuron progenitor numbers (Rossi et al, unpublished results).

Next, we asked whether increased apoptosis was responsible for the reduced parasympathetic neuron numbers. To our knowledge, and according to a recent review [283], very little is known about normal developmental cell death in the mammalian parasympathetic nervous system. We found Caspase-3+ apoptotic neurons in the developing intrapancreatic ganglia; in WT mice the occurrence of caspase-3+ neurons was 1% of all neurons at E15.5 and slightly less at the time of birth. In GFRα2-KO mice, however, the number of caspase-

3+ neurons increased rapidly after E15.5 to a peak level of 4% at E17.5. By the time of birth, the occurrence of apoptosis had decreased to levels comparable to WT mice. Consequently, apoptosis appears to be a major factor contributing to the intrapancreatic neuron loss in GFRα2-KO mice. Moreover, this observation also supports the hypothesis that increased apoptosis is responsible for the reduced numbers of submandibular ganglion (SMG) neurons in GFRα2-KO mice [301]. Apoptosis was observed in the WT SMG neurons and, as predicted, we found the numbers of caspase-3+ neurons increased in the late embryonic and newborn GFRα2-KO SMG, albeit to a smaller extent than in the pancreatic ganglia. The low numbers of caspase-3+ neurons in the SMG during development suggest that PCD is not a major determinant of SMG neuron numbers before birth, although it can contribute more postnatally.

As an alternative method to activated caspase-3, we used TUNEL to demonstrate apoptosis in peripheral ganglia [354]. For the final analysis, Caspase-3 was chosen over TUNEL, as TUNEL-positive cells in the peripheral ganglia were often negative for PGP9.5 (I; Supp. Fig.). TUNEL+ cells were encountered in the sympathetic ganglia (39/1058 cells), but were absent in the vicinity of enteric ganglia (0/426).This is in line with the consensus that developmental cell death is a rare event in the normally developing ENS. However, since caspase-3 may not detect all types of programmed cell death in neurons [355], it is possible that the full scale of cell death was not observed. Also, as caspase-3 is involved in most cases of apoptosis, the specific pathway leading to cell death cannot be deduced from only this one marker. Of note is the finding that, in the absence of GDNF, sympathetic neurons in culture appear to die via a pathway involving activated caspases 2 and 7 [177]. Further investigations are needed to study the possibility that similar pathways are also activated in GFRα2-KO parasympathetic ganglia.

In addition to parasympathetic ganglia, we studied the occurrence of caspase-3+ apoptosis in the enteric ganglia of the duodenum and colon, as well as in sympathetic paravertebral ganglia and DRGs at the pancreatic level. Ontogenetic cell death appears in mouse sympathetic ganglia at E15.5 and continues until early postnatal age [274]. In agreement with this, we observed caspase-3+ neurons in the sympathetic ganglia of both WT and GFRα2-KO mice, the occurrence peaking at little over 3% during E17.5. No significant differences were witnessed between the genotypes. In the DRG, where apoptosis is a relatively early event [165,167], caspase-3+ neurons were most numerous in both genotypes at E15.5 (the earliest time point studied), and were almost nonexistent at P0. These findings comply with earlier observations that GFRα2 is not required for the survival of developing sympathetic or sensory neurons [103,307].

Apoptosis has been considered almost absent from developing enteric neurons [317] and, in agreement with this, we found virtually no enteric neurons expressing active caspase-3 in either WT or GFRα2-deficient mice. The lack of caspase-3+ apoptosis, however, does not rule out the possibility that PCD does not occur in enteric neurons. For example, late stage colonic neuron precursors have been shown to die through a non-apoptotic (caspase-3 and TUNEL negative) mechanism in the absence of GDNF/GFRα1 -signaling [332]. It is possible that a similar mechanism could function in a subpopulation of GFRα2+ enteric neurons and underlie the small loss of myenteric neurons reported in GFRα2-KO mice [307]. Moreover, one cannot rule out the possibility that an increased yet still rare occurrence of caspase-3+ apoptosis in GFRα2-KO mice was missed, due to too low sampling frequency orapoptotic pathways involving executor caspases other than caspase-3. Nevertheless, our results here are in support of the view that apoptosis in the ENS is rare in both WT and GFRα2-KO mice. Although it is known that enteric neurons are capable of dying via apoptosis, for example after a damaging insult [356], it is still unclear why pancreatic and enteric neurons differ in the occurrence of natural ontogenetic apoptosis.

NRTN expression is upregulated and GDNF downregulated in the pancreas and the submandibular gland during late embryonic development, coinciding with the emerging expression of GFRα2 in the cranial and intrapancreatic parasympathetic neurons (Figure 9) [39,248,308]. At the same time, elevated apoptosis (this study) and neuron loss [301] is observed in the corresponding ganglia of GFRα2-KO mice. Thus, intrapancreatic and submandibular neurons appear to first (during migration) depend on GFRα1 and GDNF [300,301,308] and later on GFRα2 for survival. The survival promoting effect of GNDF on enteric neurons requires the PI3K/Akt-signaling, which makes this pathway a likely candidate for the similar properties of NRTN/GFRα2 [357]. A reduction in the number of SMG neurons was also observed in the NRTN-KO mice at birth, supporting the view that NRTN is the survival promoting ligand for these neurons [248,300]. While it has not been reported whether NRTN-KO mice also lack intrapancreatic neurons, it is most probable that they do. Still, the unlikely possibility remains that GDNF promotes neuron survival via GFRα2 in the pancreas. Finally, it is worth mentioning that the death inducing dependence receptor-activity of RET has been suggested to be insignificant in developing enteric neurons [331]. Whether RET induced PCD is involved in the developmental apoptosis of parasympathetic neurons, remains unknown.

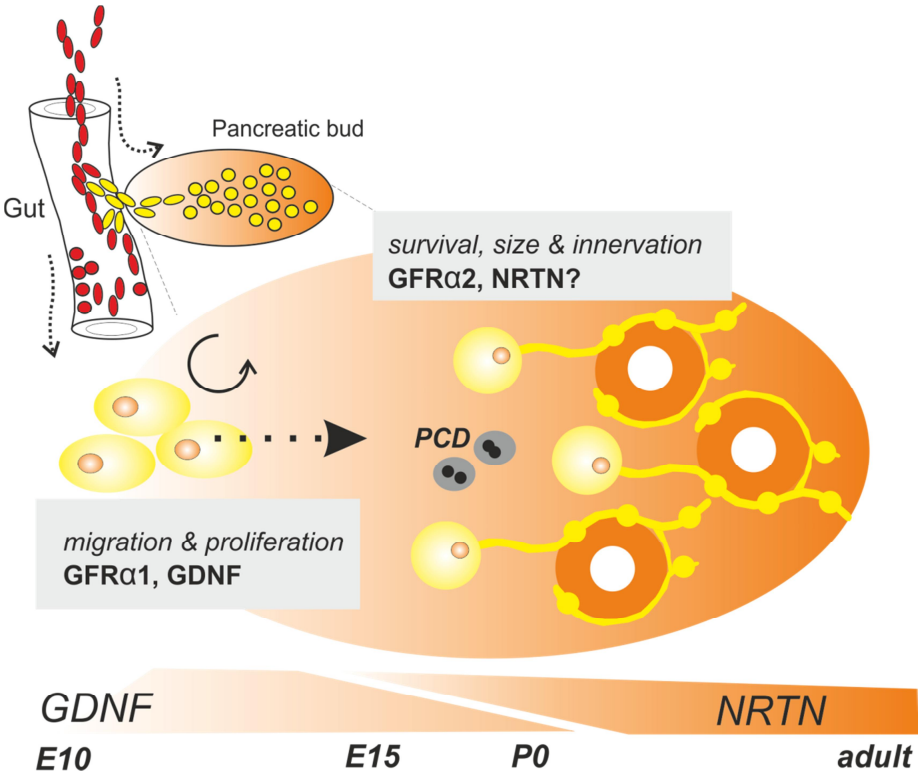

Figure 9. Parasympathetic neurons switch from GDNF/GFRα1- to NRTN/GFRα2- signaling for survival, maintenance, and target innervation. A subpopulation of enteric neural crest cells/progenitors migrates into the pancreas to become parasympathetic neurons. During this secondary neural migration the parasympathetic progenitors express GFRα1, while GDNF is expressed in the pancreatic tissue. The secondary neural migration to the pancreas also requires DCC/Netrin (not shown). After the migration, the neural progenitors switch to expressing GFRα2; this coincides with the occurrence of PCD among the parasympathetic progenitors. At the same time, GDNF expression is downregulated and NRTN upregulated in the pancreatic tissue. Modified from [5].

GFRα2 is needed for size in cutaneous C- and RA Aβ-LTMRs, and in Mrgprd+ nociceptors (II)

A previous study disclosed that GFRα2 is required for the soma size maintenance of cutaneous IB4+ nonpeptidergic nociceptors [240]. We aimed to expand on this study, and asked whether GFRα2 also regulates cell size, and possibly survival, in two other DRG neuron populations known to express RET and GFRα2: the small, unmyelinated C-LTMRs and the large, heavily myelinated RA Aβ-LTMRs [181,225,226].

We first set out to confirm that GFRα2 actually regulates cell size in the nonpeptidergic nociceptors. Here we used *Mrgprd* as a marker, as this gene has been shown to encode a G-protein coupled receptor that is specifically expressed in polymodal nonpeptidergic nociceptors [235]. Using immunohistochemistry, we found that most, if not all EGFP+ neurons expressed GFRα2 in *Mrgprd*-EGFP mouse DRGs. In GFRα2-KO;*Mrgprd*-EGFP mice, however, the EGFP+ neurons were substantially reduced in size, validating the previous findings that GFRα2 regulates nonpeptidergic nociceptor neuron size. The number of EGFP+ neurons is not reduced in GFRα2-KO DRGs, since these mice have previously been shown to retain normal numbers of IB4+/P2X3+ DRG neurons, which correspond almost fully with Mrgprd+ neurons [235,240].

Next, we proceeded to investigate the role of GFRα2-signaling in the C-LTMRs, using tyrosine hydroxylase (TH) as a specific marker for these neurons [181]. Co-staining with GFRα2 showed that nearly all of the TH+ neurons in thoracic level DRGs expressed GFRα2, representing about 1/3 of all GFRα2+ neurons. When observed in GFRα2-KO mouse DRGs, TH+ immunoreactivity appeared similar in comparison to WT, indicating that GFRα2 is not required for the expression of TH in the neurons. Also, no differences were seen in the numbers of TH+ neurons in WT and KO DRGs, which implyes that GFRα2 signaling is not required for the survival of C-LTMRs. Finally, to see if the C-LTMRs require GFRα2 for soma size, we performed a cell size distribution analysis and discovered a significant level of atrophy in the size of GFRα2-KO TH+ neurons (Figure 10).

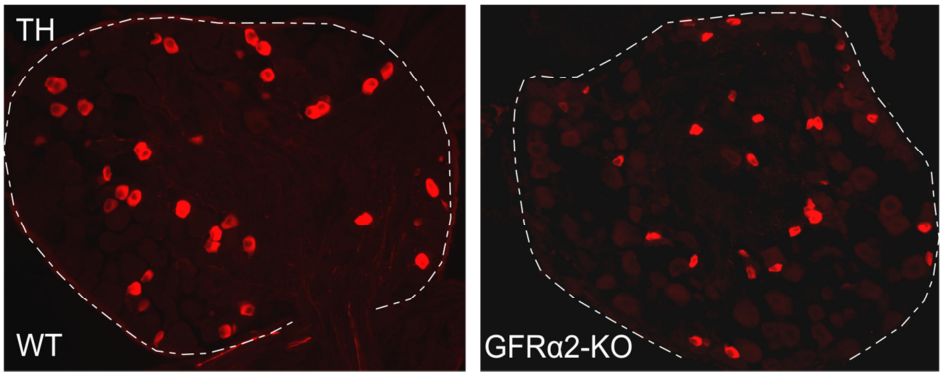

Figure 10. C-LTMRs in mouse DRGs. Representative images of WT and GFRα2-KO thoracic DRGs stained with a TH-antibody. Note the atrophied neuron somas in the KO DRG. the dashed line delineates the ganglion.

Our findings on Mrgprd+ and TH+ DRG neurons reinforce the view that GFRα2 regulates nonpeptidergic neuron size. In addition to GFRα2-KO mice, this neuronal atrophy has been observed in several RET-cKO mouse models, implying that RET-signaling underlies this phenomenon [187,246,247]. The *in vivo* ligand responsible for the trophic sup-

port on GFRα2+ DRG neurons appears to be NRTN, as these neurons atrophy and loose GFRα2 expression in NRTN-KO mice [248]. Accordingly, in mice overexpressing NRTN in skin keratinocytes, the size and number of neurons expressing GFRα2 is substantially increased [250]. Concerning neuron numbers, the general consensus is that GFRα2- and RET-signaling are not required for nonpeptidergic neuron survival [187,227,240,246]. In agreement with this, we found no sign of Mrgprd+ or TH+ DRG neuron loss in GFRα2-KO mice.

In contrast to nonpeptidergic neurons, which initiate the expression of RET and GFRα2 during late prenatal and early postnatal development, the large RET+ RA Aβ-LTMRs begin expressing these receptors already in earlier fetal stages [187,225,226]. In agreement with this, we found GFRα2-immunoreactivity in a minor population of large Mrgprd-negative DRG neurons in newborn mice. Interestingly, no immunoreactivity against GFRα2 was evident in adult myelinated DRG neurons. GFRα2 expression has been observed in the large RET+ neurons at P14 in mice [226], but other studies have indicated that in adult rodents these neurons express GFRα1 [358,359] and possibly GFRα3 [99]. Further corroborating our findings, a recent study using single cell RNA sequencing found no evidence of GFRα2 gene expression in adult early-RET DRG neurons [200]. Drawn together, these results suggest that the large RET+ neurons downregulate GFRα2 expression and begin signaling through different GFRα receptors in adulthood.

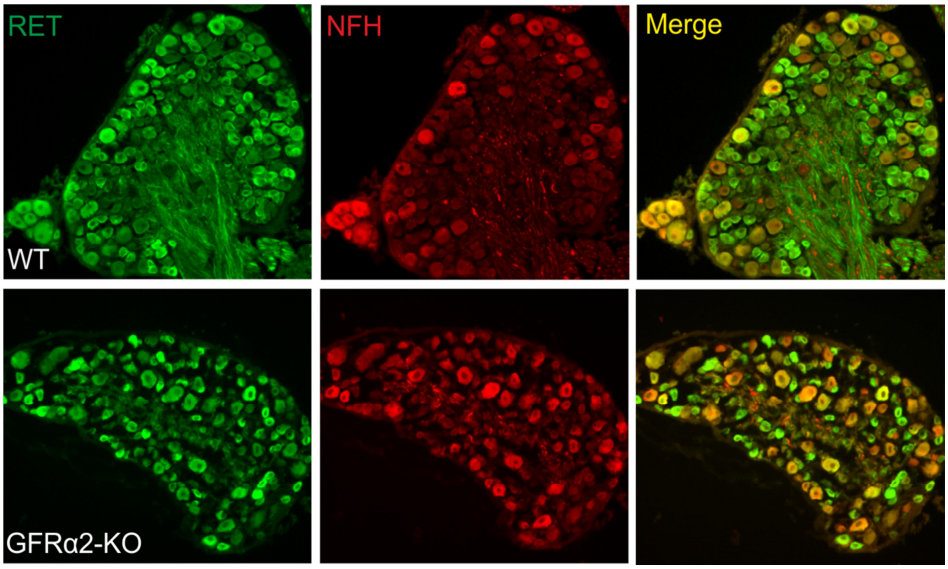

Figure 11. Large RET+ neurons in mouse DRGs. Representative images of WT and GFRα2-KO thoracic DRGs stained with antibodies against RET and NFH. The large NFH+/RET+ neurons survive in the GFRα2-KO mice, but are smaller than in WT animals.

Because the large RET+ neurons are heavily myelinated, we were able to use the expression of NFH together with RET to demonstrate these neurons in adult WT and GFRα2-KO animals. In adult DRGs, most RET+ neurons were small and NFH-negative; however, a minority of RET+ neurons were clearly NFH+ and large. Double positive neurons were also present in the KO animals, and no significant differences in their numbers were observed between the genotypes. Similarly to the nonpeptidergic DRG neurons, the average soma sizes of the RET+/NFH+ neurons were significantly smaller in GFRα2-KO

mice (Figure 11). Thus, these results indicate that, while GFRα2-signaling is not essential for the survival of early-RETneurons, it is required for them to obtain their proper size during development. Consistent with these findings, myelinated axons in the saphenous nerves of GFRα2-KO mice have previously been shown to be thinner, yet equally numerous when compared to WT animals [360]. Furthermore, specific hypertrophy of the largest myelinated fibers in the saphenous nerves have been reported in mice overexpressing NRTN in the skin [250]. Finally, large RET+ neurons have been shown to be normally present or only marginally reduced in numbers in both NRTN-KO mice and in another GFRα2-KO mouse model [132,226].

The specific signaling pathways that control cell size via GFRα2/RET have not been reported. A probable mechanism is the activation of the protein kinase Akt via PI3K, which is known to regulate cell size in mammals [361]. In support of this, the specific overexpression of PI3K and Akt in cultured E13 DRG neurons leads to the hypertrophy of neuron somas and the thickening of axons [362]. Also, PI3K activation appears to mediate the trophic effects of NGF on the soma size of sympathetic neurons [363].

GFRα2 in target innervation

GFRα2 regulates hairy skin innervation by Mrgprd+ neurons but not by C- and RA Aβ-LTMRs (II)

Accompanying the reduced soma size in IB4+ DRG neurons, GFRα2-KO mice have lost over 70% of the nonpeptidergic free nerve endings in the glabrous footpad epidermis [240]. A comparable phenotype has also been reported in RET-cKO mice [187,246]. We wanted to see if GFRα2-signaling regulates nonpeptidergic innervation of the hairy skin as well. Compatible with the phenotype seen in the glabrous skin, we observed a loss of 50-70% of EGFP+ free nerve endings in the GFRα2-KO;*Mrgprd*-EGFP back and dorsal paw epidermis. In addition, we studied the back skin of additional pairs of WT and GFRα2-KO mice using PGP9.5 immunohistochemistry, and observed a qualitatively similar difference in nerve fiber density. Given that GFRα2 is needed for their soma size, we asked whether GFRα2-signaling could be important also for the target innervation of C- and RA Aβ-LTMRs, which both project to hair follicles in the back skin.

In the WT back skin we found TH+ LLE-complexes around ~88% of small caliber hair follicles. Surprisingly, both the percentage of TH+ innervated small caliber hair follicles and the morphology of TH+ LLE were unchanged in GFRα2-KO littermates. To rule out the possibility that our TH-antibody binds the Aδ-LTMRs found on small caliber hair follicles, we performed a double staining against TH and TrkB (a marker for Aδ-LTMRs). Antibodies against the two markers bound two distinct populations of LLEs in the back skin, demonstrating the specificity of our TH-staining. Consequently, our results suggest that GFRα2 is not required for target innervation by C-LTMRs.

Whether the formation of C-LTMR LLEs is compromised in RET- or NRTN-KO mice has not been reported. GFRα2 is the only RET co-receptor expressed in adult mouse C-LTMRs [181], but it remains possible that a transient expression of another GFRα receptor could precede GFRα2 during development. Since GDNF is also expressed in the developing mouse skin and hair follicles [39,364], the possibility that C-LTMRs could use GDNF/GFRα1 mediated signaling (*in cis* or *trans*) for target innervation cannot be excluded.

In the back skin, the RA Aβ-LTMRs form LLEs around the two largest types of hair follicles (awl/auchene and guard). We focused on the largest type (guard), as these are easily distinguished from the two smaller types of follicles. NFH+ LLEs were found associated

with most (~88%) guard follicles in the WT, along with circumferential endings. Similarly to the C-LTMRs, no significant reduction in the percentage of innervated guard follicles or abnormal morphology of the endings was found in the GFRα2-KO mice. This indicates that GFRα2 is dispensable for target innervation in cutaneous RA Aβ-LTMRs.

NFH+ LLEs have been reported to be completely missing in a RET-cKO mouse at P15, suggesting that RET-signaling is crucial for the formation of RA Aβ-LTMR endings in the hairy skin [225]. In addition, another study has shown that RET- and NRTN/GFRα2-signaling is absolutely needed for the formation of Pacinian corpuscles, the end organs of another subset of RA Aβ-LTMRs [226]. Also, cutaneous NFH+ afferents have been shown to be impaired in prenatal RET-KO mice [227]. Together, these results indicate that, although they are fundamentally dependent on RET-signaling, RA Aβ-LTMRs differ in their requirement for GFRα2 for target innervation. The finding that GFRα1, but not GFRα2, is expressed in the large RET+ neurons in adult mice suggests the possibility that target innervation in the cutaneous RA Aβ-LTMRs could depend on GFRα1 mediated signaling. A recent study showed that although GFRα2-null mice show an early deficiency in the central innervation of RA Aβ-LTMRs, the phenotype is normalized during development through *in trans* signaling by GDNF/GFRα1; however, a knock-out of both GFRα2 and GFRα1 lead to a definitive loss of central innervation [132]. Therefore it is possible that a similar mechanism is able to fix the loss of cutaneous innervation of RA Aβ-LTMRs in GFRα2-deficient mice.

In addition to neurotrophic factors, a number of other attractive and repulsive cues influence the peripheral projection of the cutaneous nerve fibers [365,366]. For example, versican, a repulsive proteoglycan found in the epidermis, regulates the density of epidermal free nerve endings of nonpeptidergic IB4+ neurons [367]. Also semaphorin 3, another repulsive proteoglycan found in the epidermis, can repulse and inhibit adult sensory afferents *in vivo* [368]. NRTN is able to prevent axon growth cone collapse caused by semaphorin 3A *in vitro* [369], suggesting a possible mechanism for the loss of epidermal Mrgprd+/IB4+ nonpeptidergic free nerve endings in GFRα2-KO mice. Since the LLE palisades of C- and RA Aβ-LTMRs terminate around the hair follicles and, thus, below the epidermis, the requirement for contra-repulsive signaling via NRTN may not be critical for these neurons. Finally, if NRTN acts as an important guidance molecule for the Mrgprd+/IB4+ neurons, then it seems logical that other cues could be involved in the targeting of the RET-expressing LTMRs.

Lack of gastric mucosal cholinergic innervation and glial cells in GFRα2-KO mice (III)

To investigate the role of GFRα2 in the innervation of gastric mucosa, we first studied the expression or the GFRα2 ligand NRTN in the stomach through *in situ* hybridization. Similarly to a previous study [39], we found intense expression of NRTN in the stomach mucosa. Specifically, NRTN transcripts were found in the basal layers of the oxyntic and pyloric mucosa in both juvenile and adult mice, and no signal was seen in the smooth muscle layers of the stomach wall. Also, no above background expression of GDNF was witnessed in the gastric mucosa.

Most of the efferent innervation in the gastric mucosa consists of intrinsic cholinergic nerve fibers [339]. Accordingly, we observed dense trees of VAChT-positive nerve fibers in WT oxyntic and pyloric mucosa. Nearly all of the cholinergic fibers co-expressed GRP and many were also immunoreactive for VIP. VAChT+ fibers in the mucosa expressed GFRα2, as did the S100β-positive glial cells, agreeing with findings in the myenteric cholinergic neurons and related glial cells in the small intestine [52]. Only rare TH+ sympathetic fibers were seen in the stomach mucosa, and these did not express GFRα2, consistent with previous findings in the pancreas, for example [96]. A profound defect was

found in the VAChT/GRP/VIP-positive innervation of the gastric mucosa of GFRα2-KO mice; the density of VAChT/GRP-expressing fibers in the oxyntic mucosa was reduced by >90% in the mutant animals, while VIPergic innervation was reduced by ~80%. A similar pattern, moreover, was witnessed in the pyloric mucosa. Also, the number of S100b+ glial cells and their processes in the gastric mucosa had reduced by roughly 80% in the GFRα2-KO mice. In contrast to the deficits found in the gastric mucosa, VAChT- and VIP-positive innervation and glial cell numbers were normal in the stomach muscle layers of GFRα2-KO mice.

Our results demonstrate that NRTN/GFRα2-signaling is required for the cholinergic innervation of gastric mucosa. The deficit in innervation was observed already in two-week old animals, coinciding with a strong expression of NRTN in the gastric mucosa. As the gastric mucosa functionally and morphologically matures during the first weeks after birth [370], our findings suggest that NRTN/GFRα2 is required for the development of mucosal innervation. NRTN expression remained at high levels in adults, suggesting that it is also required for the maintenance of the cholinergic innervation in the gastric mucosa. In contrast to the mucosa, normal nerve fiber (cholinergic and noncholinergic) and glial density was observed in the gastric muscle layers of the GFRα2-deficient mice, indicating that GFRα2 is not required for the innervation of gastric muscle and myenteric plexus. In support of this, no expression of NRTN was observed in the stomach muscle layers. An open question is, whether the innervation defect in GFRα2-KO mice is exacerbated by a possible reduction in the number of neurons in the stomach myenteric plexus. GFRα2-KO mice show a small but significant reduction in the number of myenteric neurons in the small intestine. Furthermore, this area also shows the highest innervation defect in the bowel [307], suggesting the possibility of a rostrocaudal gradient in GFRα2/NRTN dependency in the GI tract. NRTN expression in the GI tract is at the highest in the stomach mucosa and it remains possible that a significant number of stomach myenteric neurons are lost in the GFRα2-KO mice. Therefore, future studies assessing the number of gastric myenteric neurons and the occurrence of apoptosis in GFRα2-KO stomach wall are warranted.

The number of S100β+ glial cells and their extensions was also heavily reduced in the mutant gastric mucosa. Interestingly, the S100β+ glial cells are still present in the sublingual salivary gland, the lacrimal gland (III; Suppl Fig 6) and the endocrine pancreas of GFRα2-KO mice (Rossi et al., unpublished), although cholinergic innervation to these targets is mostly gone [52,96]. The reasons for the differences in GFRα2-dependency among the glial cells are not known. One possibility is that the abundant remaining sympathetic and sensory innervation in the unaffected tissues may provide enough trophic signaling to support the glial cells.

Consequences of the gastric innervation deficiency

Cholinergic innervation is not required for maintenance of the gastric mucosa in mice (III)

In the rat, unilateral truncal vagotomy leads to atrophy of the oxyntic mucosa on the denervated side without affecting gastrin levels [348]. This suggests a direct trophic effect on the gastric mucosa mediated by the efferent vagus and, specifically, by the intramural neurons [371]. It has been suggested that neuropeptides, especially PACAP, are responsible for the proliferative effects of vagal activity on the gastric mucosa [371–373]. PACAP is co-expressed in the VAChT/GRP-positive nerve fiber in the gastric mucosa and, therefore, PACAP+ fibers should be missing from the GFRα2-KO gastric mucosa [374]. We found no evidence of mucosal atrophy in the GFRα2-KO mice; the mucosal thickness was com-

parable to WT littermates in the oxyntic and antral areas. Similarly, the numbers of parietal cells, ECL-cells, D-cells, and A-like cells were indistinguishable between the genotypes. Consequently, efferent cholinergic and GRP+/VIP+/PACAP+ innervation appears to be dispensable for trophic support of the stomach mucosa, at least in mice. In agreement with our findings, vagal control of proliferation has been suggested to apply only in certain species, and to play only a minor role in humans [375,376]. In contrast, a recent study reported that cholinergic innervation via muscarinic M3 receptors may promote tumorigenesis in the gastric mucosa of mice, and that vagotomy may reduce the risk of gastric cancer in human patients [377].

Cholinergic innervation of the gastric mucosa is dispensable for gastrin secretion (III)

The release of gastrin is thought to be induced mainly by dietary amino acids, amines, and calcium of ingested food and by the mechanical distension related to feeding [335,378]. Earlier studies done mostly on isolated rat stomachs have suggested that acetylcholine and GRP released from cholinergic intramural neurons are important regulators of feeding induced gastrin release [379]. Although carbachol and GRP are potent pharmacological stimulants of gastrin release in intact animals [380], the physiological role of muscarinic and GRP stimulation on gastrin release has been questioned [381,382]. To clarify the importance of mucosal cholinergic/GRPergic innervation on the physiological regulation of gastrin secretion, we measured fed and fasted plasma gastrin levels from GFRα2-KO mice and WT littermates. In both genotypes, the gastrin levels were elevated in the fed state when compared to fasted values; no differences were observed between WT and mutant mice in either feeding state.

A recent microdialysis study showed that food-induced gastrin release is ablated by vagotomy or by local infusion of the neurotoxin TTX, but increased by unilateral vagotomy [381]. The somewhat paradoxical effects of the partial vagotomy could possibly be explained by a loss of the inhibitory effects of VIPergic nerves on gastrin secretion [379]. In any case, these results imply that the effects of vagal activity on gastrin release are complex. It must be noted that TTX and vagotomy also ablate the CGRP+ afferent neural signals from the gastric mucosa, which are known to regulate gastrin release in response to luminal pH levels [383]. In contrast, the GFRα2-KO mice have an apparently normal afferent innervation of the gastric mucosa. Since the circulating gastrin levels in the GFRα2-KO mice were normal regardless of feeding state, it can be concluded that the efferent cholinergic nerve fibers in the gastric mucosa and their neurotransmitters (including GRP) may not play a physiologically important role in the regulation of normal gastrin secretion.

Normal basal acid secretion and maximal secretion capability in GFRα2-KO mice (III)

Vagotomy is known to lead to a rapid and lasting decline of basal acid secretion in both experimental animals and humans [342,345]. The effects of vagal efferent activity are communicated via acetylcholine from the intrinsic mucosal nerves. It stimulates M3 receptors on parietal cells to release acid while also inhibiting somatostatin release from D-cells, likely through M2 or M4 receptors [341]. Accordingly, M3R-KO mice show a high gastric juice pH and a reduced level of basal acid secretion [344].

We hypothesized that basal acid secretion should be compromised in GFRα2-KO mice, since they lack almost all of the cholinergic innervation of the gastric mucosa. Surprisingly, the intragastric pH levels in GFRα2-KO mice after an overnight fast were low, similar to levels seen in WT littermates. As expected on the basis of these findings, we observed normal levels of acid content and basal acid secretion under urethane anesthesia in GFRα2-

KO mice. To rule out the possibility that the urethane anesthetic influences basal secretion levels, we also measured basal acid content in conscious mice. Consistent with the other findings, the measurements revealed no differences between the genotypes.

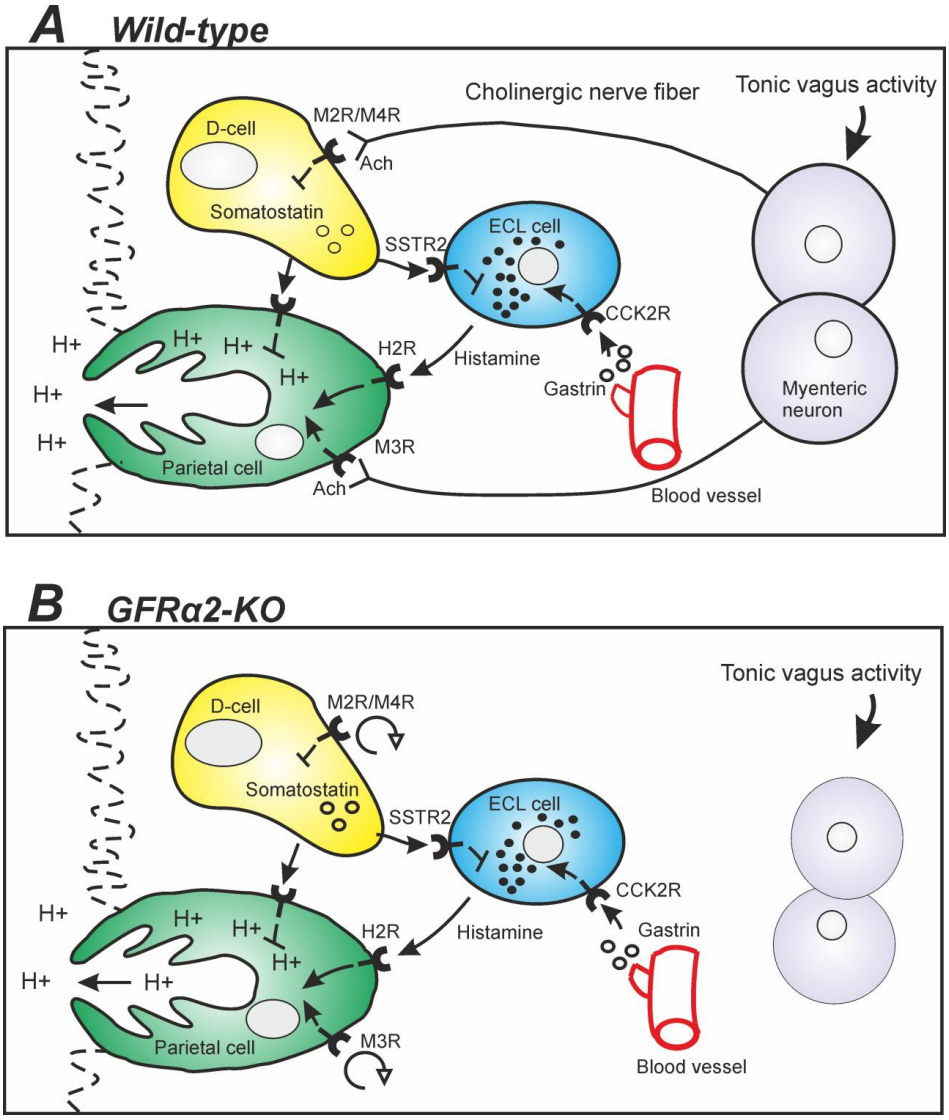

Figure 12. Cholinergic innervation of the gastric mucosa is not necessary for basal acid secretion in GFRα2-KO mice. ***(A)*** Gastrin from antral G-cells (not shown) stimulates basal acid secretion by releasing histamine from ECL cells. Somatostatin from D-cells tonically inhibits acid secretion directly from parietal cells and by inhibiting histamine from ECL cells and gastrin from G-cells (not shown). Acetylcholine released from myenteric neurons in response to tonic vagus activity promotes acid secretion via M3 receptors (M3R) on parietal cells, but also by inhibiting tonic somatostatin release from D-cells. ***(B)*** Basal acid secretion in GFRα2-KO mice is not impaired presumably partly because of compensatory upregulation of muscarinic receptor constitutive activity (circular arrows). CCK2R, gastrin receptor; H2R, histamine H2 receptor, M2-4R, muscarinic receptors; SSTR2, somatostatin receptor. Modified from publication III.

To see if the remaining (<10%) cholinergic innervation in the oxyntic mucosa was able to influence acid secretion, we used 2-deoxyglucose (2-DG) to bring about a central hypoglycemia induced vagus activation in WT and GFRα2-KO mice [384]. As expected, this treatment induced a robust (~8x) acid secretion response in WT but not in the KO mice. A possible explanation for the normal basal acid secretion in GFRα2-KO mice is that residual acetylcholine from the muscle layers reaching the parietal cells in combination with a possible sensitization of the muscarinic receptors, could lead to normal levels of basal acid secretion. In fact, when stimulated with the muscarinic agonist carbachol, the GFRα2-KO mice showed a 2-fold higher response in acid secretion compared to WT mice. This effect was likely due to receptor sensitization and not increased expression, since the gastric M1-M5 receptor mRNA levels were not altered in the KO mice. Interestingly, a similar increased responsiveness to muscarinic agonists has been reported in the cavernosal smooth muscle of GFRα2-KO mice [385]. However, when treated with the ganglionic blocker hexamethonium – an agent known to mimic the effects of surgical vagotomy [386,387] – no effects were seen on basal acid secretion in GFRα2-KO mice. In contrast, basal acid secretion was significantly reduced in WT mice exposed to hexamethonium. The results from these experiments show that the GFRα2-KO mice have a complete loss of functional efferent innervation of the gastric mucosa, but nevertheless maintain a normal basal acid secretion (Figure 12).

When we treated WT mice with the muscarinic antagonist atropine, a significant reduction in basal acid secretion was induced. Interestingly, atropine also reduced basal acid secretion in GFRα2-KO mice to a similar degree. Muscarinic receptors are able to signal in a constitutive manner (i.e. they remain active even without a bound ligand) [388], and atropine as an inverse agonist is able to inhibit both stimulated and constitutive activity of these receptors [389]. That atropine reduces basal acid secretion also in GFRα2-KO mice implies that constitutively active muscarinic receptors on parietal cells and/or D-cells may facilitate basal acid secretion *in vivo*.

In addition to maintaining normal basal acid secretion, an intact vagus is also required for histamine stimulated acid secretion, but the mechanism is unclear [347,349]. We found that acid secretion in GFRα2-KO mice after histamine treatment was comparable to WT littermates, suggesting that the acid secretion capacity in the KO mice is not reduced. This is in contrast to findings in M3R-KO mice, where the acid secretory response to histamine has decreased by 50%. The possibility of constitutive activity of muscarinic receptors in the parietal cells of GFRα2-KO mice could explain why they are able to respond to histamine stimulation. Previous studies done on isolated mouse stomachs have also shown that parietal cells are able to respond to histamine even when nerve activity in the gastric wall is blocked with TTX [390]. A drawback in our experimental setup was the lack of a dose response study using histamine. Therefore, the possibility that acid secretion response to a low dose of histamine in the GFRα2-KO mice is affected cannot be ruled out. Although ECL cells are known to lack muscarinic receptors, the vagus nerve appears to have an influence over ECL cell sensitivity to physiological levels of gastrin [391], possibly via neuropeptides such as PACAP and VIP, which are known to elicit histamine release from ECL cells [392]. In our preliminary experiments, pentagastrin induced a lower acid output in GFRα2-KO mice than in WT mice, supporting the view that ECL sensitivity is under neural control.

CONCLUSIONS

The main findings in the work presented here were:

(i) PCD is a normal part of parasympathetic neuron development in mice. GFRα2-signaling regulates parasympathetic neuron survival in pancreatic and submandibular ganglia during late embryonic development, and lack of GFRα2-mediated signaling results in the profound loss of intrapancreatic neurons through apoptosis. In contrast, apoptosis in the ENS appears to be rare and is not increased in the absence of GFRα2, suggesting that the number of enteric neurons is not determined by PCD.

(ii) In spinal sensory ganglia, GFRα2 regulates the neuronal size, but not the innervation of hair follicles by both large RA Aβ-LTMR and small C-LTMR neurons. In contrast, GFRα2 regulates both the cell size and epidermal innervation of small C-nociceptors. RA Aβ-LTMRs express GFRα2 during early life, but lose GFRα2-expression at some point after maturation.

(iii) GFRα2-signaling via NRTN is required for the cholinergic innervation of the gastric mucosa; however, this innervation is not necessary for trophic support of the gastric mucosa or for gastrin or basal acid secretion. In addition, although vagally stimulated gastric acid secretion is lost, secretion in response to direct parietal cell stimulation remains in the absence of gastric mucosal innervation in GFRα2-KO mice.

ACKNOWLEDGEMENTS

I would like to extend my gratitude towards the following people:

Matti Airaksinen – my supervisor and mentor.

The members of the Airaksinen group, especially Kaija Berg, Tiina Laakso, Marika Markkanen, and Pavel Uvarov.

My co-authors: Meri Lähteenmäki, Jari Rossi, and Karl-Heinz Herzig.

Pia Runeberg-Roos and Matias Röyttä, the pre-examiners of this thesis.

My opponent, Urmas Arumäe.

The staff and fellow researchers at the Department of Anatomy and the Neuroscience Center, University of Helsinki.

All my friends.

My wonderful family: my parents Irma-Liisa and Markku, my sisters Satu and Helena, and Helena's spouse Tomi.

The Antila family.

My beloved Hanna – my sweetheart and a great colleague.

I would also like to acknowledge the Sigrid Juselius Foundation, the Academy of Finland, the University of Helsinki, and the Novo Nordisk Foundation for providing funding for the research projects included in this thesis.

Sincerely,

Jussi Kupari

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