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NEURONAL TYPES AND THEIR SPECIFICATION DYNAMICS IN THE AUTONOMIC NERVOUS SYSTEM

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On the cover: abstract illustration of sympathetic neurons extending their axons
Credits: Gioele La Manno

NEURONAL TYPES AND THEIR SPECIFICATION DYNAMICS IN THE AUTONOMIC NERVOUS SYSTEM

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents

ABSTRACT

The autonomic nervous system is formed by a sympathetic and a parasympathetic division that have complementary roles in the maintenance of body homeostasis. Autonomic neurons, also known as visceral motor neurons, are tonically active and innervate virtually every organ in our body. For instance, cardiac outflow, thermoregulation and even the focusing of our eyes are just some of the plethora of physiological functions under the control of this system. Consequently, perturbation of autonomic nervous system activity can lead to a broad spectrum of disorders collectively known as dysautonomia and other diseases such as hypertension. Neuroblastoma, one of the most common and lethal infancy cancer, arises from defects during the embryonic development of sympathetic neurons. Despite its importance in everyday life and clinical relevance, little is known regarding the molecular mechanisms regulating the birth, differentiation and heterogeneity of the autonomic neurons. This PhD thesis aims at reducing this gap of knowledge.

In **paper I,** we describe the role of the Homeobox transcription factor HMX1 and receptor signalling in directing neuronal fate during embryogenesis. We propose a new model for sympathetic specification in which mature noradrenergic and cholinergic types emerge from a common progenitor and neuronal identity is established via mechanisms involving active repression of receptors and transcription factors directing alternative cell fates.

In **paper II,** we take advantage of high-throughput sequencing approaches to explore the heterogeneity of the sympathetic system and describe the existence of seven molecularly distinct cell types. Using a combination of retrograde and lineage tracing approaches, we describe the developmental mechanisms leading to the emergence of two specialized cell types projecting to the pilo (PEM) and nipple (NEM) erector muscles.

In **paper III**, we show that the parasympathetic nervous system, previously thought to be originated by the Neural Crest Stem Cells (NCSCs) is derived from stem-like Schwann cell precursors (SCPs) intimately associated with the extending cranial nerves during development.

All together, the data collected in this thesis provide new insights into key aspects regulating the origin and development of the autonomic nervous system and provide compelling evidence regarding the existence of specialized cell types regulating specific functions. This knowledge provides new principles on how the autonomic nervous system develops that might help to understand also its pathologies, such as neuroblastoma and dysautonomia.

LIST OF SCIENTIFIC PAPERS

I. **Furlan A**, Lübke M, Adameyko I, Lallemend F, Ernfors P
The transcription factor Hmx1 and growth factor signaling control sympathetic neurons diversification
EMBO Journal 2013 April; 32(11), 1613-1625

II. **Furlan A**, La Manno G, Lübke M, Häring M, Abdo H, Hochgerner H, Kupari J, Usoskin D, Airaksinen MS, Oliver G, Linnarsson S and Ernfors P
Visceral motor neuron diversity delineates a cellular basis for nipple- and pilo-erection muscle control
Nature Neuroscience 2016 October; 19(10), 1331-1340

III. Dyachuk V*, **Furlan A***, Shahidi MK, Giovenco M, Kaukua N, Konstantinidou C, Pachnis V, Memic F, Marklund U, Müller T, Birchmeier C, Fried K, Ernfors P, Adameyko I *equal contribution
Parasympathetic neurons originate from nerve-associated peripheral glial progenitors
Science 2014 July; 345(6192), 82-87

Publications not included in this thesis

Usoskin D, **Furlan A**, Islam S, Abdo H, Lönnerberg P, Lou D, Hjerling-Leffler J, Haeggström J, Kharchenko O, Kharchenko PV, Linnarsson S, Ernfors P
Unbiased classification of sensory neuron types by large scale single-cell RNA sequencing
Nature Neuroscience 2015 January; 18(1), 145-153

Kaukua N, Shahidi MK, Konstantinidou C, Dyachuk V, Kaucka M, **Furlan A**, An Z, Wang L, Hultman I, Ahrlund-Richter L, Blom H, Brismar H, Lopes NA, Pachnis V, Suter U, Clevers H, Thesleff I, Sharpe P, Ernfors P, Fried K, Adameyko I
Glial origin of mesenchymal stem cells in a tooth model system
Nature 2014 September; 513(7519), 551-554

Adameyko I, Lallemend F, **Furlan A**, Zinin N, Aranda S, Kitambi SS, Blanchart A, Favaro R, Nicolis S, Lübke M, Müller T, Birchmeier C, Suter U, Zaitoun I, Takahashi Y, Ernfors P
Sox2 and Mitf cross-regulatory interactions consolidate progenitor and melanocyte lineages in the cranial neural crest
Development 2012 January; 139(2), 397-410

CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetylcholine
ARTN	Artemin
ASCL1	Achaete-Scute Family BHLH Transcription Factor 1
BAX	BCL2 Associated X Protein
BMP	Bone morphogenic proteins
CHAT	Choline O-Acetyltransferase
CNS, PNS	Central, peripheral nervous system
CNTF	Ciliary Neurotrophic Factor
CreERT2	Cre recombinase – estrogen receptor T2
CT-1	Cardiotrophin 1
DBH	Dopamine Beta-Hydroxylase
E, P	Embryonic, Postnatal day
EMN	Erector mucle neuron
ENC1	Ectodermal-Neural Cortex 1
GATA	GATA Binding Protein
GDNF	Glial cell-line derived neurotrophic factor
GFL	GDNF family ligand
GFR	GDNF family receptor
Gp130	Membrane Glycoprotein 130
HAND2	Heart And Neural Crest Derivatives Expressed 2
HMX1	H6 Family Homeobox 1
INSM1	Insulinoma Associated 1
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
NA	Noradrenergic
NCSC	Neural crest stem cell
NEM	Nipple erector mucle
NGF	Nerve Growth Factor
NPY	Neuropeptide Y
NRTN	Neurturin

NT-3	Neurotrophin 3
PEM	Pilo erector muscle
PHOX	Paired Like Homeobox 2
PLP1	Proteolipid Protein 1
PRPH	Peripherin
PSPN	Persephin
RET	Ret proto-oncogene
SCG	Superior cervical ganglion
SCG10	Superior Cervical Ganglion-10 Protein
SCP	Schwann cell precursors
SOX10	SRY-Box 10
TH	Tyrosine Hydroxylase
Tho	thoracic
TLX3	T-Cell Leukemia Homeobox 3
TRK	Tropomyosin receptor kinase
VACHT	Vesicular Acetylcholine Transporter
VMAT2	Vesicular Monoamine Transporter

1 INTRODUCTION

The autonomic nervous system was first described by Walter Gaskell and John Langley in the second half of the 19th century. Later on, Walter Canon's pioneering studies on the effects of autonomic denervation identified the sympathetic and parasympathetic divisions of this system as the main regulator of the internal homeostasis (Purves, 2012). Both divisions share a similar architecture with inputs from the hypothalamus being transmitted first to pre-ganglionic neurons within the central nervous system and then to the post-ganglionic neurons in the peripheral nervous system or directly to the target organ.

Autonomic neurons regulate organ function via release of neurotransmitters. Virtually every organ (with a few exceptions discussed below) receives both sympathetic and parasympathetic innervations (Figure 1). Because autonomic neurons are tonically active, target tissues receive some input at all time. It is this complementary interaction between the two autonomic divisions that maintains body homeostasis. Despite their general similarities, there are crucial differences regarding the location of the sympathetic and parasympathetic neurons and the neurotransmitters they release.

In the presence of an external or internal stressor, the sympathetic system generates the ”fight-or-flight” response, which prepares the body to either face the stressor or flee from it. Once the stressor is removed, the parasympathetic nervous system restores the homeostasis by bringing the body to a ”rest-and-digest” state. It is believed that autonomic neurons respond in an “all-or-none” fashion independently from the quality of stimulus (Selye, 1974). However, more recent studies have provided preliminary evidence for the existence of some degree of autonomic specialization (discussed below).

In the event of alterations of normal autonomic function, a number of disease states can arise, which are collectively referred to as dysautonomia. Furthermore, sympathetic, but not parasympathetic, cells can abnormally proliferate during development and generate neuroblastoma (NB), the most common extracranial solid tumour during infancy. TRKA and ALK tyrosine kinase receptor signalling have been linked to NB prognosis as their activity can accelerate cancer cell proliferation or induce differentiation (Reiff et al 2011; Peterson, 2003). Thus, the identification of molecules regulating signalling events during normal development can be crucial for understanding the disease and to identify new therapeutic approaches.

1.1 The sympathetic division

The sympathetic pre-ganglionic neurons are located in the intermediolateral nucleus of the lateral grey column of the spinal cord, spanning from the 1st thoracic to the 3rd lumbar level. Their myelinated axons exit the spinal cord from the ventral root and enter the spinal nerve, traveling a relative short distance before contacting the post-ganglionic neurons. Alternatively, axons simply cruise through the ganglion without interruption and directly establish contact with the target organ (e.g. chromaffin cells of the adrenal medulla). Pre-ganglionic neurons exiting the spinal cord from their corresponding segment can contact several post-ganglionic chain neurons at different spinal levels (Purves, 2012).

Post-ganglionic sympathetic neurons are organized in paravertebral and prevertebral ganglia. The former form a chain of ganglia located on each side and next to the dorsal aorta and extend rostro-caudally along the entire animal axis. The latter comprises neurons organized in plexuses, located in the body cavity and the pelvis. These are the cardiac, the superior and inferior mesenteric and the celiac plexuses (Purves, 2012).

Post-ganglionic neurons extend their unmyelinated axons in the grey communicating rami to innervate a large number of target organs, including, amongst others, the smooth muscle of the blood vessels, the eye, the erector muscles of the hair follicle and the nipple, the cardiac muscle and the sweat glands. Thus they regulate a variety of physiological processes including the control of glucose levels, skin and muscle vasoconstriction and vasodilation, cardiac outflow, peristalsis, pilo- and nipple-erection, ejaculation and respiration (Figure 1).

Although the majority of organs receive both sympathetic and parasympathetic innervation a minority – sweat glands, adrenal gland and pilo- and nipple-erector muscles and blood vessels – is functionally regulated only by sympathetic inputs.

Pre-ganglionic sympathetic neurons release acetylcholine whereas most of post-ganglionic sympathetic cells release noradrenaline. A small fraction of post-ganglionic sympathetic neurons produces and releases acetylcholine and express markers closer to the parasympathetic lineage. Despite progress has been made in elucidating the molecular mechanisms leading to the specification of the sympathetic lineage, still little is know about the dynamics governing cell diversification.

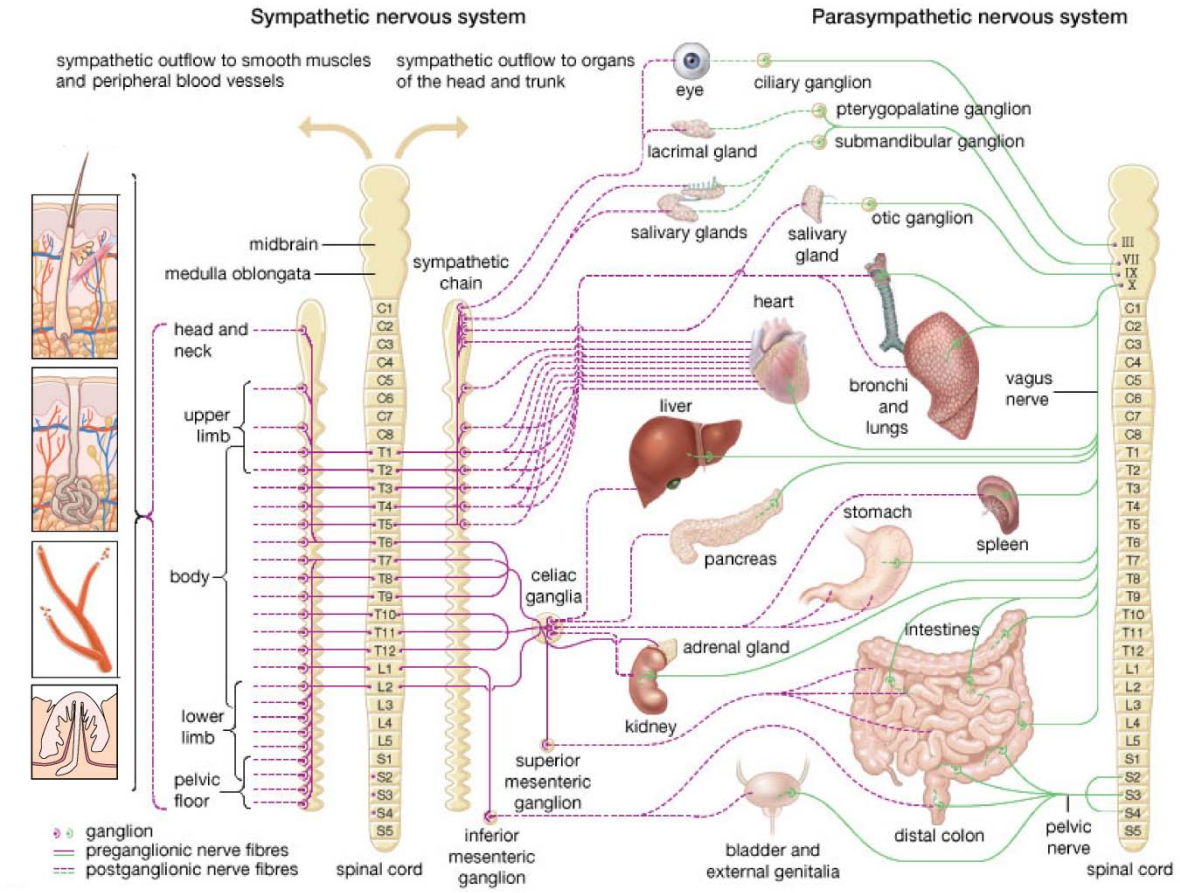

Figure 1. Schematic representation of the sympathetic and parasympathetic divisions of the autonomic nervous system and their target organs. Modified from*: Encyclopaedia Britannica, Inc*.

1.2 The parasympathetic division

Pre-ganglionic parasympathetic neurons are located in brain stem nuclei and in the 2nd to 4th sacral level of the spinal cord and regulate the function of the post-ganglionic parasympathetic neurons. The myelinated axons of the sacral pre-ganglionic neurons exit the spinal cord through the ventral root and travel into the pelvic splanchnic nerve to contact post-ganglionic neurons of the pelvic plexus, which, in turn, innervate the large intestine, rectum, bladder, gonads and external genitalia.

Pre-ganglionic neurons of the brainstem are organized into nuclei (Edinger-Westphal, superior and inferior salivary and dorsal nucleus of the vagus) and their axons travel along the associated cranial nerves (III or oculomotor, VII or facial, IX or glossopharingeal) to reach post-ganglionic neurons located in the ciliary, pterygopalatine, submandibular, otic and sublingual ganglia. Parasympathetic pre-ganglionic nerves traveling within the vagus nerve (X cranial nerve) innervate the trunk parasympathetic neurons directly embedded in the organs. Both pre- and post- ganglionic parasympathetic neurons release acetylcholine (Purves, 2012).

1.3 Development of the autonomic neurons

1.3.1 Migration of Neural Crest Stem Cells

Autonomic neurons originate from the neural crest stem cells (NCSC), a transient population of multipotent stem cells that appear at the margins of the closing neural tube. NCSC are responsible for the formation of the entire peripheral nervous system, including its glial component and some part of the head ectomesenchyme (Dupin et al, 2006; Le Douarin, 1982). Around Embryonic day (E)10, NCSC undergo an epithelial-to-mesenchymal transition and, in the trunk region, migrate along a ventral pathway to form the sympathetic primordia, a chain of cells extending rostro-caudally on both sides of the dorsal aorta (Marmigere & Ernfors, 2007). Bone Morphogenic Proteins (BMP) secreted by the aorta instruct NCSC to upregulate key transcription factors such as ASCL1 and PHOX2b and to acquire noradrenergic features, thus becoming sympathoblasts (Figure 2). According to the current view, a group of sympathoblasts in the lumbar region will later migrate more ventrally and form the pre-vertebral ganglia and the adrenal gland (Huber, 2006; Unsicker et al, 2005). In the rostral region a group of sympathoblasts will migrate rostrally and form the superior cervical ganglion (SCG) (Rubin, 1985). Studies concerning the origin of the parasympathetic nervous system are scarcer. Early studies using elegant quail-chick transplantation experiments demonstrated the neural crest origin of this division, and identified which axis contributes to each cranial parasympathetic ganglion (Le Lievre & Le Douarin, 1975). Little is known though about the migratory routes of the precursors cells and about the mechanisms responsible for their commitment to the parasympathetic lineage (Airaksinen & Saarma, 2002).

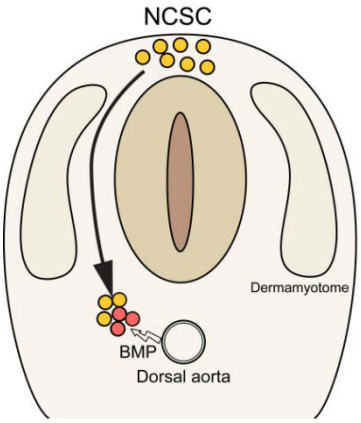

Figure 2. Schematic representation of the ventral migratory pathway of NCSC (in yellow) contributing to the sympathetic lineage (in red). Modified from: Marmigere and Ernfors, *Nature Reviews Neuroscience*, 2007.

1.3.2 BMP-induced network of early transcription factors

The correct assembly of both the sympathetic and parasympathetic ganglia requires the concerted action of some key transcription factors such as ASCL1 (also known as MASH1) and PHOX2a/PHOX2b, as discussed below. Moreover, the development of the sympathetic neurons requires the function of GATA2/3, HAND2 and INSM1 whose expressions are downstream of PHOX2a/b and ASCL1 (Francis & Landis, 1999; Goridis & Rohrer, 2002).

The cross-regulatory interactions of these factors, prompting the acquisition of the noradrenergic traits and sympathetic differentiation are summarized in Figure 3.

1.3.2.1 ASCL1

The Achaete-Acute Family BHLH Transcription Factor 1 (ASCL1) is expressed both in the CNS, where it acts as a proneural gene (Guillemot & Joyner, 1993) and in the PNS, where it seems to have several functions, being involved in proliferation, survival and differentiation of autonomic neuroblasts (Guillemot et al, 1993; Parras et al, 2002). ASCL1 expression is induced in sympathetic neuroblasts as early as E10.5 and it is downregulated by E12.5. Loss of function experiments showed that ASCL1 deficiency mildly affects the correct assembly of neuroblasts and delays the acquisition of noradrenergic traits, such as expression of TH and DBH (Pattyn et al, 2006). Moreover, *Ascl1*$^{-/-}$ sympathetic neuroblasts proliferate less (Parras et al, 2002) and the paravertebral chain appears markedly smaller at later stages (personal observation). Similarly, parasympathetic cranial ganglia of ASCL1-deficient mice are reduced in size (sphenopalatine ganglion) or completely absent (otic and submandibular ganglia) at E16.5 (Guillemot et al, 1993; Hirsch et al, 1998).

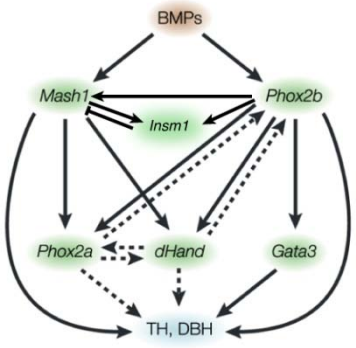

Figure 3. Transcriptional network directing sympathetic specification. Solid arrows indicate experimental confirmed regulation, dashed arrows indicate suggested interactions. Modified from: Goridis and Rohrer, *Nature Reviews Neuroscience*, 2002

1.3.2.2 PHOX2b and PHOX2a

In mice lacking PHOX2b, both sympathetic and parasympathetic development is completely aborted at early developmental stages. PHOX2b-deficient neuroblasts fail to proliferate and eventually die and no expression of noradrenergic markers such as TH, DBH or neuronal markers like the Superior Cervical Ganglion-10 Protein (SCG10), Rearranged during transfetion (RET) or Peripherin (PRPH) (Pattyn et al, 2000; Pattyn et al, 1999) is detected. PHOX2a expression is also aborted. PHOX2a but not PHOX2b expression is dependent on ASCL1, as parasympathetic ASCL1-deficient neuroblasts express PHOX2b, but not PHOX2a (Hirsch et al, 1998). Genetic ablation of PHOX2a causes only minor defects in sympathetic ganglia but parasympathetic neurons are not formed (Morin et al, 1997). Both PHOX2a and PHOX2b have been shown to be regulators of noradrenergic specification both *in vitro* and *in vivo* (Apostolova & Dechant, 2009).

1.3.3 RET signalling

A number of soluble factors and receptors are crucial for directing the events ultimately leading to the mature autonomic fate. In particular, NGF and NT3 signalling via the TRKA and TRKC receptors and the biological activity of members of the GDNF family of ligands (GFL) that signal via RET and its co-receptors are crucial for axonal extension, survival and cell type differentiation.

GFLs play a crucial role in the development of autonomic neurons. The glial cell line-derived neurotrophic factor (GDNF), Artemin (ARTN), Neurturin (NRTN) and Persephin (PSPN) form a complex binding to one of the four non-signalling alpha receptor subunits belonging to the GFL receptor family (GFRα1-GFRα4). The GFL-GFRα complex then recruits RET and initiates signalling. Although GDNF, NRTN, ARTN and PSPN preferentially bind to GFRα1- GFRα4 receptors, respectively (Airaksinen & Saarma, 2002), signalling of ARTN and NRTN via RET-GFRα1 and of GDNF through RET-GFRα2 has been described (Airaksinen et al, 1999; Rosenthal, 1999). Mice lacking RET, GFRα1 or GDNF die at birth, whereas mice knock-out for the other family members or their co-receptors are viable and fertile, but display different degrees of altered development (Airaksinen & Saarma, 2002).

1.3.3.1 RET role in parasympathetic development

RET signalling through the GDNF-GFRα1 complex is crucial for the formation of the parasympathetic nervous system. In mice knock-out for either RET, GDNF or GFRα1, migration of parasympathetic neuroblasts is affected and the otic and the sphenopalatine ganglion are completely missing (Enomoto et al, 2000; Rossi et al, 2000). In normal development, parasympathetic neurons undergo a GDNF-to-NRTN switch around birth. While RET expression is maintained, GFRα2 replaces GFRα1 expression. At the same time, parasympathetic target organs start to express Neurturin (Enomoto et al, 2000; Rossi et al, 2000). Genetic deletion of GFRα2 or its ligand only moderately affects parasympathetic neuron number. However, deficits in the soma size of these cells and in the innervation of the target organs are dramatic, with some tissues (i.e. the lacrimal gland, salivar gland, pancreas) completely devoid of parasympathetic fibers (Heuckeroth et al, 1999; Rossi et al, 2003; Rossi et al, 1999). Deletion of GFRα3 or GFRα4 does not significantly affect the development of cranial parasympathetic neurons (Airaksinen & Saarma, 2002; Nishino et al, 1999).

1.3.3.2 RET role in sympathetic development

Analysis of RET knock-out mice showed that sympathetic precursors require RET signalling for achieving proper cell migration and for axonal guidance (Baloh et al, 1998; Enomoto et al, 2001; Nishino et al, 1999), cell survival (Enomoto et al, 2001) and subtype specification (Brodski et al, 2002; Burau et al, 2004).

Sympathetic neuroblasts endogenously express RET (Enomoto et al, 2001) and GFRα3 at very early stages and artemin signalling via the RET-GFRα3 complex has been shown to be crucial for proper cell migration and axonal extension (Nishino et al, 1999), an event that is initiated when neuroblasts are still en-route towards their final destination (Rubin, 1985). Axons typically extend along the arterial vasculature and follow a gradient of Artemin, secreted first by the tissue surrounding the ganglion and then by smooth muscle cells of the arteries (Honma et al, 2002), to reach their target organs. In RET and GFRα3 deficient embryos, cell migration is impaired and axons are shorter than in control animals.

Although it is difficult to interpret to what extent RET signalling is crucial for innervation, as disrupted cell migration might at least partially account for the innervation phenotype, mice knock out for Artemin (Enomoto et al, 2001; Honma et al, 2002; Nishino et al, 1999; Schuchardt et al, 1994) recapitulate the deficits observed in RET- or GFRα3- knockout mice adding more evidence to the central role of this signalling pathway in the control of innervation.

Despite GFRα2 being expressed in sympathetic progenitors (personal observation) and at birth (Ernsberger, 2008), genetic deletion of GFRα2 does not seem to significantly affect the generation or survival of sympathetic neurons. However, postnatal sympathetic cholinergic neurons are atrophied and innervation of their targets, the sweat gland and the periosteum, is severely affected (Hiltunen & Airaksinen, 2004). Genetic ablation of GDNF – but not of GFRα1 – causes a subtle but still significant reduction in the number of sympathetic neurons (Airaksinen et al, 1999; Moore et al, 1996). Finally, no overt abnormalities were detected in GFRα4-deficient mice (Airaksinen & Saarma, 2002).

Unlike in parasympathetic cells, RET expression in sympathetic neurons is downregulated in the course of development and only a small percentage of cells expresses RET at birth. Furthermore, and similarly to what is observed in parasympathetic development, sympathetic cells also undergo a switch in neurotrophin dependence. Sympatoblasts downregulate TRKC and upregulate TRKA, switching to NGF trophic support during late embryonic development, after which they undergo terminal mitosis (Birren et al, 1993). The molecular mechanisms governing these events are not yet fully understood.

1.3.4 Survival of sympathetic neurons

1.3.4.1 TRKA, TRKC and NT3

NT3 is a neurotrophin that binds the TRKC receptor with high affinity but it is also able to activate TRKA, the receptor of NGF, *in vitro* and at very high concentrations (Davies et al, 1995).

In vitro studies suggested a role for NT3-TRKC in sustaining survival of sympathoblasts (Birren et al, 1993; Dechant et al, 1993; DiCicco-Bloom et al, 1993) and in the induction of TRKA (Verdi & Anderson, 1994; Verdi et al, 1996).

Consistently, *in vivo*, NT3 is secreted by tissues surrounding the sympathetic ganglion (Verdi et al, 1996), TRKC is expressed by early sympathoblasts (Birren et al, 1993; DiCicco-Bloom et al, 1993) and its expression progressively declines in the course of development while TRKA expression in upregulated (Birren et al, 1993).

However, loss of function experiments showed that post-mitotic sympathetic neurons are produced in numbers similar to control animals (Wyatt et al, 1997) and that the neuronal loss observed in *Nt3*$^{-/-}$ deficient embryos takes place after sympathoblasts undergo their last mitotic event, suggesting that NT3 is crucial for post-mitotic neurons rather than for neuroblasts survival.

In line with this conclusion are several observations: I) the most abundant TRKC transcript in the early sympathetic ganglion encodes for a receptor lacking the catalytic tyrosine kinase domain (Wyatt et al, 1997); II) mice lacking TRKC have normal numbers of sympathetic cells (Fagan et al, 1996; Tessarollo et al, 1997); III) a similar degree of neuronal loss is observed in the *Ngf*$^{-/-}$ embryos (Francis et al, 1999; Kuruvilla et al, 2004; Wyatt et al, 1997). All together, these data strongly suggest that NT3 actions might be mediated via TRKA, highly expressed in post-mitotic neurons, rather than TRKC, expressed by sympathoblasts.

More recently, a role for NT3-TRKA signalling in proper innervation of peripheral targets was described, as NT3-deficient neuroblasts display shorter axons and reduced final target innervation (Kuruvilla et al, 2004), suggesting that the apoptosis phenotype displayed by NT3-deficient embryos might be caused by a lack of trophic support from the target organ.

In summary, although it has been convincingly proven that NT3 signalling via the TRKA receptor is important for neuron survival, the fact that NT3 is secreted by sympathetic intermediate target organs like blood vessels (Francis & Landis, 1999) at the time when they receive sympathetic innervation from TRKC^{+} sympathoblasts, makes it reasonable to hypothesise that NT3-TRKC signalling might have a yet to be identified role in sympathetic development.

1.3.4.2 TRKA and NGF

Pioneering work from Rita Levi-Montalcini and Victor Hamburger showed that neuronal survival depends on target innervation (Levi-Montalcini, 1987) and led to the formulation of the neurotrophic hypothesis, according to which, to ensure proper innervation of target organs, neurons are first overproduced embryonically and subsequentely trimmed in numbers postnatally when their axons reach the target organ and competition for neurotrophins determines which neurons will survive or undergo apoptosis (Oppenheim, 1991).

Nerve growth factor (NGF) secreted by target organs (Korsching, 1993) binds its receptor TRKA in the membrane of sympathetic axons in proximity of the target organ. This event causes the internalization of the NGF-TRKA complex into endosomes and activates retrograde transport to the cell body. A central role for actin modifiers recruitment in directing NGF-TRKA, but not NT3-TRKA, retrograde signalling and survival has recently been discovered (Harrington et al, 2011). NGF-TRKA signalling is not only crucial for sympathetic neuron survival (Miller & Kaplan, 2001; Reichardt, 2006) but also for final target innervation. Mice lacking TRKA or NGF display a near complete lack of sympathetic neurons at birth and axons do not reach nearby targets (Crowley et al, 1994; Fagan et al, 1996). Due to the perinatal mortality of these mutants, efforts to address the full role of NGF-TRKA signalling in target innervation were long hindered. More recently, mouse knockout models for the proapoptotic factor BAX, resulting in the prevention of neuronal death, were generated. Compound knockouts for BAX and TRKA showed that many sympathetic targets had mild to severe degrees of innervation impairment (Glebova & Ginty, 2005). Hence, NGF signalling via TRKA is fundamental for post-mitotic neuron survival and important for final target innervation. The finding that not all target organs were affected by a lack of TRKA suggested that other molecules must be involved in the process. Potential candidates are members of the GDNF family signalling via RET. In line with this, GDNF is able to induce neurite extension *in vitro* and its expression was reported in sympathetic targets (Glebova & Ginty, 2005).

1.3.5 Differentiation of sympathetic cells

1.3.5.1 Lessons from the cholinergic type

In the adult sympathetic ganglion, the majority of neurons are noradrenergic and only a small subset is cholinergic. In rodents, cholinergic neurons of the stellate and rostral ganglia innervate the sweat glands (sudomotor neurons) or the membrane covering the external

surface of the bones, also known as periosteum (Anderson et al, 2006; Asmus et al, 2000; Ernsberger & Rohrer, 1999).

Decades of research on the mechanisms at the basis of the development of the cholinergic neurons have provided solid evidence that sudomotor cholinergic properties originate from a trans-differentiation process. Prospective sudomotor neurons are originally noradrenergic neurons that after reaching their target organ, the sweat glands of the paw, undergo a transient phase when both noradrenergic and cholinergic traits are present within the same neurons, before the cholinergic phenotype is stabilized and the noradrenergic markers are downregulated (Asmus et al, 2000; Guidry & Landis, 1998; Landis & Keefe, 1983).

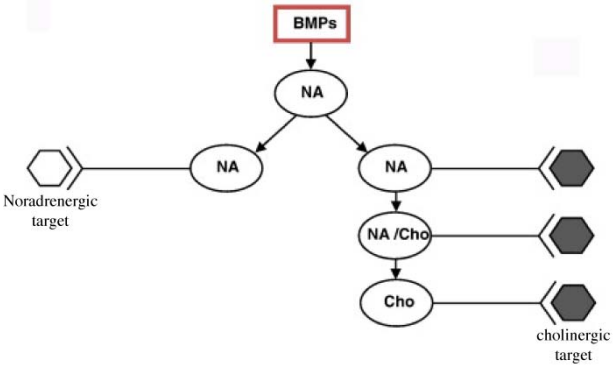

Figure 4. Classical target-dependent model for cholinergic differentiation. NA: noradrenergic; Cho: cholinergic. Modified from: Apostolova and Dechant, *Autonomic Neuroscience*, 2009.

Transplantation experiments, sympathetic co-culture with sweat glands and analysis of the Tabby mice, which are devoid of the sweat glands, convincingly demonstrated that this event takes place post-natally and that it is target-dependent (Guidry & Landis, 1995; Landis et al, 1988).

Some of the members of the neuropoietic cytokine family (LIF, CNTF, CT-1) were first considered excellent candidates for mediating the switch (Ernsberger & Rohrer, 1999). These molecules signal via complexes formed by receptor subunits gp130 and LIFRB.

Several lines of experiments, including CT-1 neutralization experiments and genetic deletion of CNTF, LIF or both did not show innervation defects in the sweat glands (Francis et al, 1997). On the other hand, deletion of the gp130 receptor subunit caused a complete loss of cholinergic innervation in the sweat glands and a reduction of cholinergic neurons in the stellate ganglion (Stanke et al, 2006) suggesting that there might be more than one member of this family acting in cooperation to mediate the switch. Altogether these findings led to the formulation of the so-called classical target-dependent model of cholinergic differentiation (Figure 4). Analysis of RET knockout mice showed that RET signalling is fundamental for the maintenance of embryonic cholinergic neurons but not for

the early induction of CHAT and VACHT markers (Burau et al, 2004). It is still not clear whether prospective cholinergic neurons simply die or switch to a noradrenergic fate.
Interestingly, CHAT/VACHT expression has been reported in rodent paravertebral ganglia (Schutz et al, 1998) at embryonic stages, considerably before neuron-target contact. Considering that at E11.5 virtually all sympathetic neurons express TH, these data suggest the *in vivo* existence of $TH^{+}VACHT^{+}$ ”bimodal” or ”hybrid” cells. Furthermore, it is possible that more than one type of cholinergic neuron exists and that these might have different molecular signatures, functions and developmental histories.

1.4 Heterogeneity of autonomic neurons

Selye’s doctrine of non-specificity describes the autonomic nervous system as largely non-specific, stating that the nature of the stressor does not play a major role in the activation of the sympathetic fight-or-flight response and that all elements involved in this response are unspecific (Selye, 1974). However, more recent studies have collected evidence questioning this view and suggesting a higher degree of finesse, where different stimuli activate specific and dedicated post-ganglionic neurons. Retrograde tracing studies, analysis of the electrophysiological properties of sympathetic dendrites and immunohistochemical interrogation of neuropeptide expression have supported this view (Elfvin et al, 1993; Ernsberger, 2001; Gibbins, 2013; Gibbins, 1991).
Furthermore, it was shown that during standing up, selective constriction of muscles, but not of skin, blood vessels takes place (Jänig, 2006). Conversely, cold stimuli induce a selective vasoconstriction of blood vessels in the skin, along with pilo-erection (goose bumps) and erection of the nipple (Clapham, 2012; Jänig, 2006; Ootsuka & Blessing, 2005). Pilo- and nipple-erection are also triggered by emotional stimuli, like fear or music (a phenomenon called ”frisson”), whereas nipple but not pilo erection is also stimulated by mechanical stimuli such as suckling during breast-feeding (Benedek & Kaernbach, 2011; Birkenfeld & Kase, 1994; Colver & El-Alayli, 2015). Although collectively these observations strongly suggest that the sympathetic response is highly diversified in its elements, no definitive evidence has yet been provided proving the existence of distinct molecular neuronal types *in vivo*.

2 RESULTS AND DISCUSSION

2.1 Paper I

In this paper we investigate the origin of the molecular mechanisms regulating sympathetic differentiation. The traditional view proposed that the cholinergic identity is established via a neurotransmitter switch after interaction with the target organ. However, our data show that sympathetic neuroblasts express both noradrenergic (TH, DBH, VMAT2) and cholinergic markers (RET, TRKC, VACHT, CHAT, TLX3) and thus display a ”hybrid” phenotype. In line with this, our genetic tracing analysis confirmed that post-mitotic noradrenergic and cholinergic neurons are in fact both derived from this common cell type. We then describe the role of the homeobox transcription factor HMX1 in repressing TLX3 and RET expression and in induction and maintenance of TRKA, a marker of post-mitotic noradrenergic neurons. Consistently, genetic ablation of HMX1 led to a failure in repressing RET and TLX3 and expression of TRKA was significantly reduced. Although deletion of HMX1 did not cause an increase in the number of $CHAT^{+}$ neurons, the percentage of neurons expressing VIP and SST, markers normally associated with the cholinergic type, was increased in the mutant ganglia. Furthermore, we report a dramatic decrease in the innervation of pilomotor erector muscles. Finally, we suggest a role for TRKC and RET signalling in regulating the emergence of the cholinergic type through mechanisms of cross-regulation, resulting in HMX1 repression and TLX3 expression.

Our data clearly show that bimodal precursors originate both noradrenergic and cholinergic neurons. Expression of TRKA is indicative of the emergence of post-mitotic noradrenergic neurons, which are numerous already at E15.5. At this early stage, it is rather unlikely that the axons already reached their target organs and in some cases, the target organogenesis has not started yet (e.g. errector muscles of hair follicle or nipple). We show that approximately one day prior to TRKA upregulation, HMX1 expression is detected in $RET^{+}TRKC^{+}$ neuroblasts and that genetic deletion of HMX1 prevents TRKA expression.

This finding, together with the proposed role for RET and TRKC signalling in shaping cell identity, suggests that neuronal identity is established via mechanisms involving active repression of receptors and transcription factors directing alternative fate (HMX1 for noradrenergic, RET and TRKC signalling for cholinergic), rather than by direct interaction with the target organ (Figure 5).

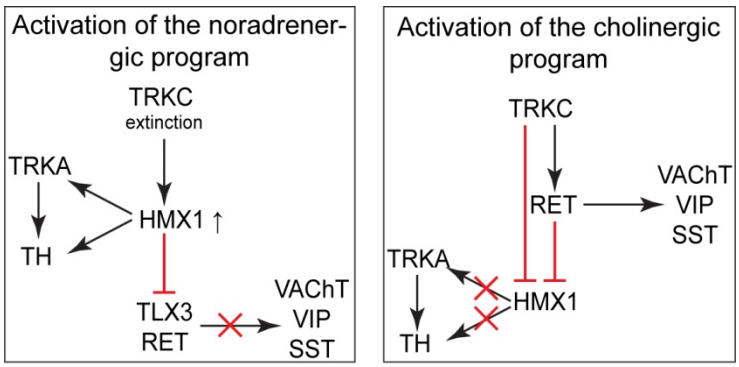

Figure 5. Schematic representation of the genetic programs defining target-independent sympathetic cell fate. From: Furlan et al, *EMBO journal*, 2013

We report that the innervation of the erector muscle of the hair follicle is dramatically reduced in mice lacking HMX1. A possible explanation could be that the absence or delay of TRKA expression in post-mitotic neurons causes a lack of trophic support and consequent cell death. However, although $TRKA^+$ neurons are still reduced at birth, we report an increased number of neurons in the HMX1 mutant newborn mouse. One possible explanation is that TRKA expression is not completely abolished but simply reduced and that residual levels are sufficient for survival. Alternatively, prolonged RET signalling might allow mutant cells to survive or direct sympathetic axons to other targets and to alternative sources of NGF. Intriguingly, the RET and TRKA signalling pathways are involved in the regulation of neuroblastoma differentiation. TRKA expression is lost in malignant forms of the disease while activation of RET signalling in NCSC-derived organs can lead to tumour formation (Miyake et al, 1994; Nakagawara et al, 1993; Takahashi, 2001). Thus, since HMX1 represses RET and upregulates TRKA expression, its role in neuroblastoma differentiation should be investigated.

2.2 Paper II

In this paper we investigate the heterogeneity of the sympathetic nervous system. We carried out single-cell RNA sequencing on post-natal day (P)27-34 sympathetic neurons from the stellate and paravertebral ganglia and identified seven neuronal types. Based on known markers, we were able to conclude that five groups were noradrenergic whereas two were cholinergic. RET expression is downregulated in noradrenergic post-mitotic neurons starting at E14.5 (Furlan et al, 2013). Interestingly, transcriptomics data showed that some of the adult noradrenergic neurons re-introduced RET expression (RET^+TRKA^+ neurons). Quantification of the soma size of RET^+TRKA^+ neurons showed that NA2 and NA4-NA5 types were bigger than other noradrenergic neurons. Analysis of transgenic reporter mice combined with retrograde and cell fate tracing showed that RET^+TRKA^+ neurons are

specialized TRKA$^+$ neurons, born embryonically, and that the NA2 and NA5 neuron types project to the nipple erector (NEM) and pilo erector (PEM) muscles, respectively. Thus, these neurons are ”erector muscle neurons” (EMNs). RET signalling requires the presence of its co-receptors and ligands (Airaksinen & Saarma, 2002). Transcriptomics data showed that NA2 neurons express GFRα3 whereas NA5 neurons express GFRα2. Consistently, NEM and PEM smooth muscle cells express ARTN and NRTN, respectively. We showed *in vitro* that ARTN, NRTN and NGF can induce RET expression in TRKA$^+$ post-mitotic neurons at P0.

PROX1 has recently been identified as a marker of proliferative sympathetic neuroblasts in chick (Holzmann et al, 2015). In an attempt to understand the mechanisms leading to the emergence of the EMNs we genetically ablated PROX1 in the neural crest lineage using *Wnt1-Cre* transgenic mice. PROX1-deficient neuroblasts exit the cell cycle prematurely, upregulate TRKA expression and commit to an EMN-like phenotype when the axons extended by the neuroblasts are still en route towards their final target organs. At this time, ARTN secreted by smooth muscle cells of the blood vessels guides axonal elongation (Honma et al, 2002). Early TRKA$^+$ neurons of PROX1-deficient embryos respond to artemin by prematurely re-introducing RET expression. Finally, we show by genetically ablating the *Ret* gene from neurons expressing TRKA or knocking out its co-receptor GFRα2 that RET signalling is important for development of EMNs and for proper innervation of their targets. The implications of the data presented in this paper are discussed below.

2.2.1 New cell types

Our data show an unpredicted degree of finesse for a system which has long been considered to respond to all types of stimuli in an ”all-or-none” fashion. We show that specialized groups of neurons are responsible for the functional activation of erector pili (PEM) and nipple erector muscles (NEM) sympathetic targets. This proof of concept finding has broader implications in the context of autonomic neuropathies (dysautonomia), a wide spectrum of disorders that arise from abnormalities in the response of the autonomic nervous system and can affect all organs, compromising their function. These disorders can be genetic or acquired and, to date, the available treatments are mostly focused on alleviating the symptoms. The existence of specialized cell types serving specific organs might open for more focused treatments. In order for that to happen, it is of pivotal importance to discover the physiological function of the other cell types presented in this article (Figure 6). Our analysis showed the presence of a large group of NA3 small-sized

cells expressing NPY and representing roughly 50% of the total cells in the ganglion. These are likely to innervate the blood vessels to regulate vasoconstriction. Large NA4 cells express GFRα2 and ENC1. Interestingly, these cells are particularly abundant in the stellate and first thoracic ganglia (tho1-4), whose neurons mainly innervate the cardiac muscle (Purves, 2012).

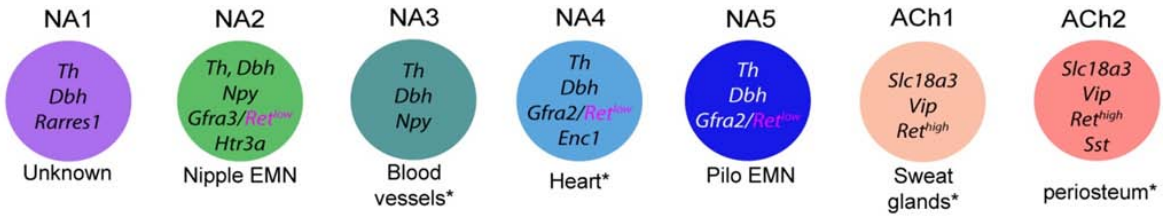

Figure 6. Sympathetic neuronal types. NA2 and NA5 are EMN neurons and project to the NEM and PEM, respectively. For the other types, the proposed target tissue is indicated by the asterisk (*). Modified from Furlan et al, *Nature Neuroscience*, 2016

NA1 neurons represent a molecularly very distinct cell type which localizes uniquely in the stellate and superior cervical ganglia and might therefore innervate head organs. Finally, our RNA-sequencing data showed the existence of two molecularly distinct types of cholinergic neurons. ACh1 neurons are $VIP^{+}SST^{-}$ and are present in stellate and paravertebral ganglia. ACh2 neurons are $VIP^{+}SST^{+}$ and are almost absent in the stellate ganglion, but present in the thoracic ganglia (tho1-12). The presence of two molecularly distinct cholinergic types suggests the existence of dedicated circuits. It is tempting to speculate that the ACh1 cells are sudomotor neurons whereas ACh2 neurons might innervate the periosteum. Future studies should address the developmental dynamics of these cell types.

2.2.2 Post-natal specialization of noradrenergic neurons

It has been convincingly shown that cholinergic neurons arise via a noradrenergic-to-cholinergic transition soon after birth. Here, we provide evidence for the existence of similar mechanisms at the basis of the specialization of EMNs. Post-mitotic noradrenergic neurons are generated as early as E14.5, when RET is downregulated and TRKA expression begins (Furlan et al, 2013). However, their specialization to EMNs is not apparent until P6, when RET is re-introduced, and it is not complete at least until P11, when their soma size has become consistently bigger than other non-EMN RET-negative neurons. Intriguingly, at the time of embryonic noradrenergic specification, and until early post-natal stages, PEM and NEM organs are not yet formed as they undergo organogenesis between P1 and P3. Soon after, muscle cells of the target organs upregulate NRTN and ARTN expression and final target innervation begins (Figure 7).

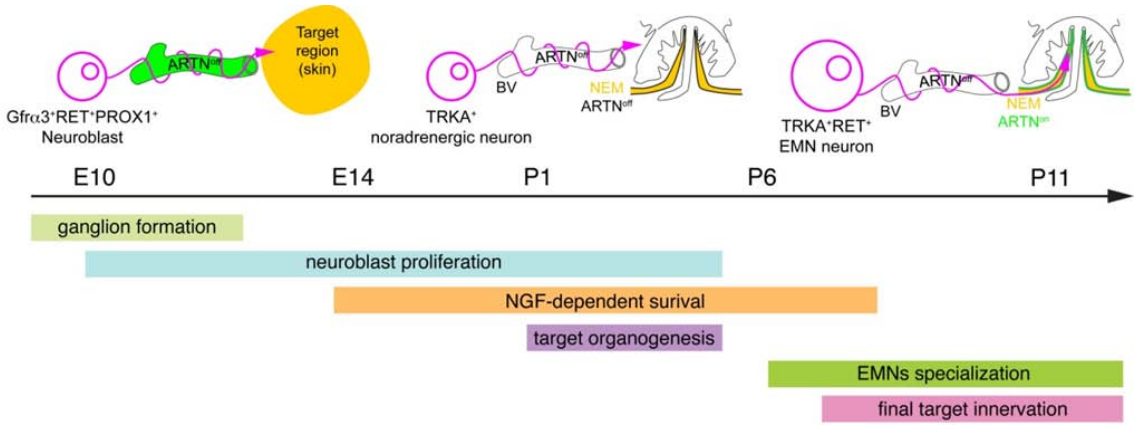

Figure 7. Summary of the events leading to specialization of NEM neurons (and in general EMNs) from TRKA$^+$ neurons. Based on: Furlan et al, 2013, 2016. Layout inspired by Glebova and Ginty, *Annual Reviews Neuroscience*, 2005.

We show that GFLs can instruct newborn TRKA$^+$ neurons to upregulate RET expression *in vitro*. Hence, it is reasonable to propose that the premature emergence of EMNs in PROX1-deficient embryos could be due to exposure of blood vessel-derived ARTN. All together these data suggest a role for the organ in final differentiation of noradrenergic neurons. Future efforts should be directed to clarify the mechanisms of this induction.

2.2.3 PROX1$^+$ sympathoblasts

The transcription factor PROX1 was first described to have a critical role for lymphatic system development (Wigle & Oliver, 1999). PROX1 is expressed in proliferative neuroblasts during sympathetic development of the chick (Holzmann et al, 2015). Our results confirm this finding in mouse and show a role for PROX1 in cell cycle regulation and differentiation. Proliferation of sympathoblasts was reported to be virtually completed at late embryonic stages (Gonsalvez et al, 2013). However, we find that approximately 16% of all cells in the newborn ganglion are PROX1$^+$ and are still dividing, suggesting that a considerable amount of neurons are generated in the post-natal period. We show that PROX1$^+$ progenitors express TH, whereas post-mitotic noradrenergic neurons maintain TH and upregulate TRKA expression, making TRKA a more reliable marker for the identification of the mature noradrenergic type.

2.2.4 RET role in EMNs development and target innervation

In order to address the role of RET signalling in the emergence of the EMN types, we deleted RET in TRKA expressing cells, that is, in all post-mitotic noradrenergic neurons. This approach allowed us 1) to circumvent the postnatal lethality shown by full RET KO animals and 2) to investigate specifically the role of RET signalling when it is re-expressed

in the post-natal period, without affecting the embryonic RET-driven events such as migration, proximal innervation and cell survival (Enomoto et al, 2001). Our results showed that *Ret* deletion does not affect the proportion of EMNs but it is important for cell size expansion of EMNs, which display a smaller soma size compared to control animals and to RET-negative neurons within the same ganglion. Furthermore we report that lack of RET cause a mild yet consistent deficit in sympathetic innervation of the PEM and NEM. Analysis of *Gfra2*$^{-/-}$ mice confirms that lack of RET signalling by NRTN-GFRA2 complex recapitulates the phenotype observed in the conditional *Ret*$^{-/-}$ mice. These results are in line with previous studies showing a role for GFRα2 in trophic support and proper innervation of autonomic target organs (Hiltunen & Airaksinen, 2004) and once again confirm that the target innervation is a multifactorial complex event requiring the action of more than one molecule (Glebova & Ginty, 2005; Bonanomi, 2012; Charoy, 2012).

2.3 Paper III

In paper III we investigate the origin of the other major autonomic division, the parasympathetic nervous system. Unlike the other parts of the peripheral nervous system, studies on the mechanisms of parasympathetic neurons development have been scarce and, although the first elegant quail-chick transplantation experiments (Le Lievre & Le Douarin, 1975) showed that parasympathetic neurons are derived from the neural crest cells of the cranial region, little is known about the molecular mechanisms generating these ganglia.

In the head, the major sympathetic ganglia are the otic, the submandibular, the ciliary, the sphenopalatine and the sublingual ganglia, which innervate the parotid gland, the submandibular ganglion, the pupillary sphincter and lacrimal gland, the nasal mucosa, and the tongue, respectively (Enomoto et al, 2000). Using genetic tracing techniques, we show that virtually all parasympathetic neurons at E17.5 are derived from E11.5 SOX10^{+} (in *Sox10*^{CreERT2}*;R26R*^{Confetti}) or PLP1^{+} (in *Plp1*^{CreERT2}*;R26R*^{Confetti}) nerve-associated Schwann (SCPs) cells and that ASCL1 expression, a known critical regulator of parasympathetic development (Guillemot et al, 1993) is initiated in these SOX10^{+} cells starting at E11.5. Genetic ablation of *Ascl1* combined with fate tracing of ASCL1-expressing cells (*Ascl1*$^{TOM+}$ cells) was achieved generating embryos bearing two copies of the *Ascl1*^{CreERT2} allele, – *de facto* creating a full knockout for *Ascl1* – and a copy of the *Tomato* reporter protein under the control of the CAG promoter (*Ascl1*$^{CreERT2/CreERT2}$*;R26R*^{Tomato}). Injection of tamoxifen at E12.5 and analysis at E17.5 showed that ASCL1-deficient cells fail to form PHOX2B^{+}

parasympathetic neurons and instead retain glial marker expression, indicating a crucial role for ASCL1 in the glia-neuron transition.

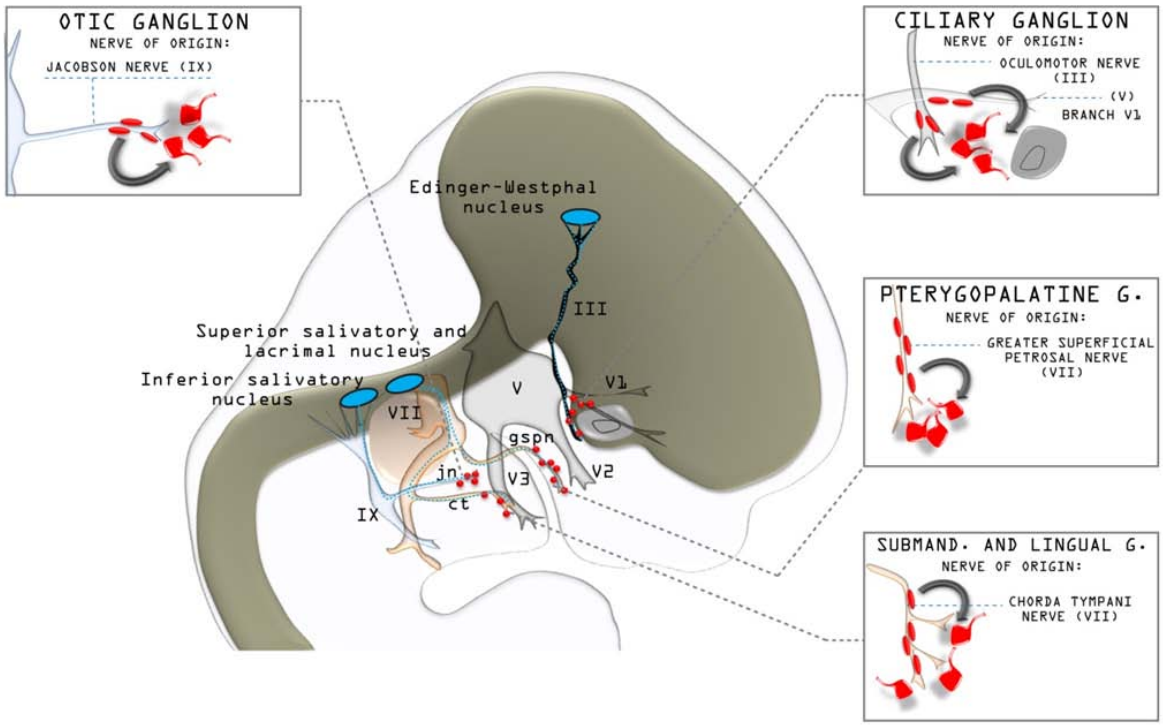

Figure 8. Scheme illustating the forming cranial parasympathetic ganglia (red cells) and their pre-ganglionic axons. ct: chorda tympani; III: oculomotor cranial nerve; V1: ophthalmic branch of trigeminal nerve;V2: maxillary branch of trigeminal nerve; V3: mandibular branch of trigeminal nerve; gspn: greater superficial petrosal nerve;jn–Jacobson's nerve; roman digits indicate cranial nerves. From: Dyachuk and Furlan et al, Science, 2014.

The dependence of the parasympathetic system formation from glial cells was confirmed by analysis of mice knockout for ERBB3 (*Erbb3*$^{-/-}$), the receptor for Neuregulin, which displayed complete absence of sphenopalatine, otic and submandibular ganglia, and by ablation of the greater superficial petrosal (Gspn) and the Jacobson's (also known as tympanic nerve, or IX cranial nerve) pre-ganglionic nerves in *Ret*$^{CFP/CFP}$ mice, which resulted in the concomitant absence of the parasympathetic ganglia they innervate (i.e. the sphenopalatine and otic ganglia) but not other cranial ganglia. Finally, the competence of glial cells to generate both neurons and glia elements of the ganglion has been tested in *Plp1*^{CreERT2}*R26R*^{Confetti} embryos. Tamoxifen injection resulted in the stochastic expression of one of the four fluorescent proteins, allowing clonal analysis of the progeny of single PLP1^{+} cells and showing they are able to generate both neuronal and glial cells, thus being bi-potent.

Our findings confirm the versatile pluripotent nature of glial cells and their capabilities to contribute to organ formation (Adameyko et al, 2009) and show that the developing nerve maintains a stem-cell niche where SCPs can, at discrete locations, and probably under the

influence of locally secreted factors, generate neuronal progenitors which will form the whole parasympathetic nervous system (Figure 8).

3 CONCLUSIONS AND PERSPECTIVES

In this thesis, we investigated the developmental mechanisms at the basis of the origin of the autonomic nervous system and of the diversity of its neurons.
We described a novel role for the transcription factor HMX1 for consolidating a noradrenergic fate, by maintenance of TRKA expression and for extinguishing cholinergic properties, via RET repression. TRKA and RET signalling pathways are involved in neuroblastoma progression (Peterson & Bogenmann, 2004). Deletion of HMX1 resulted in an increase of the number of sympathetic cells, suggesting a potential role for this molecule in forcing cell cycle exit.

Next, we took advantage of next generation sequencing tools to identify sympathetic cell types based on their molecular signature. We reported the existence of seven types of sympathetic neurons *in vivo* and showed that they regulate specific functions. The identification of novel, specialized sympathetic cell types might be useful for better understanding dysautonomia, autonomic neuropathies caused by alterations of normal autonomic function and affecting an estimated 70 million people worldwide. Intriguingly, the novel cell types identified are highly diverse in their receptor expression, suggesting the possibility that pre-ganglionic neurons might be as diverse as the post-ganglionic neurons that they control.

The finding that the differentiation and specialization of EMNs neurons is completed only after birth raises some interesting questions stretching outside of the boundaries of the autonomic nervous system. Do cells specialize after birth in a completely target-dependent manner, implicating a stochastic process, or are their fates determined already during embryonic development, which would suggest that postnatal specialization is the result of a functional adjustment of a selected set of predetermined neurons?

We showed that at E14.5, when only a few post-mitotic neurons are detected, NPY^+ neurons represent approximately half of all cells in the ganglion. Intriguingly, at P11, the percentage of NPY^+ cells is not significantly changed compared to E14.5, but at this stage nearly all cells had exited the cell cycle and EMNs neurons had emerged. The NA4-5 type represents approximately 40% of all noradrenergic neurons and do not express NPY. Thus, it seems that NA2 neurons could arise from NPY^+ cells while NA5 neurons would not. These results seem to suggest that some form of specialization is present already at early

embryonic stages. Future works should address to what extent progenitors are committed and when and how this commitment is achieved.

Lastly, we show that the sympathetic and parasympathetic divisions have different developmental histories, as parasympathetic neurons are not generated by neural crest stem cells, like the sympathetic counterpart, but are derived from nerve-associated Schwann cell precursors, via the transcriptional activity of ASCL1. Future works will need to address the role of the nerve in mediating the glial-neuron transition.

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