On the development of the parasympathetic, enteric and sacral nervous systems
Isabel Espinosa Medina

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On the development of the parasympathetic, enteric and sacral nervous systems

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Thèse de doctorat de Biologie du développement
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This manuscript contains unpublished results that should be considered confidential.
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Chapter 1

Introduction

‘[N]ature seems unaware of our intellectual need for convenience and unity, and very often takes delight in complication and diversity’, Santiago Ramón y Cajal, 1906.

Indeed, diversity and complex connectivity characterize a functional nervous system, probably the most sophisticated system of our body, which permanently receives, integrates and transmits information from the external and internal environments.

The development of the autonomic nervous system constitutes a good model to study circuit diversity and assembly. To generate a functional autonomic nervous system, neuronal and glial precursors undergo migrations, cell-cell interactions and respond to molecular signals for their survival, proliferation and differentiation. In the present work, I explore the migratory mechanisms and neuronal interactions during autonomic circuit assembly and define a differential molecular signature to distinguish the various classes of autonomic neurons.

Although it might not be “convenient”, as S.R.Cajal said, it is precisely that diversity and complexity what make the study of the development of the nervous system fascinating.

1.1 History of the autonomic nervous system

The first observations of the anatomy of the autonomic nervous system (ANS) were made by Galenos (A.D. 130-200) on pigs. He regarded the nerves connected to different organs as hollow tubes through which the animal spirit could pass and create a coordination of the organs called sympathy. Although this is far from the current understanding of the ANS
function, the term sympathy, or more precisely sympathetic, has remained till this day (Nilsson, 1983).

The terminology “autonomic nervous system” was first proposed by Langley in 1898, replacing the previous “vegetative” nervous system of Johann Christian Bell and the “organic” nervous system of Francois Xavier Bichat. Langley’s autonomic nervous system consisted of three subdivisions: the sympathetic, with central connections in the thoracic and lumbar spinal cord; the parasympathetic, with cranial and sacral connections; and the enteric division, which comprises the intrinsic neurons of the gastrointestinal tract. This general framework has remained intact for over a century but a revision resulting from our studies will be presented in Chapter 4 of this manuscript.

The role of the ANS in maintaining the stability of the internal environment of the body or “milieu intérieur” was first defined by the French physiologist Claude Bernard in 1878 and further developed by the American physiologist Walter B. Cannon in 1932, who introduced the term “homeostasis” to define the complex mechanisms that maintain an internal steady-state. Cannon also proposed that the sympathetic and parasympathetic divisions exert opposite functions on their target organs, and coined the term ‘fight or flight’ for the stress response triggered by the sympathetic division in opposition to the ‘rest and digest’ function of the parasympathetic division, under normal conditions. The idea of two opposite forces driving autonomic function had already been formulated by Gaskell in 1910: ‘The evidence is becoming daily stronger that every tissue is innervated by two sets of nerve fibers of opposite characters so that I look forward hopefully to the time when the whole nervous system shall be mapped into two great districts of which the function of the one is katabolic [breaking down], of the other anabolic [building up], to the peripheral tissues: two great divisions of the nervous system which are occupied with chemical changes of a synthetical and analytical character respectively, which therefore in their action must show the characteristic signs of such opposite chemical processes.’

Indeed, both divisions often play opposite roles on target organs, for example on the heart, although the relationship between them can be asymmetric or subtle. For instance, under normal conditions, fine-tuning of pupil size controlling the accommodation of the eye to light is largely under parasympathetic control, which is modulated on the background of a largely stable sympathetic tone. Under conditions of excitement or alarm, however, there is a shift in this balance, inhibiting pupillary constriction and increasing tone in the pupillodilator.
muscle of the iris by sympathetic fibers (Kandel, 2012), the functional usefulness of this not being readily apparent. An example of a subtle cross-talk of both divisions is the control of salivary secretion. Salivary glands respond to both parasympathetic and sympathetic stimulation with secretion and there is no autonomic inhibition under normal conditions (Emmelin, 1987; Garrett 1987). However, the protein contents of saliva are differentially modulated by both divisions (Carpenter et al., 2000; 2005; Proctor et al., 1990; Anderson et al., 1995); for instance, under stress conditions, the increase in salivary alpha-amylase has been suggested to result from sympathetic stimulation (Nater and Rohleder, 2009). Sympathetic inhibition of salivation occurs only indirectly, through vasoconstriction, and results in the dry mouth associated with anxiety or some pharmaceutical intervention (Proctor and Carpenter, 2007), again with no clear physiological advantage. Finally, some organs receive innervation exclusively from one of the divisions: piloerector muscle, sweat glands and skin vasculature are innervated exclusively by sympathetic neurons (and see Chapter 4 for more examples), whereas lacrimal glands receive parasympathetic innervation only.

Figure 1: Schematic representation of the autonomic nervous system (Kandel, 2012). Connectivity of sympathetic and parasympathetic preganglionic neurons (green) and postganglionic neurons (brown) to their major target organs in the body. Intrinsic neurons of the gastrointestinal tract form the third and enteric division, (here, as often, considered as part of the parasympathetic nervous system). (See Chapter 3).
1.2 Anatomy and function of the autonomic nervous system

The ANS is a visceral sensory and motor system that innervates cardiac muscle, smooth muscle, and glandular tissues mediating a variety of visceral reflexes. These are by and large mediated by local circuits in the brainstem and spinal cord, although they are finely tuned through their connections to more rostral control nuclei which modulate involuntary functions. The three divisions of the ANS defined by Langley differ in their anatomical disposition of the efferent (motor) pathways (Figure 1).

The sympathetic division consists of preganglionic motor neurons located in the intermediolateral column of the spinal cord extending from the first thoracic spinal segment to rostral lumbar segments. They project axons in the ventral root that initially run together with somatic motor axons in the spinal nerve, and then separate to project (in small myelinated bundles called white rami communicans) to the paravertebral ganglia of the sympathetic chains, along each side of the spinal cord. The axons of postganglionic motor neurons are largely unmyelinated and exit the ganglia in the unmyelinated gray rami communicans to project to their target tissues (Figure 2) (Kandel, 2012). Some preganglionic fibers pass through the sympathetic ganglia and project along “splanchnic nerves” to synapse on a second chain of sympathetic ganglia, the prevertebral ganglia or adrenal medulla cells at abdominal levels, the latter being functionally and developmentally related to sympathetic postganglionic cells (see below).

The main neurotransmitter used by sympathetic preganglionic neurons is acetylcholine (ACh) (Sevigny et al., 2008; Phelps et al., 1991) and thus, they express the enzyme choline acetyltransferase (ChAT). Most sympathetic postganglionic neurons use noradrenaline as neurotransmitter and express the synthetizing enzymes tyrosine hydroxylase (TH) and dopamine β hydroxylase (DBH). However, some sympathetic postganglionic neurons undergo a transdifferentiation process and end up using ACh, such as those innervating sweat glands and periosteum (Schotzinger et al., 1994; Schotzinger and Landis, 1990; Asmus et al., 2000).
The parasympathetic division consists of preganglionic motor neurons located in different brainstem nuclei which project axons through dorsolateral exit points into the cranial nerves (with the exception of the cranial nerve III or oculomotor, which projects ventrally, see Annex and Discussion in Chapter 2). These preganglionic fibers then project long distances to synapse on postganglionic parasympathetic neurons forming ganglia close to or embedded in their target tissues (Figure 3). The organisation of the sacral outflow, classically considered the second subdivision of the parasympathetic nervous system, will be discussed in Chapter 4. The main neurotransmitter used by pre- and post-ganglionic parasympathetic neurons is ACh.

To maintain homeostasis, the afferent cranial pathways provide information about the internal state of the body. Viscerosensory neurons are derived from the epibranchial placodes (D’Amico-Martel and Noden, 1983) and form the distal ganglia of several cranial nerves (VII, IX and X) from which they transmit peripheral information to the brainstem. Much of the information from the thoracic and abdominal cavities reaches the brainstem via the nodose ganglion of cranial nerve X or vagus nerve; the petrosal ganglion of cranial nerve IX or
glossopharyngeal nerve conveys visceral sensory information from the head and neck and, both of them with the cranial nerve VII or facial nerve, relay “special” visceral sensory information about taste from the oral cavity. All of these visceral sensory afferents enter the brainstem through dorsolateral points to synapse in the nucleus of the solitary tract (nTS) (Figure 3 and Annex for a detailed circuit diagram of each of the mentioned cranial nerves). Some neurons in this nucleus directly innervate preganglionic neurons in the medulla and spinal cord, triggering direct autonomic reflexes, whereas others project to the hypothalamus, amygdala and cerebral cortex to integrate them into ongoing patterns of behaviour. Note that there is another type of cranial sensory input, mediated by somatosensory neurons which are derived from the neural crest and form the proximal ganglia of the same cranial nerves (plus the trigeminal ganglion) and transmit mechanical information from the skin, skeletal muscle, mucosae and sensory organs mostly to the trigeminal nuclei in the central nervous system (Annex).

The enteric division is formed by a network of ganglia embedded on the walls of the gastrointestinal tract. It is the most complex and truly “autonomous” of the three divisions and its neuroanatomy and function will be discussed in detail in Chapter 3.

Figure 3: Anatomical organization of the cranial parasympathetic preganglionic and postganglionic axons.
1.3 Ontogeny of postganglionic autonomic neurons

a. Autonomic neurons originate from multipotent neural crest cells (NCC)

Postganglionic neurons and glia of the autonomic nervous system (ANS) derive from the neural crest (NC), a multipotent migratory population which also gives rise to cartilage and bones of the head and neck, dorsal root ganglia, endocrine cells, smooth muscle cells, tendons, and pigment cells (Theveneau and Mayor, 2012; Le Douarin and Kalcheim, 1999).

The NC was first described anatomically in the chick embryo by Hiss (1868) as a strip of cells lying between the dorsal ectoderm and the neural tube, which prompted a series of pioneering studies in amphibians by Newth (1951) (reviewed in Hörsstadius, 1950). Later on, the introduction of modern cell labelling techniques, tissue grafting as well as the advent of molecular biology and developmental genetics led to a much deeper understanding of NC ontogeny (Le Douarin and Kalcheim, 1999).

A large body of work has been devoted to the induction of the neural crest, which does not concern us directly here. In brief, NC cells (NCC) are induced in the ectoderm at the border of the neural plate by factors including BMPs, Wingless-type proteins (Wnts), and fibroblast growth factors (FGFs), secreted by the surrounding tissues (Betancur, 2010; Sauka-Spengler and Bronner, 2008; 2010). They subsequently delaminate from the neuroepithelium and undergo a partial or complete epithelium-to-mesenchyme transition (EMT) regulated by factors including Snails, Sox and Ets gene families (Thiery JP, 2009). Then NCC migrate through stereotypical routes along the body to colonize their target tissues and differentiate into a variety of cell types. The specific mechanisms regulating NC ontogeny and migration have been extensively reviewed (Kalcheim and Burstyn-Cohen, 2005; Theneneau and Mayor, 2012; Betancur et al., 2010; Le Douarin and Kalcheim, 1999; Milet and Monsoro-Burq, 2012; Prasad et al., 2012; Stuhlmiiller and Garcia-Castro, 2012a) and here I will focus on the contribution of NCC to autonomic postganglionic neurons (schematized in Figure 4).
Parasympathetic ganglia form from NCC at cranial and sacral (but see Chapter 4) levels (Figure 4, light yellow for ciliary precursors; other parasympathetic ganglia are not represented) and their development and migration routes will be discussed in Chapters 2 and 4 of this manuscript, which contain my original data on the subject. Enteric ganglia develop mostly from NCC that emerge from the caudal hindbrain (Figure 4, dark yellow from somite 1 to 7: the so-called vagal NC) with a small contribution from the sacral level (Figure 4, dark yellow caudal to somite 28), and their development will be discussed in Chapter 3, which contains my own data on the subject. Here I will review the case of sympathetic ganglia, which originate primarily from NCC at cervical and trunk levels (Figure 4, green lines) and migrate through the somites to settle near the dorsal aorta. I will deliberately emphasize the more uncertain or controversial aspects of their development, some of which have ramifications in the following chapters of this manuscript.
b. An example of autonomic neurogenesis: sympathetic ganglia formation

Sympathetic ganglia are subdivided into two series based on their anatomical disposition: paravertebral ganglia, which form a chain of 30 pairs (8 cervical, 12 thoracic, 5 lumbar and 5 sacral in humans, the exact numbers vary with species) located dorsolateral to the aorta; and prevertebral ganglia (the celiac, mesenteric and pelvic ganglia (see also Chapter 4)), which are located in the abdomen. The latter are less separate than their nomenclature suggests and form in fact a more or less continuous plexus arising from sympathoadrenal precursors that segregate from paravertebral precursors and migrate further ventrally (Huber et al., 2009 and this work, Figure 5 and Figure 26).

Figure 5: Peripheral ganglia distribution along the rostro-caudal axis of an 8 days-old chicken embryo (Le Douarin and Kalcheim, 1999). Sympathetic ganglia (red); dorsal root ganglia (green); enteric ganglia (blue) and Remack ganglion (yellow, see Chapter 4 concerning this sacral autonomic ganglion).
It is commonly accepted that NCC emerging from the neural tube at somite level 5 down to sacral levels give rise to sympathetic ganglia (Figure 4). However, the origin of the most rostral sympathetic ganglion, the superior cervical ganglion (SCG), remains controversial. Chick-quail chimera experiments indicated that SCG forms from the level of somites 5-10 (reviewed in Le Douarin and Kalcheim, 1999, without references bearing specifically on this point). However, the only published experimental data documenting a rostral limit at somite 5 comes from a study on the sympathetic innervation of the pharynx and heart in chick-quail chimeras (Verberne et al., 1999). The authors show that NCC from somite level 0 to 3 contribute exclusively glial cells to the SCG, whereas NCC caudal to somite 5 contribute neurons and glia inclusively; contribution of NCC at somite 4 to
sympathetic ganglia was not documented. On the other hand, two studies on the murine sympathetic system suggested that SCG originates from NCC emerging from the lower cervical/upper thoracic region (starting around somite 11, see Figure 6), which subsequently migrate rostrally to occupy its final position (around somite levels 4-6, see Figure 6) (Rubin, 1985; Nishino, 1999). The first study did not provide any data documenting this migration and based its speculations on the delayed cervical sympathetic development with respect to thoracic levels (Rubin, 1985). The second study showed that in GFRα3 null mice, the receptor of the neurotrophic factor Artemin, the final position of SCG is shifted caudally (around somite levels 8-10, or cervical nerves C5 to C7) between E12.5 and E14.5 days of development (Nishino, 1999; Figure 6B-C and 6E-F), and they proposed as an explanation that rostral migration of SCG precursors was impaired (citing Rubin, 1985). However, during earlier developmental stages, SCG precursors were positioned correctly in GFRα3 mutants (Nishino, 1999; Figure 6A and D) and the observed phenotype could be due to later defects, including lack of projection to the target that could lead to the atrophy or mispositioning of SCG neurons. Finally and in contrast to the previous studies, another work performed in mice in which different neural crest populations were traced with DiI, reported that the SCG originates from NCC emerging at somite levels 1-4, rostrally to the cervical neural crest, whereas more caudal paravertebral sympathetic ganglia derive from somite level 6 onward (Durbec et al., 1996). The only evidence provided by the authors, however, show a group of DiI positive cells derived from somite levels 1-4 positioned laterally to the dorsal aorta (Durbec et al., 1996, Figure 6C) before the SCG can be recognized as such, and later stages were not documented. Thus, the upper-most origin of sympathetic ganglia is not well defined and further lineage studies will be essential to resolve this.

The pathways taken by sympathetic precursors from their origin in the dorsal neural tube have been extensively characterized for trunk NCC and are twofold (Figure 7):

- The ventromedial pathway: it is taken by two consecutive waves; first, NCC migrate between adjacent somites and contribute neurons and glia to the sympathetic ganglia and later, NCC traverse the rostral-most half of the differentiating somites and further contribute to sympathetic ganglia, but also to dorsal root ganglia (DRG), and give rise to glial cells and chromaffin cells of the adrenal medulla at somite levels 18-24.
- The dorsolateral pathway: NCC migrate between the ectoderm and the somites and give rise to melanocytes. More recently a subpopulation of melanocytes has been shown to derive from nerve-associated NCC, or Schwann-cell precursors (SCPs) (Adameiko et al, 2009).

In the mouse, NCC emigrate simultaneously along both pathways, whereas in avian species the ventromedial pathway precedes that of the dorsolateral pathway by one day (Le Douarin and Kalcheim, 1999). A lot of work has been devoted to the restriction of NCC migration to the rostral-half of the somites, and incriminated repulsive cues expressed in the caudal-half: class3-semaphorins and their neuropilin receptors and ephrins and their receptors (Halloran and Berndt, 2003; Krull, 2001; Kuriyama and Mayor, 2008). The latter have also been implicated in the migration of melanoblasts along the dorsolateral pathway. Actually, ephrins act as bifunctional guidance cues: they first repel early migratory NC cells from the dorsolateral path and later stimulate the migration of melanoblasts into this pathway (Santiago and Erickson, 2002). Other molecules implicated in NCC restriction to the ventromedial pathway are Slits/Robo receptors (Jia et al., 2005).

Figure 7: Migration pathways of trunk neural crest cells (NCC). NCC emerge from the dorsal neural tube (NT) and follow a ventromedial pathway (red) in two phases (1 and 2) and give rise to paravertebral sympathetic ganglia (SG) lateral to the dorsal aorta (DA), dorsal root ganglia (DRG), prevertebral sympathetic ganglia (pSG) and adrenal medulla cells (AM). Other NCC follow the dorsolateral pathway (3, purple) and give rise to melanocytes (purple circles). NCC migrating along spinal nerves (4, red) also give rise to a subpopulation of melanocytes. Sc: sclerotome, dm: dermomyotome; n: notochord.
Sorting of NCC migrating along the ventromedial pathway to form sympathetic ganglia requires the expression of signalling molecules that attract them towards the dorsal aorta. In the chick, differential expression of CXCR4 by sympathetic precursors and not by DRG precursors, confers the first the ability to migrate to the site of SDF-1 ligand expression in the mesenchyme adjacent to the dorsal aorta. Indeed, gain- and loss-of-function experiments demonstrated that expression of CXCR4 is necessary for NCC migration to the sympathetic primordium (Kasemeier-Kulesa et al., 2010). However, casting doubt on the robustness and evolutionary conservation of this role is the fact that \textit{CXCR4} null mice have been analysed more than ten years ago, the authors reporting only slight DRG malformations without mentioning any effect on sympathetic gangliogenesis (Belmadani et al., 2005) and no further analysis of these mutants has been published to our knowledge. Moreover, in zebrafish, trunk NCC do not express \textit{CXCR4} and CXCR knock down interfered only with cranial ganglia development (Olesnicky Killian et al., 2009).

Other signalling pathway implicated in the migration of sympathetic precursors, both in chicken and mice, is the Neuregulin-1(Nrg1)/ErbB pathway. Nrg1 is an EGF-like factor that signals through the transmembrane tyrosine kinase heterodimer receptor ErbB2/ErbB3, and plays essential functions in the development of some of the major derivatives of NCC, such as Schwann cells and sympathetic neurons (Britsch, 2007). The expression of Nrg1 in the para-aortic mesenchyme attracts ErbB3/ErbB2 expressing NCC to the site of sympathetic ganglion formation and in ErbB2 or ErbB3 null mice, trunk NCC form DRG but fail to migrate further ventrally to the dorsal aorta (Britsch et al., 1998; Saito et al., 2012).

The dorsal aorta was shown to promote sympathetic differentiation, and Bone Morphogenetic Proteins (BMPs) expressed by this blood vessel, were sufficient to direct cells towards the sympathoadrenal fate where they express catecholaminergic characters (Reissmann et al., 1996; Schneider et al., 1999). More recently, inhibition of BMP signals by \textit{Noggin} in chicken showed that these molecules are also essential for SDF-1 and Nrg1 expression in the para-aortic mesenchyme, thus acting indirectly in the migration of sympathetic precursors (Saito et al., 2012). The same study showed that BMPs are directly involved in late segregation of adrenal precursors from a common pool of sympathoadrenal precursors and their secondary ventral migration to the site of adrenal medulla formation. Inhibition of BMPs by conditionally expressed dominant-negative BMPR1A after NCC have colonized the dorsal aorta resulted in the absence of the ventrally migrating adrenomedullary...
population whereas sympathetic ganglia remained intact (Saito et al., 2012 Fig. 3D-E). It has been proposed that differential expression of Crossveinless (Cv-2), known to act as a BMP antagonist in several contexts (Coles et al., 2004; Kelley et al. 2009; Moser et al., 2007), by sympathetic but not medullary precursors repress BMP signalling exclusively in the sympathetic lineage before segregation (Saito and Takahashi, 2015). However, a limitation of the study by Saito et al. (2012), is that BMPs are essential for sympathetic neuronal differentiation and in particular for the induction of Phox2b expression, in the absence of which sympato-adrenal precursors die (Schneider et al., 1999; Pattyn et al., 1999). Thus the lack of adrenomedullary cells ventral to the dorsal aorta in the absence of BMP signalling could result from the demise of these precursors.

In the chick, sympathetic cells located dorsolateral to the aorta undergo a secondary migration to their final position closer to the ventral root, which provides preganglionic innervation (Kirby and Gilmore, 1976). A recent study has reported a mechanism by which BDNF released by preganglionic axons would be essential for this secondary migration of TrkB-positive primary sympathetic neurons to their final location (Kasemeier-Kulesa et al., 2015). In this study, video-microscopy after ablation or re-orientation of the ventral root showed that sympathetic cells are attracted by their preganglionic partners when they are close to them (Kasemeier-Kulesa et al., 2015, Fig.3) and require BDNF/TrkB signalling to reach their final secondary position (Kasemeier-Kulesa et al., 2015, Fig.4). However, these manipulations were performed after the primary sympathetic ganglia initiated the dorsal migration and did not prove that the ventral root triggered it.

The formation of secondary sympathetic ganglia has not been reported in mammals (Kirby and Gilmore, 1976; Champlain et al., 1970) and BDNF or TrkB null mice did not display sympathetic ganglia defects (Fagan et al., 1996; Ernfors et al. 1994). These differences with respect to what Kasemeier-Kulesa observed in chicken were attributed to the fact that the studies on BDNF and TrkB null mice focused on the superior cervical ganglion instead of lumbar sympathetic ganglia, for which a transient expression of TrkB has been reported (Straub et al., 2007; Kasemeier-Kulesa et al., 2015). However, the fact that today there is still no report on the formation of secondary sympathetic ganglia in mammals or sympathetic defects in BDNF or TrkB null mice suggests this secondary migration is specific to aves. Moreover, it remains unknown if the secondary migration of chicken sympathetic cells is conserved at upper-cervical levels because, due to technical difficulties, the superior cervical
ganglion was not analysed in the previous studies (Kirby and Gilmor, 1976; Kasemeier-Kulesa et al., 2015), which adds to the long list of uncertainties on the ontogeny of the most rostral sympathetic ganglion.

The control of sympathetic neuronal differentiation by BMPs secreted by the dorsal aorta involves a complex transcriptional network that activates the expression of pan-neuronal and subtype-specific genes. This network includes the early autonomic determinants Phox2b, Mash1 and later expressed transcription factors such as Insm1, Hand2, Gata2/3, whose roles have been extensively reviewed elsewhere (Kameda, 2014; Rohrer, 2011).
2.1 Introduction

The development of the parasympathetic division of the autonomic nervous system is less well known than that of the sympathetic and enteric divisions. In this chapter I uncover the migration mechanism of parasympathetic precursors.

a. Anatomy and function of parasympathetic neurons

Parasympathetic innervation is mediated by hindbrain preganglionic neurons that project in cranial nerves to synapse onto postganglionic neurons in ganglia located close to or embedded within their target organs (See Annex). The often small size of parasympathetic ganglia and their scattered positions along the body axis within their target organs make them difficult to isolate and study, with the exception of the ciliary ganglion in chicken (much bigger than the equivalent in mammals), which has been by far the most studied.

Parasympathetic ganglia of the cranial region include:

- The ciliary ganglion: located in the posterior orbit, it is innervated by preganglionic neurons that project from the Edinger-Westphal nucleus in the oculomotor nerve (III cranial nerve). It innervates the ciliary muscles and the pupillary sphincter mediating the accommodation of the eye to light and distance.
- The sphenopalatine ganglion: located in the pterygopalatine fossa, it receives preganglionic innervation from neurons located in the superior salivatory nucleus projecting though a branch of the facial nerve (VII cranial nerve), the greater superficial petrosal nerve (GSPN). It innervates lacrimal glands, nasal mucosa and small salivary glands in the palate.

- The submandibular and sublingual ganglia: they also receive preganglionic innervation from the superior salivatory nucleus via corda tympani (CT), another branch of the facial nerve. They are located in close association to the submandibular and sublingual salivary glands that they innervate.

- The otic ganglion: located in the infratemporal fossa, it receives preganglionic innervation from the inferior salivatory nucleus via Jacobson’s nerve, a branch of the glossopharyngeal nerve (IX cranial nerve). It provides innervation to the parotid gland for salivation.

In the thoracic region, numerous parasympathetic ganglia appear close to their target viscera, including cardiac and pulmonary ganglia that innervate the heart and lungs. They receive preganglionic innervation from neurons located in the dorsal motor-nucleus of the vagus nerve (X cranial nerve) and in the nucleus ambiguus, via several branches of the vagus nerve.

In the abdominal region the pelvic plexus, a paired structure located close to the rectum and reproductive organs, innervates the lower bowel, the reproductive and urinary organs. It receives preganglionic innervation from both, the lumbar and the sacral spinal cord, and is thus considered a mixed sympathetic-parasympathetic plexus (but see Chapter 4).

b. Inductive signals and transcriptional determinants of parasympathetic neurogenesis

The specification and differentiation of parasympathetic neurons is mediated by local signals closely related to those acting in the sympathetic lineage. The mechanisms involved have been well characterized for the ciliary ganglion of the chicken embryo due to its accessibility (Müller & Rohrer, 2002). Ciliary precursors derive from the mesencephalic neural crest and receive secreted BMPs from the retro-orbital mesenchyme at the site of ganglion formation, which induce the expression of the neuronal determinants *Ascl-1, Phox2a* and *Phox2b*. BMP5 and 7 are essential and sufficient for ciliary neuron formation, prevented by the BMP antagonist *Noggin*, but not for the migration of ciliary precursors (Müller &
Rohrer, 2002). A similar role of BMPs might extend to mammals, where BMPs applied in vitro or to cells isolated from rat sciatic nerves induce a cholinergic phenotype, the main neurotransmitter of mature parasympathetic neurons (White, 2001).

Development of all autonomic ganglia depend on the paired-homeodomain transcription factor \textit{Phox2b} (Hirsch et al., 1998; Pattyn et al., 1999) which induces the expression of \textit{Phox2a} and maintenance of \textit{Ascl-1} and regulates the expression of DBH and TH, enzymes involved in the synthesis of noradrenaline. All parasympathetic ganglia are missing in \textit{Phox2b} KO mice, only the sphenopalatine and the submandibulary ganglia in \textit{Phox2a} KO (as a result of a non-cellular autonomous effect, Coppola et al., 2010), and in \textit{Ascl-1} KO they all form but fail to express \textit{Phox2a} and DBH, and then degenerate (Hirsch et al., 1998; Pattyn et al., 1997; Pattyn et al., 1999). Parasympathetic neurons have a transient noradrenergic phenotype (for a very small proportion of neurons in the case of the ciliary ganglion) that is substituted by a cholinergic phenotype in mature neurons. During sympathetic neurogenesis \textit{Hand2} induces the expression and maintenance of a noradrenergic phenotype (Stanzel et al., 2016). Although ciliary precursors do not express \textit{Hand2}, they can respond to the ectopic expression of \textit{HoxB8}, a marker of sympathetic precursors, with an increase in noradrenergic differentiation (Huber et al., 2012). An early differential expression of these and other not yet identified factors by parasympathetic versus sympathetic neurons might explain how the same signalling molecules (BMPs) can induce two different outcomes.

Glial cell-line derived neurotrophic factors (GDNF) also play critical roles in parasympathetic ganglia formation. The target-derived ligands GDNF and Neurturin signal through a receptor complex formed by the tyrosine kinase receptor Ret and a GPI-associated member of the GFRα family, expressed by parasympathetic precursors. In mice lacking Ret, GDNF or its high-affinity receptor GFRα1 the otic and sphenopalatine ganglia are missing, whereas the submandibulary ganglion forms and degenerates later. In mice mutants for Neurturin or its high-affinity receptor GFRα2, there is a decrease in the number of submandibular and ciliary neurons not observed in the otic or sphenopalatine ganglia, although innervation of the lacrimal gland is missing (Enomoto et al., 2000). This and other studies concluded that GDNF signalling via Ret/ GFRα1 is essential for early survival, proliferation and/or migration of parasympathetic precursors of the otic, sphenopalatine and submandibulary ganglia, whereas Neurturin via Ret/ GFRα2 exerts a late trophic support and promotes target innervation in all ganglia. Moreover, GDNF and Ret can induce a cholinergic phenotype in chick sympathetic
neurons in vitro (Brodski et al., 2002), but it remains unknown if they induce parasympathetic cholinergic differentiation in vivo.

c. Migration of parasympathetic precursors

In contrast to the sympathetic and enteric divisions, very little is known about the migration of parasympathetic precursors to their numerous and scattered positions along the body, far from their origin in the neural crest.

Only two studies provide images of the migration pathway of parasympathetic precursors of the otic ganglion, described as a ‘stream of cells’ in one of them (Takano-Maruyama et al., 2012) and said to follow the tubotympanic recess in the other (Enomoto et al., 2000). The latter shows a gradient of GDNF expression along the migration pathway, in the absence of which they fail to migrate and die. However, this interpretation needs revision as explained in the Discussion of this chapter.

Recent results from my host lab have shown that sensory neurons innervating taste buds are essential for parasympathetic ganglia formation (the sphenopalatine and the submandibular ganglia) and for the establishment of their preganglionic innervation (visceral motoneurons projecting in the facial nerve). In practice, genetic damage to the epibranchial ganglia led to the elimination of several nerves and (in a non-cell autonomous fashion) their associated parasympathetic ganglia (Coppola et al., 2010). The simplest explanation put forward by the publication was that parasympathetic precursors where guided by the nerves, and my first publication tests this hypothesis and its generality.
2.2 Publication

Parasympathetic ganglia derive from Schwann cell precursors


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Bone morphogenetic protein (BMP) family members act directly to induce Ascl1 and Phox2b (12), both of which are required for induction of the parasympathetic neuron fate (18, 19). Thus, induction of a neuronal fate driven by rising BMP levels in nerves at sites of neuronal induction could explain the derivation of neurons of the parasympathetic nervous system from progenitors that otherwise give rise to Schwann cells. This interpretation is consistent with the observation that BMP7 is expressed at the site of ciliary ganglion formation (12) and that it has been implicated in induction of parasympathetic neurons from ectoderm-nervous NCCs (20). In contrast to broad expression of PHOX2B along tissues and organs during postneural crest development, its expression is located closer to the target tissues. Thus, our data describe the origin of the parasympathetic system, in which its neurons are recruited from glial progenitors dwelling in cranial and trunk nerves in which its neurons are recruited from glial progenitors dwelling in cranial and trunk nerves and that it has a role in the development of other parasympathetic ganglia: the otic (gX) ganglion in the facial (nVII), geniculate (gVII), petrosal (gIX), and nodose (gX) ganglia that project in the cranial nerves (21). Like all other peripheral autonomic neurons, parasympathetic precursors are dispatched in the em- bryo prior to the dorsal aorta or in the walls of the gut are well charted (20). We studied how para- symptothetic precursors are dispatched in the em- bryo to arrive at sites far away from their origin. Genetic deletion of two branches of the facial nerve (nVII) eliminated their associated parasympathetic ganglia, suggesting a role for cranial nerves in parasympathetic ganglioneogenesis (21). We thus sought to analyze the role of cranial nerves in the development of other parasympathetic ganglia: the otic ganglion, which innervates the parotid gland, and the cardiac ganglia, which innervate the heart (22).

The proneural gene Neurog2 is expressed during the differentiation of viscerosensory neurons in the ganglia (23), and it regulates mRNAs coding for the cell adhesion molecule NCAM (24). We thus sought to understand how parasympathetic ganglia form close to visceral organs and what their precursors are. We find that many cranial nerve-associated crest cells coexpress the pan-autonomic marker PHOX2B along with markers of cranial nerves (25).

**ACKNOWLEDGMENTS**

We thank M. Lubke for breeding Ret mice; J.-F. Brunet, M. Wegner, and F. Lillienfeld for generously providing Phox2b Sox10, and 2B4 antibodies; H. Clevers for Confetti; H. Enomoto (RIKEN) for R26R mice; and the CLICK Imaging Facility supported by the European Research Council (ERC) advanced grant (232675) to U. Kemler, the MRC Centre for Medical Research, and the Swedish Dental Association and Stockholm County Council. The Swedish Medical Research Council, the Bertil Hållsten Research Foundation, SFO on Stem Cells and Regenerative Medicine (StaRGen), and the Knut and Alice Wallenberg Foundation (CLiC) to N.A. and M.K.S. received support from the Swedish Medical Research Council (521-2007-655), the Swedish Dental Association and Stockholm County Council, and the Swedish Cancer Foundation to W. J. Howard. M.R. was supported by the Swedish Brain Foundation, the WallenbergScholar, and Söderberg Foundation. This work was supported by the Swedish Research Council for Medicine and Health, the Swedish Cancer Foundation, the Knut and Alice Wallenberg Foundation (KAW2008.0123), Swedish Dental Society GLUSS0035 and SLSS3193, and the Ruth and Richard Julian Foundation (36918). We studied how parasympathetic ganglia form close to visceral organs and what their precursors are. We find that many cranial nerve-associated crest cells coexpress the pan-autonomic marker PHOX2B along with markers of cranial nerves (25).

**NEURODEVELOPMENT**

Parasympathetic ganglia derive from Schwann cell precursors

I. Espinosa-Medina,1, 2 E. Ottin,1, 2 C. A. Picard,1, 2 Z. Chettouh,1 S. Dynecki,1 G. G. Consalves,1 E. Coppola,1 J.-F. Brunet1

Parasympathetic ganglia form one of the three divisions of the peripheral autonomic nervous system. They are located deep in the cephalic, thoracic, and abdominal tissues, close to or embedded in their target organs, on which they exert actions opposite to sympathetic ganglia. The systems are together as essential for cardiovascular, respiratory, and digestive functions (1). Activity of parasympathetic neurons is modulated by preganglionic, or visceromotor, neu- rons, most of which are located in the hindbrain and project in cranial nerves (2). Like all other peripheral autonomic neurons, parasympathetic precursors derive from the neural crest (3). Migration pathways of sympathetic and enteric precursors toward the dorsal aorta or in the walls of the gut are well charted (20). We studied how para- symptothetic precursors are dispatched in the em- bryo to arrive at sites far away from their origin. Genetic deletion of two branches of the facial nerve (nVII) eliminated their associated parasympathetic ganglia, suggesting a role for cranial nerves in parasympathetic ganglioneogenesis (21). We thus sought to analyze the role of cranial nerves in the development of other parasympathetic ganglia: the otic ganglion, which innervates the parotid gland, and the cardiac ganglia, which innervate the heart (22).

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Fig. 1. Dependency of parasympathetic ganglia on cranial nerves. (A, C, E, and G) Views of whole-mount embryos at E11.5 stained for neurofilament and centered on cranial nerves VII, VIII, and IX (top) or IX, X, and XI (bottom) in wild-type (WT) embryos (A) and the indicated genotypes (C, E, and G). Dorsal is left, rostral up. (B, D, F, H) Immunohistochemistry for PHOX2b combined with in situ hybridization for peripherin at E13.5 on parasagittal sections through the head (top) or transverse sections through the thorax (bottom) in WT embryos (B) and the indicated genotypes (D, F, and H). In Neurog2 KO, only the somatosensory neurons of the vestibulo-acoustic (VIII) and trigeminal (T) ganglia are missing, with no incidence on parasympathetic ganglia. C, cardiac ganglia; CT, chorda tympani; ENS, enteric nervous system; G, gVII (geniculate ganglion); G*, atrophic gVII (corresponding to its somatic part); JN, Jacobson’s nerve; N, gX (nodose ganglion); O, otic ganglion; P, gIX (petrosal ganglion); yellow asterisk, vestigial nX.

Fig. 2. Stages in the formation of the otic ganglion. (A to G) Detection of TUBB3, SOX10, and PHOX2b on Jacobson’s nerve (JN on the three-dimensional rendering of a whole-mount neurofilament stain of cranial nerves V, VII, IX, and X at E10.5 in the top left) and the otic ganglion that forms at its distal end. (A and B) The most distal SOX10+ cell (arrowheads) lags behind the most advanced fibers (asterisks). (E to G) A cluster of PHOX2b+/SOX10weak cells forms a third of the way along JN, presumably corresponding to a previously undescribed parasympathetic ganglion. GSPN, greater superficial petrosal nerve; other abbreviations as in Fig. 1. Scale bars indicate 50 μm in (A) and (B) and 100 μm for (C) to (G).
diptheria toxin chain A action (9) was too slow for the required time frame, we adapted a technique originally developed in Caenorhabditis elegans (30), conditionally expressing a toxic allele of the sodium channel ASIC2α (11) under the promoter of Pain-like homedomain 2α (Phox2α) (fig. S4). In Pgk::Cre; Phox2a::GFP embryos (where Cre is expressed in the germ line), precursors of visceral sensory neurons should be killed in the epibranchial placodes and visceromotor neurons upon exit of the cell cycle, according to the expression schedule of Phox2α (22). As predicted, both types of neurons were absent in embryonic day 11.5 (E11.5) Pgk::Cre; Phox2a::GFP pups (fig. S5). The main branch of nVII persisted (fig. 1, A and G), most likely formed by the projection of somatosensory neurons that are located in the proximal part of gVII and do not express Phox2 genes (33); nIX and Jacobson’s nerve were missing (fig. 1, A and G) and so was the otic ganglion (fig. 1, B and H). As to nX, it was reduced to a vestigial ramus (fig. 1, A and G), and cardiac ganglia failed to form (fig. 1, B and H). The agenesis of parasympathetic ganglia in Pgk::Cre; Phox2a::GFP embryos was non–cell-autonomous: When we recombined the Phox2α::GFP locus selectively in premigratory neural crest with a Wnt1::Cre allele (14)—thus, in sympathetic and parasympathetic precursors—sympathetic ganglia were absent at E13.5 (fig. S6), but parasympathetic ganglia were unaffected (fig. 1, B and H). They degenerated only one day later (fig. S6), in line with the fact that they start expressing Phox2α at E12.5, that is, 2 days later than sympathetic ganglia (15). Therefore, the absence of cardiac ganglia in Pgk::Cre; Phox2a::GFP embryos at E13.5 is most likely secondary to the atrophy of nX. In summary, the Neuron2 KO and Pgk::Cre; Phox2a::GFP mutations each delete a different set of cranial nerves cell-autonomously, and the corresponding parasympathetic ganglia fail to form. We conclude that parasympathetic ganglia formation depends on the cranial nerves that innervate them.

This dependency suggests that the nerves form before the ganglia and then recruit the ganglionic cells. To gather evidence for this, we monitored the formation of Jacobson’s nerve and the behavior of neural crest cells in its vicinity by combined detection of βIII-tubulin (TUBB3), SOX10 [an early marker for neural derivatives of the neural crest (16)], and PHOX2b [a general determinant of autonomic ganglia (17)]. Between E10 and E11 (Fig. 2, A to C), in short sequence, Jacobson’s nerve emerged from the distal pole of gIX and projected toward its endpoint, close to gVII, accompanying by SOX10 cells with elongated nuclei, which lagged behind the most advanced fibers (Fig. 2, A and B) and whose numbers progressively increased. The pan-autonomic determinant Phox2b was switched on along the nerve in a salt-and-pepper manner between E10.5 and E11 (Fig. 2, B and C) and was expressed by all SOX10+ nerve-associated cells at E11.5 (Fig. 2D). Between E12.5 and E13.5, the otic ganglion anlage, first visible as a buildup of cells around the distal end of the nerve (Fig. 2E), condensed and became demarcated from the nerve, which was concomitantly depleted of cells (Fig. 2, F and G). At the same time, Phox2b and Sox10 expression domains became mutually exclusive, PHOX2b weak or absent in most nerve-associated cells, and SOX10 weak in most PHOX2b+ ganglion cells. Nerve-associated...
and ganglion cells at this stage had most likely switched off Phox2b or Sox10, respectively, because all nerve-associated cells equally expressed both genes a day earlier (and see below). The observed sequence of events suggests that the SOX10+ cells, which then become SOX10+/PHOX2b+ double-positive, migrate along the nerve and reach in that way the site of ganglion formation, although a directed proliferation could also play a role. Accordingly, in Neurog2 KO, where Jacobson’s nerve is absent (Fig. 1), the stream of cells was absent (Fig. S8). This scenario is not unique to the otic ganglion, because a similar sequence was evident for the sphenopalatine and cardiac ganglia (fig. S7).

Schwann cell precursors, which later give rise to myelinating and nonmyelinating Schwann cells, occupy embryonic nerves from E11 (fig. S7). Therefore explored whether the nerve-associated cells, which later give rise to myelinating and nonmyelinating Schwann cells, occupy embryonic nerves from E11 (fig. S7). The observed sequence of events suggests that the SOX10+ cells, which then become SOX10+/PHOX2b+ double-positive, migrate along the nerve and reach in that way the site of ganglion formation, although a directed proliferation could also play a role. Accordingly, in Neurog2 KO, where Jacobson’s nerve is absent (Fig. 1), the stream of cells was absent (Fig. S8). This scenario is not unique to the otic ganglion, because a similar sequence was evident for the sphenopalatine and cardiac ganglia (fig. S7).

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Thus, parasympathetic ganglion neurons are derivatives of multifated Schwann cell precursors, known to give rise in vivo to melanocytes (23) and endoneurial fibroblasts (24) in addition to Schwann cells. The neuronal potential of these precursors, reported in vitro or after back transplantation for sciatic nerve–derived cells (25, 26), is achieved during the normal development of cranial nerves. Accordingly, the numerous locations of parasympathetic ganglia throughout the head and trunk are specified by the pattern of cranial nerve outgrowth. A parsimonious way of hooking up pre- and postganglionic partners in development and, conceivably, of synchronizing their appearance in evolution.

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS
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Materials and Methods
Figs. 53 to S1
References (27–33)
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VESSEL FORMATION
De novo formation of a distinct coronary vascular population in neonatal heart

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The postnatal coronary vessels have been viewed as developed through expansion of vessels formed during the fetal period. Using genetic lineage tracing, we found that a substantial portion of postnatal coronary vessels arise de novo in the neonatal mouse heart, rather than expanding from preexisting embryonic vasculature. Our data show that lineage conversion of neonatal endocardial cells during trabecular compaction generates a distinct compartment of the coronary circulation located within the inner half of the ventricular wall. This lineage conversion occurs within a brief period after birth and provides an efficient means of rapidly augmenting the coronary vasculature. This mechanism of postnatal coronary vascular growth provides avenues for understanding and stimulating cardiovascular regeneration following injury and disease.

Coronary artery disease causes myocardial infarction, the leading cause of death worldwide. How coronary arteries develop is a fundamental biological question with important ramifications for human health and disease (1). Defining the developmental pro-

grams that give rise to the coronary arteries will provide critical information for regenerative approaches to congenital and adult heart disease (2–4). Most previous studies of coronary developmental origins have focused on the midgestation stage, when coronary vessels initially form
Supplementary Materials for

Parasympathetic ganglia derive from Schwann cell precursors


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This PDF file includes:

Materials and Methods
Figs. S1 to S11
References
Materials and Methods

Histology.

In situ hybridization, immunochemistry and wholemount immunochemistry have been described in (5).

Wholemount immunofluorescent staining was adapted from (27). The embryos at stage E10.5 were fixed overnight in 4% paraformaldehyde (in PBS), serially dehydrated in methanol, and frozen at -80°C. The embryos were freeze-thawed five times, rehydrated, and blocked overnight at 4°C in blocking buffer (5% DMSO, 0.3% Triton X100, 5% FCS in PBS). Primary antibodies were applied in blocking buffer for 3 days at 4°C. Embryos were washed 5 times in blocking buffer at room temperature, and secondary antibodies were then applied for 2 days at 4°C. Finally, embryos were washed 5 times in blocking buffer at room temperature.

Embryos were cleared following the 3DISCO protocol (27), and imaged with an Ultramicroscope (light sheet microscopy, LaVision BioTec), or cleared in 80% glycerol/PBS, and imaged using a SP5 confocal microscope (Leica). 3D reconstructions were performed using the IMARIS imaging software.

Immunofluorescence on sections was performed on embryos fixed for 3 hours in 4% paraformaldehyde (in PBS). For cryostat sections, embryos were embedded in a 15% sucrose/7.5% gelatine mix, frozen at -80°C and sectioned (12µm) using a Leica Cryostat. Primary staining was performed overnight on sections in a PBS-Triton-FCS solution (PBS, 0.1% Triton, 20% FCS). The slides were washed three times, 10 minutes in PBS-Triton (0.1% triton), and secondary staining was performed in PBS-Triton-FCS, 2 hours at room temperature. Slides were mounted with Fluoromount (Sigma) and analyzed with a SP5 Leica confocal microscope. For floating sections, embryos were embedded in 3% agarose-PBS, and cut using a Leica Vibratome. Sections (200µm) were collected in PBS. Staining was performed in washing buffer (PBS, 1% BSA, 0.1% TritonX100, 0.05% SDS). Primary staining was performed overnight. Embryos were washed 3 times for 1 hour in washing buffer and secondary antibodies were applied overnight. Sections were cleared in 80% glycerol/PBS, and imaged using a SP5 Leica confocal microscope.

The antibodies used are listed below. Antigen retrieval, by boiling for 10 minutes in sodium citrate (10mM) was needed for optimal labeling with the anti-Islet antibody.

Probes

The probes for Cadherin19 and PLP-1 (cDNA from Source Bioscience, clones #IRCKp5014H0217Q and #IRAvp968G0365D, respectively) were synthesized following the distributor’s information. The other probes used were peripherin, Ascl1, Neurog2, Tbx20, and βIII-tubulin.
Antibodies

The following primary antibodies were used for immunochemistry and immunofluorescent staining.

- anti-2H3 (NF), Mouse, 1/500, Hybridoma Bank (#2H3)
- anti-bIII Tubulin (TuJ1), Mouse, 1/500, Covance (#MMS-435P)
- anti-bgal, Rabbit, 1/400, Cappel (#55976)
- anti-bgal, Goat, 1/400, Serotec (#4600-1409)
- anti-dsRed, Rabbit, 1/500, Clontech (#632496)
- anti-ErbB3, Guinea Pig, 1/2500, (gift of T. Müller and C. Birchmeier)
- anti-FoxD3, Guinea Pig, 1/100, (gift of T. Müller and C. Birchmeier).
- anti-GFP, Chicken, 1/500, Aveslab (#GFP-1020)
- anti-Islet1/2, Mouse, 1/400 (40.2D6 and 39.4D5, Hybridoma Bank)
- anti-NeuN, Rabbit, 1/250 Millipore (#ABN78)
- anti-Oct6, Rabbit, 1/1000, (gift of M. Wegner).
- anti-p75, Rabbit, 1/100, Promega (#G3231).
- anti-Phox2b, Rabbit, 1/500
- anti-Phox2b, Guinea Pig, 1/500
- anti-S100, Rabbit, 1/100, Dako (#Z0311)
- anti-Sox10, Goat, 1/250, Santa Cruz (#SC-17342)
- anti-Sox2, Goat, 1/200, Santa Cruz (#SC-17320)

The following secondary antibodies were used:

- anti-rabbit Cy3, 1/500, Jackson Immunoresearch Laboratories (#711-165-152)
- anti-rabbit A488, 1/500, Jackson Immunoresearch Laboratories (#711-545-152)
- anti-goat Cy3, 1/500, Jackson Immunoresearch Laboratories (#705-165-003)
- anti-goat Cy5, 1/500, Jackson Immunoresearch Laboratories (#705-605-003)
- anti-rabbit Cy3, 1/500, Jackson Immunoresearch Laboratories (#711-165-152)
- anti-guinea pig Cy3, 1/500, Invitrogen (#A-11073)
- anti-chicken A488, 1/500, Jackson Immunoresearch Laboratories (#103-645-155)
- anti-mouse Cy3, 1/500, Jackson Immunoresearch Laboratories (#715-165-150)
- anti-mouse A488, 1/500, Invitrogen (#A-21202)
- anti-mouse Cy5, 1/500, Jackson Immunoresearch Laboratories (#715-175-150)
Mouse strains.

The following mouse strains were used in this study:

**Neurog2 KO (8):** knock in of CreERT2 into the Neurog2 locus, allowing the expression of a tamoxifen inducible Cre from the Neurog2 promoter and creating a null allele

**Neurog1 KO (28):** null allele of Neurog1

**Wnt1 ::Cre (29):** transgenic line expressing Cre under the control of the 3’ enhancer of Wnt1

**Phox2b ::Cre (13):** BAC transgenic line expressing Cre under the control of the Phox2b promoter

**Phox2b ::Flpo (20):** BAC transgenic line expressing FLP recombinase under the control of the Phox2b promoter

**Rosa^{tdT} (Rosa^{lox-stop-lox-tdTomato}) (30):** knock in line expressing the reporter gene tdTomato from the Rosa locus in a Cre-dependent manner

**Ptk::Cre (31):** transgenic line expressing Cre in the germ line

**RC::Fela (21):** double-responsive indicator line expressing GFP after FLP-mediated recombination and LacZ after FLP- and Cre-mediated recombination under the control of the CAG promoter in the Rosa locus.

**Phox2aASIC2a:** knock in line expressing the toxic G430 mutant of the ASIC2a cation channel (described in fig. S4). To genotype the Phox2aASIC2a line the following primers were used:

- **Forward:** GCACCCCTGGAGGAGATTGCCTG
- **Reverse:** CTTTAGGTATTTCCGCTCCCCTGC

For the Tamoxifen induction of the Neurog2-Cre^{ERT2} inducible line, a mix of Tamoxifen (10 mg/ml, Sigma, T5648) and Progesterone (5mg/mL, Sigma, P3972) was prepared in corn oil (Sigma, C-8267) at 37°C (protected from light) until completely dissolved. This solution (10 µl per gram of body weight) was administered by intragastric gavage to pregnant female at 8.5 or 10.5 days of gestation.
Absence of the petrosal ganglion and atrophy of the nodose ganglion in Neurog2 KO

Immunofluorescence for Phox2b and Tubb3 on sagittal sections at equivalent levels of a wild type (left panel) and Neurog2 KO (right panel) embryo at E11.5. Inset: Phox2b labeling of JN-associated cells. In the mutant both the petrosal (P) and geniculate (G) ganglia are absent while the nodose ganglion (N) is merely atrophic (N*). Consequently, all parts of the glossopharyngeal nerve are missing, including Jacobson’s nerve (JN) and its associated Phox2b+ cells. OV: otic vesicle
Precursors of parasympathetic ganglia never express Neurog2.

Native tdTomato fluorescence combined with immunofluorescence for Phox2b and Islet1 on parasagittal sections of E13.5 Neurog2\textsuperscript{CreERT\textsubscript{2};Rosa\textsuperscript{tdT}} embryos treated with tamoxifen at E8.5 (left panels) or E10.5 (right panels). Neurog2 is expressed in precursors of the somatic sensory neurons in dorsal root ganglia and cranial visceral sensory neurons, as previously described (7, 32, 33), but neither in those of cranial somatic sensory neurons (trigeminal, jugular and superior ganglia), as previously described (28), nor in precursors of parasympathetic ganglia (sphenopalatine and otic) and the enteric nervous system, as shown here. DRG: dorsal root ganglia; ENS: enteric nervous system; G: geniculate ganglion; J/S: fused jugular and superior ganglia; O: otic ganglion; P/N: fused petrosal and nodose ganglia; Sp: sphenopalatine ganglion; T: trigeminal ganglion. Asterisk: blood vessel with blood cells. Scale bar: 100µm.
Neurog1/2 double knock-outs lack all sensory ganglionic neurons in the head.

Combined immunofluorescence for Phox2b and Islet1/2 on parasagittal sections through the head of wild type (WT) and Neurog1/2 double knock-out embryos at E13.5 showing the lack of trigeminal (T), geniculate (G), petrosal/nodose (P/N) and jugular/superior (J/S) ganglia. Scale bars: 100µm.
Structure of the *Phox2a*<sup>ASIC2a</sup> locus.

The *Phox2a*<sup>ASIC2a</sup> allele was created by homologous recombination in ES cells. A cDNA for rat ASIC2a harboring the G430F mutation (11) was inserted between the first and second exon of the *Phox2a* locus, preceded by a floxed EGFP-Neo-STOP-IRES cassette, itself preceded by a splice acceptor site. Upon Cre-mediated recombination *ASIC2a* is transcribed from the *Phox2a* promoter and translated from its own ATG.
The Phox2a<sup>ASIC2a</sup> allele kills visceral sensory and branchio-visceral motor neurons.

Transverse section through the head of E11.5 Pgk::Cre;Phox2a<sup>ASIC2a</sup> embryos treated for in situ hybridization for Tbx20 (two upper panels) or immunohistochemistry for Phox2b (four lower panels). In the hindbrain of Pgk::Cre;Phox2a<sup>ASIC2a</sup> embryos only the youngest post-mitotic precursors of the facial nucleus (dark blue arrowhead) are detected, as they switch on Phox2a (thus ASIC2a), but neither the older, caudally migrating ones (black arrowhead), nor the superior salivatory nucleus cells born in r6 (light blue arrowhead). The geniculate (G) and the nodose (N) ganglia are missing. ICA: internal carotid artery; OV: otic vesicle; T: trigeminal ganglion.
Parasympathetic ganglia form then degenerate in Wnt1::Cre;Phox2a^{ASIC2a} embryos.

Transverse section through the trunk of embryos with the indicated genotypes stained with a Phox2b antibody. Sympathetic ganglia, visible in wild type and Wnt1::Cre E13.5 embryos, are deleted in Wnt1::Cre;Phox2a^{ASIC2a} embryos at E13.5, while parasympathetic ganglia are present in Wnt1::Cre;Phox2a^{ASIC2a} at E13.5 but have degenerated by E14.5. A: dorsal aorta; B: bronchi; E: esophagus; C: cardiac ganglia; SC: sympathetic chain.
Parasympathetic precursors line several cranial nerves at midgestation

Parasagittal sections through embryos stained by immunofluorescence for Phox2b, Sox10 and Tubb3 at the indicated stages. (A,B) Phox2b<sup>+</sup>/Sox10<sup>+</sup> cells occupy the greater superficial petrosal nerve (GSPN), a branch of the facial nerve that innervates the sphenopalatine parasympathetic ganglion, as early as E11.5 (A). At E13.5, as Phox2b expression has become exclusive of that of Sox10, the cells have accumulated distally in a smooth proximo-distal gradient to form the anlage of the ganglion (B). (C) A mixture of Sox10<sup>−</sup>/Phox2b<sup>−</sup> and Sox10<sup>+</sup>/Phox2b<sup>+</sup> cells coat the vagus nerve (X) at E11.5 and Sox10<sup>−</sup>/Phox2b<sup>−</sup> cells have invaded two of its cardiac branches (cardiac nerve, CN). (D) At E12.5 these cells increase in numbers and switch on Phox2b. (E) At E13.5 they have formed a cardiac ganglion (CG). Scale bar: 100µm (A,B); 80µm (C-E)
Parasympathetic ganglia derive from the neural crest
Sections through an E13.5 embryo where the RC::Fela reporter has been recombined with a FLP deleter allele and a Wnt1::Cre and labeled by immunofluorescence for Phox2b and nlsLacZ: the vast majority of parasympathetic ganglionic cells (here in the otic ganglion, left panel) have undergone Cre-mediated recombination, thus have a history of Wnt1 expression in line with their origin in the neural crest, just like enteric ganglionic neurons (middle panel) and sympathetic ganglionic neurons (right panel).
Expression status of neuronal markers and proneural bHLH factors by precursors of parasympathetic neurons

Immunofluorescence (A,B) or combined immunohistochemistry and in situ hybridization (C-J) for the indicated markers. (A-C) Phox2b⁺ cells that line Jacobson’s nerve (JN) do not express the neural markers NeuN (A) (unlike placodal-derived sensory neurons of the geniculate ganglion (G), lower panel) or Tubb3 (B,C) (lower panels: close up in (B), expression of Tubb3 in the nearby petrosal ganglion (P) as a positive control in (C)). (D-G) Parasympathetic precursors in Jacobson’s nerve or the greater superficial petrosal nerve (GSPN) do not express the bHLH proneural genes Ascl1 (D,E) or Neurog2 (F,G) but those that have reached the forming otic (O) or sphenopalatine (S) ganglia upregulate Ascl1 (D,E), but not Neurog2 (F,G). (H-J) At E13.5 expression of Ascl1 is even stronger (H,I) and the small ganglion along Jacobson’s nerve (See Fig.2 E-G) also expresses Ascl1 (arrowhead in J)
Cranial nerve-associated cells with a history of \textit{Phox2b} expression

Sections through the greater superficial petrosal nerve (A,C-E) or JN (B) at E16.5 showing the expression of tdTomato (from the \textit{Rosa}^{\text{dIIR}} reporter allele) triggered by \textit{Phox2b::Cre} (A-C) —revealed either with an anti-dsRed antibody (A,B) or detection of the native fluorescence (C)— or the expression of GFP (from the \textit{RC::Fela} reporter allele) triggered by \textit{Phox2b::FLPo} (D,E) and revealed with an anti-GFP, and stained by DAPI or the indicated antibodies. In (A) and (B) tdTomato expression (red) shows that a fraction of the cells that coat the nerve have expressed \textit{Phox2b} (arrowheads). The use of a different recombinase and different reporter show the same result in (D). The sections in (C) and (E) reveal nerve-associated cells from the \textit{Phox2b::Cre} or \textit{Phox2b::Flpo} lineage respectively, that still express Phox2b at this stage. Note that in (D) and (E) many GFP positive fibers are seen emanating from Phox2b\textsuperscript{+} viscerosensory and visceromotor fibers that project in the greater superficial petrosal nerve. Scale bars: 10μm.
Upper limb nerve-associated cells with a history of *Phox2b* expression

Sections through the upper limb at the indicated ages showing the presence of nerve associated cells with a history of Phox2b expression (yellow arrowheads in the three upper panels) that have switched on the Schwann cell precursor marker S100 at P7 (lower panels).
References and Notes


2.3 Discussion

a. Nerve-associated SCPs generate parasympathetic neurons in vivo

Schwann cell precursors (SCPs) are neural crest derivatives able to migrate long distances associated to peripheral nerves and are essential for motor and sensory neuron trophic support and nerve fasciculation (Jessen & Mirsky, 2005). They were previously thought to give rise only to non-neuronal cells in vivo. These included Schwann cells, endoneurial fibroblasts (Joseph et al., 2004), a subpopulation of melanocytes (Adameiko et al., 2009) and more recently a subpopulation of mesenchymal stem cells that produce pulp cells and odontoblasts during rodent tooth morphogenesis (Kaukua et al., 2014).

In this study we provide evidence that SCPs can generate neurons in vivo, a capacity that had been only reported in vitro (White et al., 2001; Morrison et al., 1999). Moreover, we show that cranial nerves that carry parasympathetic precursors are precisely those that project to the future ganglion, and thus specify the locations of their target ganglia in the body, a parsimonious way of circuit assembly. This mechanism differs from the one described for sympathetic ganglia derived from trunk neural crest cells (see Introduction), and suggests that parasympathetic ganglia, associated to more specialized and restricted territories than sympathetic ganglia, could have appeared later in evolution and used preformed structures for their guidance: cranial nerves carrying sensory information, and later preganglionic innervation, that in some cases at least is guided by sensory nerves (Coppola et al 2010; Outin et al, unpublished data).

Another study, published back to back to our own and using different genetic approaches, provided further evidence that parasympathetic neurons derive from SCPs (Dyachuk et al., 2014). However, some of their strategies deserve critical examination. First, the authors, using an Ascl1CreERT2 inducible line partnered to a Rosa26RTomato reporter line, claimed that parasympathetic precursors were found associated to nerves at E11.5 and E12.5 (Dyachuk et al., 2014, Fig.2F, I, J and Fig.3A and C). However, after detailed inspection of their figures, the only labelled cells are very close (greater superficial petrosal nerve in Fig.2F and 3C) or at the place of ganglion formation (in the case of the ciliary, otic and submandibular ganglia in Fig. 2I, J and 3A), in line with our results in which Ascl1 is not
expressed in migrating Phox2b+ precursors (Espinosa-Medina et al., 2014, Fig. S9). This is consistent with previous studies showing that parasympathetic ganglia form normally but later degenerate in Ascl1/KO mice (Hirsch et al., 1998). Moreover, the major group of Tomato+ cells described by the authors, also at the site of ganglion formation, are ciliary precursors that, unlike all other parasympathetic ganglia happen not to depend on their preganglionic nerve (the oculomotor nerve) (unpublished results, see Discussion-Part c).

Second, the authors took advantage of the selective elimination of certain nerves and their associated parasympathetic ganglia in Ret KO mice (GSPN-sphenopalatine ganglion and JN-otic ganglion) and the presence of others (CT-submandibular/lingual ganglia and oculomotor nerve-ciliary ganglion), as a proof that parasympathetic ganglia arise from nerve-associated precursors (Dyachuk et al., 2014, Fig S3). However, Ret was previously shown to be expressed by parasympathetic precursors (Enomoto et al., 2000) and post-migratory crest cells (Pachnis et al., 1993) and the phenotype observed could be due to a cell-autonomous effect. A more convincing evidence would required a control in which Ret is conditionally inactivated in neural crest cells. I have myself performed this experiment using a RetLox/Lox line (obtained from Dr. Enomoto in Japan) partnered with a Wnt1::Cre line to inactivate Ret in premigratory neural crest cells. My results show that parasympathetic precursors do not require Ret for their migration (Fig.8 A-B) or gangliogenesis (Fig.8 C-D), contrary to what was previously described (Enomoto et al., 2000), and thus the phenotype observed in Ret constitutive mutants is indeed due to the absence of specific nerve branches to which parasympathetic precursors associate. However, a later role of this signalling pathway cannot be excluded, as other Ret ligands such as Neurturin and its co-receptor GRFα2 have being proved essential in parasympathetic ganglia trophic support and late axogenesis (Enomoto, 2000; Rossi, 2000).
Figure 8. Parasympathetic precursors migrate and form ganglia in the absence of Ret signalling in neural crest cells. (A and B) E11.5 Whole Mount immunofluorescence showing various cranial nerves (2H3 staining in blue) and their associated SCPs (Sox10 staining in red) in both wild-type (A) and Wnt1::Cre;Ret\textsuperscript{Lox/Lox} (B) embryos. (C and D) E13.5 Phox2b immunohistochemistry showing the presence of all parasympathetic ganglia in both wild-type (C) and Wnt1::Cre;Ret\textsuperscript{KO} (D) embryos. OV: otic vesicle; VII: facial nerve; GSPN: greater superficial petrosal nerve; CT: corda tympani; JN: Jacobsons’ nerve; Cg: ciliary ganglion; Og: otic ganglion; Slg: sublingual ganglia; Smg: submandibular ganglion; Spg: sphenopalatine ganglion.

b. Migration mechanisms of parasympathetic precursors

The mechanism of migration of parasympathetic precursors is another example of axophilic migration, the best known being the migration of GnRH-1 neurons along olfactory axons (Wray, 2010) and the migration of SCPs along the lateral line axons in zebrafish (Gilmour et al., 2002). Focusing on the latter, several studies have pointed to the role of ErbB2 and ErbB3, which together form a heteromeric tyrosine kinase receptor for the ligand Nrg1-type III, as responsible for SCP migration, proliferation and myelination (Lyons et al.,
In mice mutants for ErbB2, ErbB3 or Nrg-1-Type III, SCPs failed to colonize peripheral nerves at early stages of development (Meyer and Birchmeier, 1995, 1997; Riethmacher et al., 1997; Morris et al., 1999; Woldeyesus et al., 1999). To provide evidence of the glial origin of parasympathetic neurons, Dyachuk et al. have analysed ErbB3 KO mice and shown a complete absence of parasympathetic ganglia at postnatal day 1 (Dyachuk et al., 2014, Fig 3K-P). However, this conclusion was risky because even if SCPs themselves express ErbB3 (Dyachuk et al., 2014, Fig.3; Espinosa-Medina et al., 2014, Fig. 3) ganglionic cells too, and the phenotype observed could be due to a late, i.e. perinatal, death of parasympathetic precursors. To test whether parasympathetic precursors require ErbB3/Nrg-1 signalling for their migration, I conditionally inactivated the ErbB3 ligand Nrg-1 in viscerosensory and motor neurons projecting to the site of ganglia formation using a P2b::Cre line (since both viscerosensory and visceromotor neurons express Phox2b) partnered to a Nrg-1Lox/Lox line (obtained from Dr. C.Birchmeier in Germany). My unpublished results demonstrate that the ErbB3/Nrg1 system is required for the migration of parasympathetic precursors (SCPs) to the site of ganglia formation. In the absence of ErbB3/Nrg-1, cranial nerves project along their usual path, but are completely empty of neural crest cells and defasciculate (See Chapter 3, Figure 16), consistent with previous studies in zebrafish (Gilmour et al., 2002).

Another mechanism implicated in parasympathetic gangliogenesis could be directed proliferation of the progenitors. I have tested this possibility by performing an EdU ‘click’ staining protocol (Figure 9) to look for dividing parasympathetic precursors along nerves. Preliminary data show that between E11.5 and E12.5 around 30% of precursors along the nerve and at the ganglion site proliferate (Figure 9, B and C). The lack of a proliferation gradient from the nerve to the ganglion anlage or of an increase in proliferation during early development suggests that proliferation is not the major mechanism of parasympathetic gangliogenesis. However, proliferation might be required after the migration of parasympathetic precursors and previous to differentiation, as previously demonstrated in the lateral line in zebrafish in vivo (Lyons et al., 2005). Finally, it would be interesting to explore the signals mediating parasympathetic neuron differentiation from a common pool of SCPs at particular locations. Target-derived BMPs might be involved, as previously described for ciliary gangliogenesis (Rohrer et al., 2002).
Figure 9. Proliferation of parasympathetic precursors. (A and B) Immunofluorescence against Tubb3 (nerve fibers in blue), Phox2b (pan-autonomic determinant in red) and EdU stain (S phase) on the Jacobson’s nerve at the indicated stages. Around 30% of total Phox2b+ cells along the nerve and the ganglion anlagen at both stages are EdU+. (C) Principle of EdU detection by ‘click’ reaction (B. Buck et al., 2008).

c. The case of the ciliary ganglion

The ciliary ganglion deserves special mention because, although it is historically the model of parasympathetic ganglia, it actually differs from all the others by many features. 

First, from an anatomical standpoint, it belongs to the parasympathetic ‘midbrain outflow’ and not to the ‘bulbar outflow’, unlike the others (Gaskell, 1885 and 1920; Langley, 1921). As first described by quail-chick transplants (Narayanan and Narayanan, 1978), it derives from the mesencephalic neural crest, whereas all other parasympathetic ganglia derive from the hindbrain neural crest (and the sacral neural crest, but see Chapter 4). It is innervated by fibers that emerge from the midbrain Edinger-Westphal nucleus and course in the oculomotor nerve, which exits the brain through a ventral root. In contrast, all other parasympathetic ganglia receive preganglionic innervation from fibers leaving the hindbrain.
through dorsal roots (except sacral ones, but see Chapter 4). This by itself could explain a different relationship of dorsally emerging neural crest cells with the oculomotor nerve from that of bulbar crest with hindbrain derived nerves. Moreover, ciliary preganglionic neurons of the Edinger-Westphal nucleus depend on Phox2a and not on its paralogue Phox2b, unlike all other preganglionic parasympathetic neurons (Pattyn et al., 1997) and therefore cannot be considered as standard cranial visceromotor neurons.

Second, from a physiological standpoint, the ciliary ganglion does not have a bona fide visceral i.e. homeostatic role like all other parasympathetic ganglia: it responds to light stimulation (a somatic sensation) through its connections in the optic tectum, causing pupillary constriction (Loewy, 1990; W. Neuhuber and F. Schrödl, 2011).

Third, unlike all other parasympathetic ganglia, it expresses the transcription factor Islet-1 (Huber et al., 2013).

Fourth, from a developmental standpoint, in contrast to all other parasympathetic ganglia and contrary to what has been claimed (Dyachuk et al., 2014, Fig. 2E-F), the ciliary ganglion does not depend on its associated nerves to form. Ciliary precursors migrate from the mesencephalic crest (unpublished results, Figure 12 A) and coalesce behind the optic vesicle before and independently from the oculomotor nerve (which carries the preganglionic innervation as well as motor innervation to extra-ocular muscles) (Figure 11 A). In the absence of the oculomotor nerve (in Pgk::Cre; Phox2aASIC embryos) or even in the absence of both the oculomotor and the trigeminal nerve that provides somatosensory innervation (in Neurogenin-1KO;Pgk::Cre;Phox2aASIC embryos), the ciliary ganglion is present (unpublished results, Figs. 10 and 11) (a small caveat is that we could not simultaneously destroy the abducens nerve, a somatic motor nerve).

Fifth, from a molecular standpoint, the conditional inactivation of ErbB3 in neural crest cells in Wnt1::Cre;ErbB3Lox/Lox embryos impaired the migration of mesencephalic derived ciliary precursors and correlativelly the ciliary ganglion was absent, although the oculomotor nerve was present (Fig.12, B, D and G). Thus, ErB3/Nrg1 signalling is essential for the formation of all parasympathetic ganglia, but in the case of the ciliary ganglion it acts in a non-nerve dependent manner. The most probable explanation would be that ciliary precursors express ErbB3 and are guided by target-derived Nrg1, thus it would be interesting to inactivate Nrg1 in mesenchymal cells to test if it results in the same phenotype as the one observed. This situation would liken the development of the ciliary ganglion to that of the sympathetic chain.
Figure 10. The ciliary ganglion forms in the absence of the oculomotor nerve. (A and B) E11.5 Whole Mount immunofluorescence showing various cranial nerves (2H3 staining in blue), neural crest cells (Sox10 staining in red) and differentiating ciliary precursors (Phox2b staining in green, arrows) in the indicated genetic backgrounds. In the absence of the oculomotor nerve (asterisk in B), ciliary precursors are present (B, arrow and inset). (C and D) E13.5 Phox2b immunohistochemistry showing the presence of the ciliary ganglion in both wild-type (C) and Pgk::Cre;Phox2aASIC (D) embryos (red arrowheads). IIIIn: oculomotor nerve, V: trigeminal nerve, OV: optic versicle, E: eye.
**Figure 11. The ciliary ganglion forms in the absence of the trigeminal and oculomotor nerves.** (A and B) E10.5 Whole Mount immunofluorescence showing various cranial nerves (2H3 staining in blue), neural crest cells (Sox10 staining in red) and differentiating ciliary precursors (Phox2b staining in green, arrows) in the indicated genetic backgrounds. In the absence of both the ophtalmic branches of the trigeminal nerve and the oculomotor nerve (asterisk in B), ciliary precursors are present (B, arrow). (C and D) E13.5 Phox2b immunohistochemistry showing the presence of the ciliary ganglion in both wild-type (C) and Ngn1KO; Pgk::Cre; Phox2aASIC (D) embryos. Although present, ciliary neurons seem atrophic and disorganized (higher magnification panel in D), but this phenotype was not always observed and is most probably due to the late death of Phox2a expressing neurons. IIIn: oculomotor nerve, V: trigeminal nerve, OV: optic versicle, E: eye.
Figure 12. Ciliary precursors depend on ErbB3 to migrate. (A-D) Whole Mount immunofluorescence showing various cranial nerves (2H3 staining in blue), neural crest cells (Sox10 staining in red) and differentiating ciliary precursors (Phox2b staining in green). Mesencephalic neural crest cells migrate in chains (arrows in A) and coalesce behind the optic vesicle (arrow in C) before projection of the trigeminal and oculomotor nerves in wild type embryos (A and C). Mesencephalic crest cells stall (arrow in B) and fail to coalesce behind the optic vesicle (asterisk in D) in Wnt1::Cre;ErbB3^{Lox/Lox} embryos. (F and G) Phox2b immunohistochemistry staining of ciliary neurons at E13.5 (F). The ciliary ganglion is absent in Wnt1::Cre;ErbB3^{Lox/Lox} embryos (red arrow in G). III: oculomotor nerve, V: trigeminal nerve, OV: optic vesicle, E: eye.
Chapter 3

Development of the enteric nervous system

3.1 Introduction

a. Anatomy and function of the enteric nervous system

The enteric nervous system (ENS), the intrinsic innervation of the gastrointestinal tract (GI), is the largest and most complex division of the autonomic nervous system. It controls gut motility, exchange of fluids, blood flow and secretion of gut hormones. The human enteric nervous system has around 500 million neurons (Furness, 2012) within at least 18 functional classes (Brookes, 2001). Enteric neurons form groups of interconnected ganglia in different layers of the gut specialized in various functions: the myenteric (Auerbach’s) plexus, between muscle layers, controls peristaltic movements whereas the submucosal (Meissner’s) plexus coordinates secretion, absorption and smooth muscle movements, but is absent in the oesophagus. The ENS is also composed of glial cells, actively involved in gastrointestinal neurotransmission, the maintenance of the mucosal barrier integrity and which may serve as a link between the nervous and immune systems (Rühl, 2005) (Figure 13). Enteric neurons can act autonomously to control gut motility, as shown by studies on extrinsically denervated intestines (Bayliss and Starling, 1899; Langley and Magnus, 1905; Furness et al., 1995). Because of this and of its richness in neuronal types, the ENS is often referred to as “the second brain” (Gershon, 1995).
The ENS also receives extrinsic sympathetic and parasympathetic innervation that play an essential role in controlling esophageal and gastric motility and coordinate activity between distant regions of the GI. Parasympathetic preganglionic fibers project from the dorsal-motor nucleus of the vagus nerve (dmnX) in the hindbrain to enteric ganglia in the oesophagus, stomach and small intestine through the vagus nerve (Xth cranial nerve) and from the sacral nucleus in the spinal cord to the colon and rectum through pelvic nerves (but see Chapter 4). Sympathetic fibers originate from abdominal prevertebral ganglia including the coeliac, superior and inferior mesenteric ganglia, and project mainly to the myenteric and submucosal plexuses, and the blood vessels (Uesaka et al., 2016 and see Chapter 4-Publication and Discussion).

Figure 13. Schematic representation of the enteric nervous system (reviewed in Rescigno, 2008; adapted from Furness and Costa 1980). Upper-half of a transverse section through the post-gastric ENS showing the myenteric and submucosal plexuses within different layers, their extrinsic innervation and presence of intrinsic glial cells.

b. Ontogeny of the enteric nervous system

Most of our understanding on the origin of the ENS comes from studies performed on the 1950s and 1970s on avian embryos. Pioneering neural crest ablation experiments in the chick (Yntema and Hammond (1954) and generation of chick-quail chimeras (Le Dourain and Teillet, 1973) demonstrated that the neural crest adjacent to somites 1-7, known as the “vagal neural crest”, gives rise to the majority of the ENS. Moreover, this and subsequent studies demonstrated that the sacral neural crest (derived from the neural tube posterior to somite 28)
also contributes enteric and glial cells to the hindgut (Le Dourain and Teillet, 1973; Burns and Le Dourain, 1998). The vagal and sacral ENS origin is conserved in mammals (Durbec et al., 1996; Kapur, 2000; Wang et al., 2011).

Colonization of the GI is an organized process that takes around 25% of the gestation period (Kapur et al., 1992; Wallace and Burns, 2005). Vagal neural crest cells emerge from the dorsal neural tube at embryonic day E8.5 in the mouse, migrate through the caudal branchial arches to the pharyngeal region and reach the foregut at E9-9.5 (Burns et Le Dourain, 1998; Anderson et al., 2006a). Then, vagal derived enteric neural crest cells (ENCC) colonize the gut rostrocaudally, a process that is completed by day E15.5. During the colonization of the small intestine, this region is closely juxtaposed to the proximal colon and, in mice, a sub-population of ENCC takes a transmesenteric short-cut by-passing the caecum to colonize the colon directly (Nishiyama et al., 2012). Sacral NC cells emerge from the neural tube at E9-9.5, migrate ventrally and form pelvic ganglia (Serbedzija et al., 1991; and see Chapter 4), and from E13.5 follow pelvic postganglionic nerves to colonize the hindgut (Wang et al., 2011). In the chick, sacral NC cells also form an extrinsic plexus, called the nerve of Remak, where they reside for some days before entering the hindgut (Burns and Le Dourain, 1998). Sacral NC cells colonize the hindgut after the arrival of their vagal counterparts, but do not rely on them for the invasion as shown after ablation of the vagal neural crest in the chick (Burns et al., 2000).

The migratory behaviour of ENCC has being extensively studied in explants of gut from mice or chick embryos (Duckenbrod and Epstein, 2005, 2007, 2009; Young et al., 2004b). These studies have shown that ENCC migrate in chains and, unlike trunk NC cells, do not retain the same neighbours as migration proceeds (Young et al., 2014). While the highly proliferative ENCC wavefront migrates into unpopulated gut regions, less proliferative rearguard cells populate already colonized regions by migrating non-directionally (Theveneau and Mayor, 2011). Initial size of the pre-enteric population is essential for ENCC migration as demonstrated after partial ablation of the neural crest that lead to reduced speed of the wavefront (Young et al., 2001) and distal agangliosis of the GI tract (Burns, 2000; Barlow, 2008; Druckenbrod and Epstein, 2005), which could be relevant to the common developmental defect observed in Hirschsprung disease (see below). The previous studies mostly focused on the colonization of the distal bowel, but the early migration from the neural tube to the foregut is still poorly understood.
c. **Signalling pathways involved in enteric nervous system development**

Development of the ENS involves various processes including migration, proliferation, survival and glial and neuronal differentiation, which are regulated by diverse molecular mechanisms.

The major and best-studied signalling pathway required for ENS development is the GDNF-GFRα1/Ret signalling pathway. The tyrosine kinase receptor Ret and its co-receptor GFRα1 are expressed by ENCCs (Pachnis et al., 1993; Enomoto et al, 1998) whereas their ligand GDNF is expressed by the gut mesenchyme (Trupp et al., 1995). Ret signaling supports ENS precursor survival, proliferation, migration, differentiation, and neurite growth (Lake and Heuckeroth, 2013). Moreover, mutations in *Ret* are associated with the vast majority of cases of Hirschsprung disease, a frequent developmental disorder of the ENS resulting in intestinal agangliosis over variable lengths, usually distal, and consequent megacolon upstream of the deganglionated segment (Emison et al., 2010). In mice mutants for Ret, GDNF or GFRα1, ENCCs fail to colonize the gut beyond the oesophagus and stall at the proximal stomach (Heanue and Pachnis, 2007). Of notice, the oesophageal nervous system appears only partially dependent on Ret/GDNF signalling; *Ret* KO mice display an atrophic but not absent oesophageal nervous system (Durbec et al., 1996; Yan et al., 2004), in *GFRα1* KO small number of neurons were found in the stomach (Cacalano et al., 1998) and in *GDNF* KO embryos neurons were found in the oesophagus and stomach walls (Sanchez et al., 1996). This suggests developmental differences between the oesophageal and post-oesophageal nervous systems, which have not been explored so far (see 3.1.d).

Another signalling pathway involved in enteric development is the endothelin-3 (EDN3)-endothelin B G-protein-coupled receptor (ET-BR) pathway, EDN3 being expressed by the gut mesenchyme and ET-BR by ENCCs and some mesenchymal cells (Barlow et al., 2003; Leibl et al., 1999; Lee et al., 2003a). This pathway acts downstream of Sox10 during
the colonisation of the gut (Stanchina et al., 2006) and it is involved in progenitor-state maintenance and in ENCC migration (Nagy and Goldstein, 2006; Sidebotham et al., 2002). Mice mutants for EDN3 or ET-BR display aganglionic megacolon (Hosoda et al., 1994; Lee et al., 2003). In humans, isolated mutations in EDN3 or its receptors are associated with a very small number of cases of Hirschsprung disease (Alves et al., 2013), although interactions between mutations in Ret and EDNRB are central to the genesis of the disease (McCallion AS et al., 2003).

In addition to the molecules triggering the colonization of the GI, the Slit/Robo signalling pathway has been suggested to prevent trunk NCC from entering the gut (Zuhdi et al., 2015). Secreted Slit proteins are repulsive axon guidance cues that act through Robo transmembrane receptors and have been implicated in trunk neural crest repulsion (De Bellard, 2003). Loss-of-function experiments using a dominant-negative form of the Robo receptor expressed by chick trunk NCC showed that in the absence of Slit signalling, sympathoadrenal precursors (NCC derivatives from somite levels 18-24) are able to colonize the gut, but not sympathetic NCC at more rostral levels (Zuhdi et al., 2015). The authors of this study concluded that Slit molecules act to prevent sympathoadrenal precursors from entering the gut, whereas at more rostral trunk levels, NCC are repulsed by other factors. However, their analysis of Slit (1-3) mice mutants has limitations. The authors used the general neural crest marker Sox10 to visualize enteric precursors and reported the presence of positive cells exclusively in the gut mesenchyme of Slit null mutants at mid-trunk levels, which were absent in the WT (Zuhdi et al., 2015, Figure 5 A-C). However, Sox10 is also expressed by vagal NCC (Anderson et al., 2006), which in normal conditions colonize the gut before the embryonic stage analysed by Zuhdi et al (2015) (E12.5), who did not provide any explanation for the total absence of Sox10+ cells in the gut of WT embryos. Moreover, the authors claimed that Slit mice mutants presented larger sympathetic ganglia than the WT, but did not provide any quantification. Thus, further evidence is necessary to prove the role of Slit/Robo during enteric nervous system formation in mammals, and the role of other molecules that prevent the migration of NCC caudal to somite 7.

d. The colonization of the oesophagus.

The oesophageal nervous system (OENS) is the least well-studied region of the ENS, surprisingly since in the current scenario of one rostral-to-caudal invasion of the gut wall, it represents the first stage, on which all downstream events presumably depend. As mentioned
before, several mutant backgrounds lack enteric ganglia only distal to the stomach while oesophageal neurons are spared or only partially affected, suggesting a different developmental mechanism for the most rostral portion of the ENS.

To our knowledge, only three studies have addressed specifically the formation of the OENS. The first one (Durbec et al., 1996), using whole mount neural crest labelling and lineage tracing with DiI injection in mice, proposed that the vagal neural crest is subdivided in two domains: the vagal neural crest proper (somite level 1-5), which would contribute neurons to the entire GI tract as well as the superior cervical ganglion (SCG, but see critical examination in Introduction-1.3.b), and the upper trunk neural crest (somite level 6-7), known origin of sympathoblasts, which would colonize the oesophagus specifically (and contribute to dorsal root ganglia). As this partition parallels the known pattern of dependence on Ret of the ENS and sympathetic chain, the authors speculated that the vagal neural crest is Ret dependent whereas the “trunk” contingent is not, without actually testing this hypothesis on Ret KO mice. Their conclusion was incorporated in the 1999 edition of the Neural Crest (Le Dourain and Kalcheim), who subsumed both populations under the general label of “vagal crest” (somite level 1-7).

The second study, using chick-quail chimeras, defined in greater detail the contribution of NCC from specific axial levels of the neural tube to the ENS (Burns et al., 2000). Their results show a rostro-caudal gradient whereby the most anterior vagal crest (somite level 1-2) give rise to pre-umbilical enteric neurons (being more numerous in the oesophagus and sparse in the pre-umbilical intestine) while the most caudal vagal crest (somite level 6-7) form post-umbilical ganglia only. When grafts where performed at the intermediate levels of somite 3-5, NCC formed enteric ganglia along the entire gut. These conclusions resonated with the results of a third (prior) study, by Epstein et al. (1994), who performed fate-mapping studies in the chick by injecting replication-deficient virus containing the reporter LacZ into somites at the time of neural crest migration. They proposed that NCC from somite levels 3-6 were the major source of enteric precursors whereas when somites 1 and 2 were injected, positive LacZ cells were present exclusively in the foregut, including the oesophagus. However, they found very few cells at the level of the oesophagus compared to Burns et al. (2000) and the explanation proposed by both authors was that only a small percentage of NCC was labelled by the virus, the injection being performed after most NCC had already migrated passed the somites.
The results of both studies in chicken are in direct contradiction to the previous study in mice (Durbec et al., 1996), and a difference between species is the only explanation suggested by the authors. This illustrates that the formation of the OENS is not well understood.

My unpublished results point to the existence of two distinct populations of neural crest making up the “vagal neural crest”, born at different rostro-caudal levels, following different migratory routes and endowed with different fates. They resolve previous contradictions between mice and chicken on the origin of the oesophageal nervous system.
Dual origin of enteric nervous system in vagal Schwann cell precursors and cervical sympathetic-like crest

by I. Espinosa-Medina and J.-F. Brunet
a. Materials and Methods

Histology

In situ hybridization and immunochemistry have been described (Coppola et al., 2010). Immunofluorescence on cryostat or vibratome sections was performed as previously described (Espinosa-Medina et al., 2014). Wholemount immunofluorescent staining using the 3DISCO method was adapted from Ertürk et al., 2012 as previously described (Espinosa-Medina et al., 2016). Wholemount immunofluorescent chicken chimeras were cleared using ScaleA2 solution as previously described (Hama et al., 2012).

Antibodies and probes

α-2H3 (NF), Mouse, 1:500, Hybridoma Bank (#2H3)
α-bIII Tubulin (Tuj1), Mouse, 1:500, Covance (#MMS-435P)
α-Phox2b, Rabbit, 1:500 (Pattyn et al., 1997)
α-Phox2b, Guinea Pig, 1:500 (Dubreuil et al., 2009)
α-Sox10, Goat, 1:250, Santa Cruz (#SC-17342)
α-βgal, Rabbit, 1:400, Cappel (#55976)
α-HuC/D Mouse, 1:200, Invitrogen (MABN153)
α-GFP, Chicken, 1:500, Aveslab (#GFP-1020)
α-HNK-1, Mouse, 1:50, Hybridoma Bank (#3H5)

α-rabbit Cy3, 1:500, Jackson Immunoresearch Laboratories (#711-165-152)
α-rabbit A488, 1:500, Jackson Immunoresearch Laboratories (#711-545-152)
α-goat Cy3, 1:500, Jackson Immunoresearch Laboratories (#705-166-147)
α-goat A647, 1:500, Jackson Immunoresearch Laboratories (#705-606-147)
α-mouse Cy3, 1:500, Jackson Immunoresearch Laboratories (#715-165-150)
α-mouse A488, 1:500, Invitrogen (#A-21202)
α-guinea pig Cy3, 1:500, Invitrogen (#A-11073)
α-chicken A488, 1:500, Jackson Immunoresearch Laboratories (#103-545-155)

Immunohistochemical reactions were processed with the Vectastain Elite ABC kits (PK-6101 and PK-6012; Vector Laboratories) as indicated by the manufacturer and colour development was performed using DAB (3,3’-Diaminobenzidine).

The following probes were used:

The probes for Cadherin19 and PLP-1 (cDNA from Source Bioscience, clones #IRCKp5014H0217Q and #IRAVp968G0365D, respectively) were synthesized following the distributor’s information. The other probe used was Peripherin (obtained from M.M.Portier, Collège de France).

Transgenic Mouse lines

-Phox2b::Cre (D’Autréaux et al., 2011): BAC transgenic line expressing Cre under the control of the Phox2b promoter.

-Phox2b<sup>LacZ/+</sup> (Pattyn et al., 1999): Knock in line expressing the reporter gene LacZ from the second exon of the Phox2b locus, which is disrupted and lead to a null phenotype in Phox2b<sup>LacZ/LacZ</sup> embryos.
-Ret<sup>CFP CRE</sup> (Uesaka et al., 2008): Knock in line comprising floxed human Ret9 cDNA and CFP reporter in the first exon of the mouse Ret locus. Activation of Cre recombinase results in the removal of floxed Ret9, generating a CFP-knockin (Ret-null) allele.

-Wnt1::Cre (Danielian et al., 1998): transgenic line expressing Cre under the control of the 3' enhancer of Wnt1.

-Pgk::Cre (Lallemand et al., 1998): transgenic line expressing Cre in the germ line.

-Phox2<sup>ASIC2a</sup> (P2aASIC) (Espinosa-Medina et al., 2014): Knock in line expressing the toxic G430 mutant of the ASIC2a cation channel under the control of Phox2a promoter.

-ErbB3<sup>Lox/Lox</sup> (Sheean et al., 2013): Knock in line comprising floxed human ERBB3 cDNA in the exon 12 of the mouse ErbB3 locus. Activation of Cre recombinase results in the removal of floxed ERBB3, generating a ErbB3-null allele, which lacks the same coding sequences as the previously described ErbB3<sup>Δ</sup> allele (Riethmacher et al. 1997).

-Hrg<sup>α</sup> (Nrg1<sup>Lox/Lox</sup>) (Li et al., 2002): A floxed allele of the Nrg-1 gene containing loxP sites flanking exons 7 (containing sequence alterations), 8 and 9 was generated. Activation of Cre recombinase results in the removal of floxed Nrg1, generating a Nrg-1 null allele.

-Neuregulin<sup>ΔEGF-lacZ</sup> (Nrg LacZ/+) (Meyer et al., 1997; Meyer and Birchmeier, 1995): Knock in line expressing the reporter gene LacZ from the exon of the Neuregulin1 locus.

**Generation of chicken chimeras**

Transgenic chicken expressing the GFP reporter ubiquitously (McGrew et al., 2004) were obtained from the Roslin Institute (University of Edinburgh). Chicken chimeras were generated via transplantation of discrete segments of the neural tube, including the neural crest, from GFP+ donors to WT hosts as previously described (Delalande et al., 2015).

All animal studies were done in accordance with the guidelines issued by the French Ministry of Agriculture and have been approved by the Direction Départementale des Services Vétérinaires de Paris.

**Statistical analyses**

Quantification of oesophageal neurons and measurements of the surface occupied by enteric neurons in the post-gastric ENS were performed by use of FIJI software. The similarity of variances between each group of data was tested using the $F$ test. Statistical analysis was performed using unpaired two-tailed $t$ test. Results are expressed as %mean/WT ± SEM.
b. Results

i. Presence of enteric precursors along the vagus nerve in Ret and Phox2b mutants.

Several genetic backgrounds completely block the formation of the post-gastric enteric nervous system (ENS) while preserving, to an extent, or for a while, the oesophageal nervous system (OENS). Examples include Ret and Phox2b null mice (Durbec et al., 1996; Pattyn et al., 1999).

I re-examined the differential colonization the OENS and post-gastric ENS in those mutants, on whole mounts and transversal sections of Ret and Phox2b mutant embryos at E10.5, stained for the general NC marker Sox10 and the neurofilament marker 2H3 (Figure 14). As previously demonstrated, enteric precursors were absent from the post-gastric digestive tube of Ret and Phox2b mutants (Figure 14: d’, f’), whereas OENS precursors were present (Figure 14: c’, e’).

A striking feature of both phenotypes was that the region colonized by enteric precursors was coextensive with the vagus nerve (Xth cranial nerve) down to the future stomach (Figure 14, A-I). I confirmed that the neural crest cells (NCC) in the oesophagus were enteric precursors by co-labelling them for the reporter CFP in the RetCFP knock-in line or for the reporter LacZ in the Phox2bLacZ knock-in line, and for the general NC marker Sox10, demonstrating that these cells were double-positive (Figure 14, insets-c’, e’).

ii. The oesophageal nervous system depends on the vagus nerve that carry Schwann cell precursors.

The previous results suggested that the vagus nerve itself (instead of Ret or Phox2b) might be playing a role in the migration of oesophageal precursors, just like cranial nerves do of parasympathetic precursors (Espinosa-Medina et al., 2014). Evocative of such a mechanism, the Sox10+ and Phox2b+ cells that covered the vagus nerve co-expressed the Schwann-cell precursors (SCPs) markers Cadh-19 and PLP-1 (Figure 20). I also found enteric precursors co-expressing these markers in the walls of the oesophagus, which might have detached from the nerve to reach their final positions (Figure 20, insets in B and C).
To test whether the vagus nerve was required for OENS formation, I genetically damaged the nerve by using the same strategy as in Espinosa-Medina et al., 2014 (Figure 1G), i.e. the expression of the toxic allele of the sodium channel ASIC2a expressed from the promoter of Phox2a. When we recombined the Phox2a^{ASIC2a} line with a P_{gk}^{::Cre} line (where Cre is expressed in the germline), both viscerosensory and visceromotor neurons were absent and the vagus nerve was reduced to a vestigial ramus (Espinosa-Medina et al. (2014), Figure 1G; and this work, Figure 15 B, white arrowhead). At E11.5, NCC associated with the vagus nerve in the P_{gk}^{::Cre};Phox2a^{ASIC2a} mutant were massively reduced (Fig.15 B), and correlatively, the OENS was atrophied (Fig.15 D) by 36%, as shown by quantitative analysis of OENS neurons at E13.5 (Fig.15, E). To ensure that this was not due to a cell-autonomous effect, I selectively recombined the toxic ASIC2a channel in NCC by using a Wnt1^{::Cre} line crossed to the Phox2a^{ASIC2a} line. In this mutant background, OENS neuronal numbers were similar to the WT (Figure 15, E), most likely because Phox2a is not yet switched on in enteric neurons at this stage (Pattyn et al., 1997).

Thus, part of the OENS arises from SCPs and depend on the vagus nerve, like parasympathetic ganglia do of the vagus and other cranial nerves (Espinosa-Medina et al., 2014).

iii. **Axonal Neuregulin-1 is necessary for the migration of parasympathetic and oesophageal precursors along their associated cranial nerves.**

The migration of SCPs along peripheral nerves depends on the Nrg1/ErbB3 signaling (Meyer and Birchmeier, 1995, 1997; Riethmacher et al., 1997; Morris et al., 1999; Woldeyesus et al., 1999). Because parasympathetic neurons derive from SCPs and ErbB3 null mice lack parasympathetic ganglia at postnatal stages (Dyachuk et al., 2014) (see Discussion-b in Chapter 2 of this manuscript), it is likely that Nrg1 mediates the migration of parasympathetic precursors along their associated cranial nerves. Similarly, OENS precursors could be guided by the vagus nerve to the oesophagus. To test this hypothesis, I conditionally inactivated Nrg1 in neurons projecting their axons in cranial nerves by using a Phox2b^{::Cre};Nrg1^{lox/lox} line (for a more detailed explanation see Discussion-b, Chapter 2) in which I analyzed the migration of parasympathetic and OENS precursors along their associated nerves by co-staining E11.5 mutant and WT embryos for Sox10, Phox2b and 2H3, and looked...
for the presence of parasympathetic ganglia and OENS neurons at E13.5 by in situ hybridization using the neuronal marker Peripherin and the autonomic marker Phox2b.

My results show that the ErbB3/Nrg1 system is required for the migration of parasympathetic precursors (SCPs), to the site of ganglia formation. In the absence of ErbB3/Nrg-1, cranial nerves project along their usual path, but are completely empty of neural crest cells and defasciculate (Figure 16-D and I), consistent with previous studies in zebrafish (Gilmour et al., 2002). Moreover, in this mutant background parasympathetic ganglia do not form (Figure 16- E, F and J), demonstrating that the absence observed by Dyachuck et al. at postnatal stages in constitutive ErbB3 null mice (Dyachuk et al., 2014, Fig.16) was due to a migration defect rather than the late death of parasympathetic neurons. Similarly, OENS precursors associated to the vagus nerve were massively depleted in Phox2b::Cre;Nrg1\textsuperscript{lax/lax} mutants (Figure 16-L and N) and correlatively, the OENS was later atrophied by 46% (Figure 16-Graph). To exclude a cell-autonomous effect (enteric precursors being themselves Phox2b+), I verified that OENS precursors do not express Nrg1 by lineage tracing using a Nrg1\textsuperscript{LacZ/+} reporter line (embryos obtained from Dr. C. Birchmeier in Germany) (Figure 21), and that normal OENS numbers were present when Nrg1 was specifically inactivated in NCC in Wnt1::Cre;Nrg1\textsuperscript{lax/lax} mutants (Figure 16-Graph).

Thus, all parasympathetic ganglia and about half of the OENS require the Nrg1 ligand expressed on fibers of Phox2b+ neurons projecting in their associated cranial nerves.

iv. **Nerve-independent, cervical sympathetic-like neural crest cells contribute enteric neurons to the whole gastrointestinal tract.**

In contrast with the OENS, the post-gastric ENS was unaffected by either damage to the vagus nerve (Pgk::Cre;Phox2a\textsuperscript{ASIC2a}) or the block of Nrg1 signalling from the vagus nerve (Phox2b::Cre;Nrg1\textsuperscript{lax/lax}) at E13.5 (Figure 22) and, as previously shown, about half of the OENS was still present in these mutant backgrounds. This suggested that a second population invades the gut in a nerve-independent way.

To gather evidence for this, I analysed the migration of NCC at the earliest stages of enteric colonization (between E9.5-E10 in the mouse) in wild type embryos. My results showed a population of NCC, ventromedial and caudal to the incipient vagus
nerve and its associated SCPs, that seemed to invade the foregut in continuity with the upper sympathetic chain anlagen, at cervical levels (Figure 17). This cell population was previously noticed by Durbec et al., (1996), who showed, by in situ hybridization using a c-Ret probe in E9- E10.5 WT embryos, a transiently common pool of Ret+ progenitors in the mesenchyme between the dorsal aorta and the foregut, which would then split in two groups and contribute cells to the SCG and the ENS (Durbec et al., 1996, Figure 5). However, their interpretation on the contribution to the enteric nervous system differs from ours, as will be discussed later.

The sympathetic neural crest migrates independently of its preganglionic nerves (Yntema and Hammond, 1954), under the influence of Nrg1, expressed by the mesenchyme (Saito et al., 2012). As a consequence the sympathetic chain is massively atrophic in ErbB3 knockouts ((Britsch S. et al., 1998) and Figure 23). In line with a common origin of part of the ŒNS and the post-gastric ENS with the sympathetic chain, I found that both were depleted by about 70% in Wnt1::Cre;ErbB3lox/lox mutants (Figure 18), in contrast to an earlier suggestion that there was no ENS phenotype in ErbB3 nulls (Britsch S. et al., 1998).

Thus, nerve-dependent OENS precursors rely on axonal Nrg1 whereas cervical, “sympathetic-like” NCC, are nerve-independent but nevertheless rely on ErbB3 signalling. In consequence, Wnt1::Cre;ErbB3lox/lox mutants showed a greater atrophy of the OENS and ENS with respect to the other mutants in which only one population was affected (compare graphs in Figures 15, 16 and 18).

v. Dual origin of enteric neurons in vagal and cervical sympathetic-like neural crest cells is confirmed by lineage tracing using chick chimeras.

To confirm two distinct origins and migration patterns for the NCC contributing neurons to the OENS or to all the gastrointestinal tract, I performed lineage tracing in chicken in collaboration with the lab of Alan Burns at UCL in London. We generated chicken chimeras by grafting different neural tube levels, including the NC, of GFP+ chicken donors into the same levels of WT hosts and
analysed the migration of enteric precursors during the early colonization of the foregut (E3.5) with respect to the path of the vagus nerve.

NCC grafted at somite level 1-2 reached the foregut in association with the vagus nerve (Figure 19-A). By contrast, NCC grafted at somite level 3-7 (cervical NCC) did not follow the nerve (which would require a rostral detour before entering the pharynx) but instead used a short cut, migrating in streams oriented ventrally, directly from their origin into the foregut (Figure 19-B). Transverse sections through E3.5 chimeras showed that NCC from 1-2 grafts followed a dorsolateral pathway associated to the vagus nerve to enter the oesophagus and did not contribute cells to the upper sympathetic chain (Figure 19 a, white arrowhead), whereas NCC from 3-7 grafts followed a ventromedial pathway, contributing first to the upper sympathetic chain and migrating from there ventrally around the dorsal aorta into the oesophagus (Figure 19 c, green arrowhead). Enteric precursor at the level of the stomach were all GFP+ in 3-7 grafted embryos, but only few GFP+ were present in that of 1-2 grafted embryos, which showed a higher proportion of GFP- / HNK-1+ precursors (HNK-1 was used as a general NCC marker) (Figure 19-b and d).

Of note the neural tube at the level of somite 3-7 did not give rise to the motor component of the vagus nerve (Figure 19, white arrow in B), so that the corresponding portion of the neural crest has really nothing “vagal” about it.

Thus, together with the information obtained in mice, these results confirm a dual contribution to the enteric nervous system in the previously labelled “vagal” region: Schwann cell precursors (emerging from NCC at somites 1-2) which are vagal stricto sensu, since they invade the vagus nerve and migrate along its fibres, and cervical “sympathetic-like crest” cells (emerging from NCC at somites 3-7) which contribute part of the oesophageal nervous system and most of the post-gastric enteric nervous system.

vi. Perspectives

As explained in the Introduction of this manuscript (Introduction-1.3.b), the upper limit of the neural crest that contributes to the superior cervical ganglion (the rostral-most sympathetic ganglion) is not clearly defined. The results presented above
show that the cervical NCC emerging at somite level 3-7, collectively gives rise both to sympathoblasts and enteric neurons, but it remains to be shown whether it is all “sympathetic” *stricto sensu*, or the most rostral part of it only “sympathetic-like” by its pattern of migration. For that, I plan to analyse chicken chimeras grafted at single somite levels, starting at somite 3, and co-label them for the sympathetic marker TH along with GFP used as a reporter.

Moreover, I plan to analyse the final contribution of NCC grafted at vagal (*stricto sensu*) or cervical levels to the enteric nervous system along the whole gastrointestinal tract of E7 chicken chimeras, to ensure that our results are consistent with previously published data in chicken (Burns et al., 2000).
c. **Main Figures**

**Figure 14. Presence of enteric precursors along the vagus nerve in Ret and Phox2b mutants.** (A, B, D, E, G, H) Sagittal views of whole E10.5 mouse embryos from the indicated genetic backgrounds stained for the general NC marker Sox10 and the neurofilament marker NF. The position of the stomach primordium is indicated by a dashed circle. (C, F, I) Insets showing Sox10+ enteric precursors alongside the vagus nerve. (a'-f') Transversal sections through the gut at the level indicated in the whole mounts (upper panels), stained for Sox10, NF and DAPI. Insets in c’ and e’ show higher magnifications of cells co-labelled for the reporter CFP in the Ret$^{CFP}$ line or for the reporter LacZ in the Phox2b$^{LacZ}$ line, and for Sox10, demonstrating that these cells are double-positive. NCC are absent from the post-gastric ENS in both Wnt1::Cre;Ret$^{Lox/Lox}$ and Phox2b$^{LacZ/LacZ}$ mutants but they are present in the OENS alongside the vagus nerve up to the stomach (D-I; c’-f’) while compared to the WT (A-C;a’-b’). Ht: heart; O: oesophagus; mg: midgut; X: vagus nerve; XII: spinal accessory nerve; DRG: dorsal root ganglia.
Figure 15. The oesophageal nervous system depends on the vagus nerve that carry Schwann cell precursors. (A and B) Sagittal views of the post-otic region of whole E11.5 embryos of the indicated genetic backgrounds immunostained for the indicated antibodies. In the absence of viscerosensory and motor neurons in the Pgk::Cre;P2aASIC mutant, the vagus nerve is reduced to a vestigial ramus (white arrowhead in B). (C and D) Transversal sections through the oesophagus (O) of E13.5 embryos of the indicated genetic backgrounds immunostained for the pan-autonomic determinant Phox2b. The OENS is atrophied in the Pgk::Cre;P2aASIC. (E) Quantitative analysis of Phox2b+ OENS neurons in the indicated mutant backgrounds. Pgk::Cre; P2aASIC embryos show a 36% significant reduction of OENS neurons (64%±1.6 versus 100% ± 0.9. P = 0.001. n = 4 embryos), whereas Wnt1::Cre;P2aASIC did not show a significant reduction with respect to the WT (90%±4.2 versus 100% ± 1. P = 0.898. n = 4 embryos). IX, X and XII: cranial nerves.
Figure 16. Axonal Neuregulin-1 is necessary for the migration of parasympathetic and oesophageal precursors along their associated cranial nerves. (A, D, G, I, K-N) Sagittal views of cranial nerves and associated parasympathetic (A, D, G, I) or enteric (K-N) precursors immunostained for Phox2b, Sox10 and NF. Parasympathetic precursors fail to colonize their associated cranial nerves in Phox2b::Cre;Nrg1lox/lox mutants (white arrowheads in D and I). (B, C, E, F, H and J) Correlatively, parasympathetic ganglia are absent in Phox2b::Cre;Nrg1lox/lox mutants (asterisks in E, F and J), as seen in transversal sections of the cranial region of E13.5 mutant embryos immunostained for Phox2b (brown) and hybridized for Peripherin, which stains cranial ganglia (blue). (L and N) OENS precursors are depleted from the vagus nerve fibers of E10.5 Phox2b::Cre;Nrg1lox/lox mutant (white arrowhead) and correlatively, the OENS is atrophic at E13.5 (right panel, transverse sections through the oesophagus (O) immunostained for Phox2b). Graph: Quantitative analysis of Phox2b+ OENS neurons in the indicated mutant backgrounds. Phox2b::Cre;Nrg1lox/lox embryos show a 46% significant reduction of OENS neurons (54%±2.6 versus 100%±4.28. P = 0.004. n = 3 embryos) whereas Wnt1::Cre;Nrg1lox/lox do not show a significant reduction with respect to the WT (105%±4.77 versus 100%±4. P = 0.842. n = 3 embryos). G: geniculate ganglion; JN: Jacobson’s nerve; CT: chorda tympani; VII: facial nerve; Gg: geniculate ganglion; Og: otic ganglion; Smg: submandibulary ganglion; Spg: Sphenopalatine ganglion; Ht: heart; st: stomach; DRG: dorsal root ganglion; X, XII: cranial nerves.
Figure 17. Colonization of the foregut by cervical sympathetic-like crest cells. (A) Sagittal view of a whole E10 WT embryo immunostained for NF (blue), Phox2b (green) and Sox10 (red). Two optic sagittal z-sections from the boxed-area in A are shown in the lower panels (A’ and A’’). Sox10+ NCC associated to the first vagal fibers (X) appear superficially and separated by a gap (arrowhead in A’) from a caudally located NCC population. This population is in continuity with the forming sympathetic chain (SC, Phox2b+ Sox10+ cells) and appears to migrate to the region where the future stomach (st) will form (green arrowhead in A’’). (b-d) Transversal sections through the regions indicated in A, of a E10 WT embryo stained for NF (pink), Phox2b (green) and Sox10 (red). (b) Sox10+ NCC dorsolateral to the pharynx (ph) are present close to the forming vagus nerve (X). Phox2b+ Sox10- cells are still delaminating from the placodes (white arrowheads) to contribute viscerosensory neurons to the X nodose ganglion. (c-d) Two transversal sections show the sympathetic-crest migrating laterally and ventral to the dorsal aorta (DA) into the oesophagus (O), while the vagus nerve fibers have not reach that region yet.
Figure 18. Defective enteric nervous system in the absence of ErbB3 signalling. (A-D) Sagittal views centred in the enteric region of whole E10.5 mouse embryos from the indicated genetic backgrounds stained for Sox10 (red), Phox2b (green) and NF (blue). OENS precursors are depleted from the vagus nerve fibers of E10.5 Wnt1::Cre;ErbB3lox/lox mice (white arrowheads in B and D). (a'-b') Transversal sections through the OENS of E13.5 mouse embryos of the indicated genetic backgrounds immunostained for Phox2b. Left graph: Quantitative analysis of Phox2b+ OENS neurons in the indicated mutant backgrounds. Wnt1::Cre;ErbB3lox/lox embryos show a 68% significant reduction of OENS neurons (32%±0.6 versus 100%±4.2. $P = 0.003. n = 3$ embryos). (c'-d') Transversal sections through the post-gastric ENS of E13.5 mouse embryos of the indicated genetic backgrounds immunostained for Phox2b. Right graph: Quantitative analysis of the surface occupied by Phox2b+ ENS neurons in the indicated mutant backgrounds. Wnt1::Cre;ErbB3lox/lox embryos show a 63% significant reduction of OENS neurons (37%±0.8 versus 100%±0.6. $P = 0.004. n = 3$ embryos).
Figure 19. Lineage tracing shows a dual origin of enteric neurons in vagal and cervical sympathetic-like neural crest (A and B) Sagittal views of whole E3.5 chicken GFP chimeras stained for GFP and tubulin-3 (Tubb3); the axial level of the graft is indicated in green in the drawings of the left. Chicken chimeras grafted at somite levels 1-2 show GFP+ NCC associated to the vagus nerve in their way to the foregut. (a-d) Transversal sections through the oesophagus or stomach (the exact levels are indicated in A and B) stained for GFP, the general neural crest marker HNK-1 and Tubb3. (a and b) In chimeras grafted at somite levels 1-2, GFP+ NCC colonize the oesophagus (O) following the vagus nerve and contribute partially to the stomach, where we also find GFP-/HNK-1+ cells derived from the host. Grafted NCC do not contribute to the upper sympathetic chain (white arrowhead in a) located lateral to the dorsal aorta (DA). (c and d) In chimeras grafted at somite levels 3-7, GFP+ NCC emerge from the sympathetic crest (green arrowhead in c) and migrate lateral and ventral to the DA into the oesophagus, which also receives vagal associated NCC from the host (GFP-/HNK-1+ cells). NCC derived from this axial level form the majority of neurons found in the stomach (GFP+HNK+) (d).
d. Supplementary figures

**Figure 20. Vagal-associated enteric precursors share the molecular signature of Schwann cell precursors (SCPs).**  
(A) Sagittal view of the foregut of a whole E11.5 WT embryo. Vagal-associated NCC alongside the oesophagus (O) express the general NC marker Sox10 (blue) and already differentiated enteric neurons express the autonomic determinant Phox2b (green). The vagus nerve fibers are labelled by NF (red).  
(B and C) Sagittal sections through the foregut of WT embryos stained for the indicated probes and antibodies. Enteric precursors alongside the oesophagus express the SCPs markers PLP-1 and Cadh19. Insets in B and C show higher magnifications of double-positive enteric precursors present in the oesophageal wall. O: oesophagus; st: stomach.
Figure 21. Absence of Nrg1 expression in OENS neurons. Transversal section through the oesophagus of an E11.5 Nrg1LacZ/+ embryo immunostained for Sox10 (which labels enteric precursors at this stage), 2H3 (neurofilament, labelling vagus nerve fibers) and β-galactosidase (stains cells expressing the Nrg1 reporter LacZ). Vagus nerve fibers are LacZ+ whereas enteric precursors are LacZ-. Insets on each panel show a higher magnification of a Sox10+/LacZ- /NF- cell found in the oesophageal wall surrounded by NF+/LacZ+ vagal fibers.
Figure 22. Presence of the post-gastric ENS after vagus nerve damage or axonal Nrg1 signalling block.  

**Lower panels:** transversal sections through the post-gastric ENS of the indicated mutant backgrounds immunostained for Phox2b.  

**Graph:** Quantitative analysis of the surface occupied by Phox2b+ ENS neurons in the indicated mutant backgrounds. There are no significant differences between the measures for ENS neurons of the mutants with respect to the WT.

- **Wnt1::Cre;P2aASIC** 83%±1 versus 100±0.6. $P = 0.05$; $n = 4$;  
- **Pgk::Cre;P2aASIC** 93%±1.2 versus 100±0.8. $P = 0.283$; $n = 4$;  
- **Phox2b::Cre;Nrg1lox/lox** 81%±0.6 versus 100±0.6. $P = 0.07$; $n = 5$;  
- **Wnt1::Cre;Nrg1lox/lox** 93%±0.5 versus 100±0.6. $P = 0.1$; $n = 3$.  

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Figure 23. Defect of sympathetic chain formation in the absence of ErbB3 signalling. Sagittal views of whole E10 mouse embryos of the indicated genetic backgrounds stained for NF (blue), Phox2b (green) and Sox10 (red). The vagus nerve (X) is starting to form and we observe a gap (white arrow) between Sox10+ NCC associated to the nerve and a caudal population of NCC (green arrow) emerging from the forming sympathetic chain (SC) in the WT embryo. The sympathetic crest is massively depleted in the $Wnt1::Cre;ErbB3^{Lox/Lox}$ embryo (red arrowhead).
Figure 24. The depletion of Phox2b+ enteric neurons in ErbB3/Nrg1 mutants is corroborated by Sox10 staining. Transversal sections through the oesophagus of E13.5 mouse embryos of the indicated mutants backgrounds stained for the general NC marker Sox10 (blue), the neuronal marker HuC/D (green) and the autonomic determinant Phox2b (red) (Upper panels). At this stage of development, almost no Phox2b+ co-express HuC/D whereas they co-express Sox10+. We observe a similar depletion of enteric precursors in the indicated mutant backgrounds by Phox2b or Sox10 staining (Lower panels).
3.3 Discussion

a. Dual origin of the enteric nervous system

In this study I show a dual contribution to the enteric nervous system from vagal SCPs and sympathetic-like cervical crest. Around half of the OENS originates from SCPs arising from the rostral-most vagal NC (at somite level 1-2), which reach the foregut guided by the vagus nerve. The other half of the OENS arises from sympathetic-like crest cells (at somite level 3-7) which migrate independently of the vagus nerve, along the classical ventromedial pathway. The situation is consistent between mice and chicken embryos.

The idea that enteric precursors follow the vagus nerve to reach the oesophagus is not new. Indeed, the first studies to identify a “vagal origin” for the enteric nervous system were based on the observation of neuroblasts closely associated to vagal fibers in the oesophagus (Kuntz, 1910; Stewart, 1919). However, these authors were vague (Stewart, 1919) or erroneous as to their origin (Kuntz, 1910, proposed the ventral neural tube). Later, the settling of this issue by Yntema and Hammond (1954) and Le Dourain (1973), who demonstrated an origin in the NC, eclipsed these old studies, while leaving open the question of the mechanism of guidance, which was not addressed by the ablation or chimera studies. Later studies in human and mice embryos again showed the presence of neuroblasts associated to the vagus nerve at the level of the oesophagus and stomach, and proposed that these cells used the nerve as a track on which to migrate to the bowel (Okamoto and Ueda, 1967; Webster, 1973). However, the presence of NCC in the foregut (around E9 in the mouse) prior to the first vagal fibers (E10) (Natarajan et al., 2012) seemed to put the nail in the coffin of the notion that the vagus nerve would serve as a scaffold for enteric neuronal precursors (Baetge and Gershon, 1989; Anderson et al., 2006).

Thus, from those early observations of enteric precursors associated to the vagus nerve, only the term “vagal” has remained, without much justification for it, except a rough registration with the roots of the vagal nerve. My results show that there is actually a much more precise one, since the foregut is in part colonized by vagal SCPs. On the other hand, the neural tube at the levels of somites 3-7 does not contribute motor fibers to the vagus nerve, nor vagal SCPs (Figure 19 B, white arrow). Thus, it would seem more appropriate to reserve the term “vagal crest” for NC associated to the vagus nerve, (that is coming from levels of somites 1-2), and
coin a new term to define the NC emerging at the level of somites 3-7, such as “sympathetic-like crest”.

Along with our previous study on the development of parasympathetic ganglia (Espinosa-Medina et al, 2014), my results on the origin of half of the OENS unveil another situation where SCPs give rise to neurons in vivo. Another recent study revealed that a subset of SCPs (lineage traced with a $Dhh::cre$ line, which induces recombination at ~E13 almost exclusively in the Schwann cell lineage (Jaegle et al., 2003)) invades the gut alongside the extrinsic nerves at midgestation and gives rise to enteric neurons at postnatal stages (around 5% in the small intestine and 20% of neurons in the large intestine) (Uesaka et al., 2015). Vagal SCPs invade the foregut much earlier (between E10 and E10.5) and could have been spotted with a $Dhh::cre$ line. To address the final distribution of SCP-derived enteric neurons from earlier stages of development, the Cre recombinase should be expressed under the control of a promoter directing the establishment of the SCP pool, such as Erk1/2 (Newbern et al., 2011).

The notion of an overlap between the crest giving rise to the upper sympathetic ganglia and part of the enteric nervous system, was already proposed by Durbec et al. (1996), but our conclusions as to its final contribution is opposite to theirs. Apart from a DiI tracing experiment constrained by the limited lifespan of mice embryos in vitro, Durbec et al. (1996) used the fact that $Ret$ KO mice lacked both the SCG (but not other sympathetic ganglia) and the post-gastric ENS, to propose that they derive from a common NC precursor pool (at the level of somites 1-4). On the other hand, since the OENS and lower sympathetic ganglia are both present in $Ret$ KO mice, they speculated that they originate at the same NC level (somites 6-7), without testing it experimentally. Actually, a more recent study showed that Ret deficiency affects the formation of all sympathetic ganglia and that the SCG is in fact present in $Ret$ KO mice, although atrophic and in a more caudal location (Enomoto et al., 2001). The authors speculated that this aberrantly located SCG was misinterpreted as being absent in the previous report by Durbec et al., (1996).

In the current study I also propose a dual origin for the OENS, but in contrast to Durbec et al. (1996), I show that while the rostral-most NC (the “vagal crest” proper) follows the vagus nerve and colonizes mainly the OENS, the “sympathetic-like crest” originating from somite levels 3-7 is the major contributor to the entire enteric nervous system. This is consistent with the data by Burns et al., (2000) which demonstrated that NC at somite levels 1-2 contribute...
neurons mainly to the OENS whereas most caudal NCC contribute neurons to all the gastrointestinal tract, with the novelty that these two levels correspond to two clearly separated migration pathways and mechanisms. Our study also suggests that there is no difference between mice and chicken in that respect.

b. Molecular determinants of the migration of enteric neurons

In the previous study I show that ErbB3/Nrg1 signalling is essential for enteric nervous system development. This signalling system has been previously implicated in the development of sympathetic ganglia, SCPs and parasympathetic ganglia (see Introduction and Chapter 2 of this manuscript). However, no defects were apparent in ErbB2 null and ErbB2 conditional KO mice or in ErbB2 and ErbB3 mutant zebrafish (Britsch et al., 1998; Honjo et al., 2008). There is just one study reporting a reduction of enteric ganglia in E13.5 mice lacking ErbB3 (Erickson et al., 1997), but the only piece of evidence provided by the authors, a staining of enteric neurons in the stomach of the KO and WT mice for Peripherin (Erickson et al., 1997; Figure 8-B and D), was rather unconvincing. This might explain that this paper has never been cited for the defect of enteric neurons, and that ErbB3 was mostly omitted from reviews of signalling pathways involved in ENS development.

On the other hand, Nrg1 has been identified as a Hirschsprung’ disease (HSCR) susceptibility locus in GWASS, and has been implicated as a modifier of Ret-dependent HSCR risk (Garcia-Barcelo et al., 2009; Gunadi et al., 2014; Phusantisampanet al., 2012). More recently, a study reported the expression of ErbB3/Nrg1 factors in enteric neurons and glia in the adult human colon, supporting a previously claimed role in the survival and maintenance of the enteric nervous system (ENS) at postnatal stages (Barrenscchee et al., 2015; Crone et al., 2003).

Here I provided evidence that ErbB3/Nrg1 are implicated in the colonization of the gut by enteric precursors. First, there is a massive depletion of NCC in both Nrg1 and ErbB3 mutants alongside the vagal pathway during the early colonization of the foregut. Second, axonal Nrg1 is required for the ontogeny of half of the OENS, derived from vagal SCPs. Third, ErbB3 mutants show an atrophy of around 70% of both the OENS and post-gastric
ENS, suggesting this signalling system is implicated in the migration of both vagal SCPs and sympathetic-like enteric precursors.

The discrepancy between our findings and those showing no defects of ErbB3/Nrg1 signalling in enteric nervous system development (Britsch et al., 1998; Honjo et al., 2008) is puzzling. Species differences between zebrafish and mice could account for the discrepancy with Honjo et al. (2008), but not with Britsch et al., (1998). The authors of the latter study used a Ret probe to label enteric neurons in ErbB3 null and WT embryos at E12.5 and saw no phenotype, although quantifications were not performed. I counted enteric neurons labelled for Phox2b, a pan-autonomic determinant (Pattyn et al., 1999). The possibility that Phox2b is downregulated in neurons which would still express Ret, rather than cells being absent is made unlikely by detection of an equal number of cells expressing Sox10, a general neural crest marker, along with Phox2b along the GI of E13.5 ErbB3 mutant embryos (Figure 24). The deficit in enteric neurons at E13.5 is consistent with the early migration defect of the precursors, observed for both the vagus-associated (Figure 18, B and D) or the sympathetic-like precursors (Figure 23).

Unfortunately, the conditional ErbB3 and Nrg1 mutants used in our study do not survive beyond E16, so that we cannot know whether the defects observed are maintained or compensated later.

c. Anatomical, functional and evolutionary considerations

The partial difference in origin of the OENS and the ENS parallels anatomical and physiological differences between the oesophageal and the gastrointestinal systems; while the main role of the former is to transport the bolus to the stomach by peristalsis, gastrointestinal functions involve secretion of digestive enzymes, gut hormones, absorption of nutrients and generation and voiding of faeces. While the gastrointestinal walls include smooth muscles organised in an outer longitudinal and an inner circular layer that contract to allow peristalsis, the oesophagus wall consists of varying amounts of striated fibers mixed with smooth muscle in mammals, but not in birds, which only have smooth muscle (Neuber et al., 2006). The origin of the striated oesophageal muscle has been a matter of debate for a long time, because contrary to the smooth muscle, it is not of somitic mesodermal origin. A recent study has
solved the question by demonstrating that oesophageal striated muscle in mammals originates in the cardiopharyngeal mesoderm, which also gives rise to second heart field derivatives and head muscles (Gopalakrishnan et al., 2015). It is speculated that the absence of mastication in avians could have supported the evolution of a purely smooth muscle lined oesophagus with greater distensibility for rapid ingestion of coarse food, which is further processed in the avian gizzard (or posterior stomach).

Correlatively, the oesophageal and post-gastric intrinsic nervous systems have a different anatomy: the post-gastric ENS is formed by two plexus of interconnected ganglia (the myenteric and submucosal plexuses), whereas the OENS only has a myenteric plexus between muscle layers, in line with its limited or absent secretory function (Mashimo and Goyal, 2006).

One could thus speculate that the anatomical and functional differences found between the oesophagus and the gastrointestinal tract might be the result of different developmental events, which would have allowed the incorporation of new elements to the system: vagal-SCPs could give rise to subtypes of enteric neurons more involved in OENS function than in post-gastric ENS function. To test this possibility, we should first develop a method to trace the two different NC populations described in our study. For this purpose one could use specific enhancers of the transcription factor FoxD3 that distinguish cranial and vagal/trunk NCC (Simoes-Costa and Bronner, 2016; Simoes-Costa et al., 2012), or an enhancer of HoxB3 (b3IIia, which drives expression exclusively in the NC from somite levels 1-7) (Chan et al., 2005), or of HoxB5, mostly expressed in the NC caudal to somite level 3 in mice (M K M Kam et al., 2014).

Finally, the spatial overlap in the origin of most of the ENS and the sympathetic chain that I demonstrated, extends to the late contribution to the intestine documented by Uesaka et al., (2015), and to the sacral contribution, since, according to our study on the identity of the sacral nervous system (See Chapter 4; Espinosa-Medina et al., 2016), the sacral crest gives rise to sympathetic postganglionic neurons.
Chapter 4

Identity of the sacral autonomic outflow

4.1 Introduction

a. The sacral outflow: anatomy and function

The sacral autonomic nervous system has been classified as one of the two components (with the cranial one) of the parasympathetic nervous system (Langley and Anderson, 1895; Langley, 1921). It consists of preganglionic neurons located in the intermediolateral column of the sacral spinal cord (segments S2-S4 in humans, the exact location varies with species) that exit through the ventral root and project in the pelvic nerves to innervate postganglionic neurons in the pelvic plexus. This plexus is a paired structure situated on both sides of the bladder, close to the rectum and the vagina in the female. It is connected to the prevertebral sympathetic chain by the hypogastric nerve. Its particularity resides in it being considered a mixed sympathetic-parasympathetic plexus because of its dual input: it receives sacral parasympathetic innervation through the pelvic nerves and lumbar sympathetic innervation through fibers projecting in the hypogastric nerve (see Introduction Fig.1; and Fig. 25 below). Postganglionic pelvic neurons innervate the third most distal portion of the colon, the bladder and genital organs, mediating storage and voiding functions and reproductive behaviours.
Figure 25. Connectivity of the pelvic ganglion (diagram from Keast, 2006). Pelvic postganglionic neurons receive lumbar sympathetic and sacral parasympathetic inputs, and in some species a single neuron can receive both (De Groat et al., 1979). Postganglionic axons project in the penile/cavernous nerve, the accessory nerves and other smaller nerves project to the rectum and bladder (not shown).

The view of a bipartite ‘cranio-sacral’ parasympathetic nervous system is a century old and stemmed from several anatomical, physiological and pharmacological similarities of the sacral with the cranial outflow. From an anatomic standpoint, it was argued that, like in the cranial parasympathetic outflow, the sacral target territory was less diffuse than that of the thoracic sympathetic outflow (which projects to all blood vessels in the body) and, importantly, lacked projections to the paravertebral sympathetic chain: Gaskell noted in 1886 that there was no *rami communicans* below the second lumbar root and that was his main argument to draw a parallel with the cranial visceral nerves, which have no connexion to the sympathetic chain. Another major argument introduced by Langley was pharmacological: the shared sensitivity to muscarinic antagonists of the sacral and cranial outflows, (Langley, 1921) i.e. the cholinergic nature of their ganglia. However, it took only a decade or so to find several examples of cholinergic neurons in bona fide sympathetic ganglia, so that this argument was weak, in retrospect (Dale, H. 1935; Schotzinger et al., 1994; Huber et al., 2012). Moreover it is now accepted that some neurons with lumbar input, thus sympathetic, are cholinergic (Keast, 2006).
On the other hand, a number of features are at odd with the parasympathetic nature of the sacral pathway:

- Unlike other parasympathetic neurons embedded in their target tissues, many pelvic neurons are located quite far from their targets (in human, many centimeters), similar to neurons in the prevertebral chain (Keast, 2006), with which they are in fact in anatomical continuity (Figure 26).

- Sacral and thoracic preganglionic neurons share a location in the mediolateral spinal cord and a ventral exit point for their axons, that differs from the more varied topography and dorsal axonal exit point of cranial preganglionic neurons (See Introduction, Figures 2 and 3).

- A dual lumbo-sacral input is not specific to the pelvic ganglion but shared by the inferior mesenteric ganglion, which is nevertheless considered exclusively sympathetic (Crowcroft and Szurszewski, 1971).

- Schematics often represent the sacral input to the rectum as disynaptic (preganglionic spinal neurons synapsing directly on enteric myenteric neurons), but several studies show that it is predominantly (Olsson et al., 2006) if not exclusively (Fukai and Fukuda, 1985) trisynaptic (preganglionic spinal neurons synapsing on pelvic postganglionic neurons and these synapsing on myenteric neurons), thus sympathetic-like.

- Some neurons have been found to have a dual sacral and lumbar input, making them impossible to classify.

For all these reasons and a few more which are physiological and will be discussed after the publication, the division of the pelvic nervous system into sympathetic and parasympathetic has always been awkward, and deserved to be re-examined. We did this by using genetic and developmental criteria to define cell types.
b. Ontogeny of cranial and spinal preganglionic motor neurons

Four classes of motor neurons can be distinguished in the spinal cord and hindbrain: somatic motor (SM) neurons that innervate most skeletal muscles, branchiomotor (BM) neurons that innervate muscles derived from the branchial arches, and visceromotor (VM) neurons of two classes that innervate, respectively, sympathetic and parasympathetic ganglia. The distinction between the latter classes is often overlooked, and the flawed idea that the parasympathetic preganglionic are cranial equivalents of the sympathetic ones in the spinal cord, inherited from the XIXth century, is still implicit in the nomenclature.

Hindbrain VM and BM neurons arise from the progenitor domain that flanks the floorplate, the p3 domain, whereas the dorsally adjoining pMN domain produces SM neurons (Guthrie, 2007). BM and VM precursors then migrate dorsally and project through dorsolateral exit points whereas SM precursors remain in a ventral position and leave the neuroepithelium ventrally (Briscoe et al., 1999). In contrast, all spinal motor neurons, somatic and visceral, are thought to derive from the pMN domain (Phelps et al., 1991; Jessell, 2000).
Subsequently, spinal VM precursors migrate dorsally to form the intermediolateral preganglionic column (PGC) and some also move medially between the PGC and the central canal (described in rat thoracic and upper lumbar preganglionic sympathetic neurons in Markham and Vaughn, 1991). Both spinal VM and SM fibers exit the spinal cord through the ventral root (Kandel, 2012).

These distinctions are paralleled by genetic developmental pathways. Hindbrain BM and VM neurons differentiate from progenitors that express Nkx2.2, Nkx2.9 (Briscoe et al., 1999; Pabst et al., 2003), Tbx2/20 (Kraus et al., 2001) and depend on Phox2b to form (Pattyn et al., 2000), whereas SM neuronal fate in the pMN domain of both the hindbrain and spinal cord is controlled by Pax6, Nkx6.1 and Nkx6.2, Olig2 and do not depend on Phox2b (Pattyn et al., 2000, Ericson et al., 1997; Sander et al., 2000; Vallstedt et al., 2001; Osumi et al., 1997). Under the influence of these early determinants, postmitotic SM neurons express Hoxb9, Isl1/2, Lhx3/4 (Jessel, 2000; Tsuchida et al., 1994; Tanabe et al., 1998). The development of spinal (sympathetic) VM neurons requires a low level of expression of Foxp1, as opposed to a high level required for the diversification of spinal motor neuronal precursors into ventral SM neurons (Dasen et al., 2008).

I combined and extended this set of criteria that distinguish between cranial and spinal VM neurons to explore the sacral contingent, largely unexamined in the literature. One of the few studies suggests that sacral preganglionic neurons might share their origin with thoracolumbar ones (Phelps et al. 2002) implying that sacral and cranial VM neurons would have very distinct ontogenies and inviting to revisit their common classification as parasympathetic (see Publication below).

c. Ontogeny of postganglionic pelvic neurons

The literature on the development of the pelvic ganglion is rather sparse. Like all other autonomic ganglia, pelvic postganglionic neurons are neural crest derivatives and emerge from the lumbosacral neural tube, caudal to somite 28 (Yntema and Hammond, 1955; Serbedzija et al., 1991; Wang et al., 2011). Pelvic precursors migrate ventrally around the hindgut before coalescing between the hindgut and urogenital sinus to form pelvic ganglia in rodents (or plexus in humans) (Kapur, 2000; Anderson et al., 2006; Wang et al., 2011). In the chick, the “ganglionated nerve” of Remak, an anterior continuation of the pelvic plexus that
extends upward along the intestine, has been better studied than the latter and is classically considered parasympathetic (Browne, 1953; Yntema and Hammond, 1955).

Studies on the development of the enteric nervous system have provided some evidences on the early formation of pelvic ganglia in mammals and Remak nerve in the chick, as they represent a secondary source of enteric neurons and glia that migrate along postganglionic fibers to colonize the hindgut (Le Dourain and Teillet, 1973; Burns and Le Dourain, 1998; Burns and Le Dourain, 2001; Kapur, 2000; Wang et al., 2011). In this context, it has been shown that postganglionic pelvic and Remak neurons do not depend on Ednrb (previously implicated in neural crest migration of enteric precursors, Druckenbrod, 2009), GFRa1 or RET (Cacalano, 1998) to project axons to the hindgut. In contrast, genetic deletion of Neurturin or GFRa2 leads to a reduction in pelvic cholinergic neurons and an atrophy of the mucosa of their target tissues (Wanigasekara et al., 2004; Wanigasekara and Keast, 2005; Yan and Keast, 2008).

More recently a genome-wide screen has identified 155 murine genes expressed in pelvic ganglia showing an early regional segregation of progenitors and differentiating neurons. Among the documented expression patterns, the authors show that some subpopulations express the transcription factors Hand1, Gata2 and Lmo1 whereas others express Rtn4, Ndrg2 and Ndrg4 (Wiese et al., 2012). Moreover, they describe a central progenitor domain characterized by the expression of Sox10 surrounded by differentiating neurons, a segregation pattern previously observed in sympathetic, ciliary and dorsal root ganglia (Müller and Rohrer, 2002; Callahan et al., 2008; Tsarovira et al., 2008; Nishi et al., 2010).

In the following study, we define a whole set of differential genetic and developmental features for cranial parasympathetic neurons and thoracic sympathetic neurons, pre and post-ganglionic and re-examine the sacral outflow in this light.
The sacral autonomic outflow is sympathetic


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conclude that the soluble UGT85B1 interacts with both CYP79A1 and CYP71E1, but that it is not necessary for CYP79A1-CYP71E1 complex formation (Fig. 4E). CYP79A1, CYP71E1, CYP98A1, and POR2b are situated very close together at the ER surface and have comparable pairwise FRET values (Fig. 4F and table S1I). All microsomal P450s require electron donation from POR; therefore, it is not surprising that CYP98A1 is proximal to the dhurrin biosynthetic enzymes (Fig. 4, A, B, and D). UGT85B1 was situated close to the nonpartner ER membrane proteins, CYP98A1 and POR2b, when CYP79A1 and CYP71E1 were coexpressed (table S12).

A prerequisite to understanding how cells coordinate diverse metabolic activities is to understand how the enzyme systems catalyzing these reactions are organized and their possible enrollment as part of dynamic metabolons. Efforts to maximize product yield from genetically engineered pathways (14–17) would benefit from this information. In this study, we showed that the dhurrin pathway forms an efficient metabolon. CYP79A1 and CYP71E1 form homo- and hetero-oligomers, which enable recruitment of the cytosolic soluble UGT85B1 (Fig. 4G). UGT85B1 regulates the flow of t-tyrosine and stimulates channeling between CYP98A1 and CYP71E1. Efficient metabolic flux and channeling require an overall negatively charged lipid surface and may provide an additional means for regulating the dynamic assembly necessary to respond swiftly to environmental challenges. A similar organization may characterize the biosynthetic pathways of other specialized metabolites as well.

REFERENCES AND NOTES
11. Materials and methods are available as supplementary materials on Science Online.

ACKNOWLEDGMENTS
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SUPPLEMENTARY MATERIALS
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27 May 2016; accepted 4 October 2016
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cranial autonomic outflow is sympathetic
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A kinship between cranial and pelvic visceral nerves of vertebrates has been accepted for a century. Accordingly, cranial preganglionic neurons are considered parasympathetic, as are their targets in the pelvic ganglia that prominently control rectal, bladder, and genital functions. Here, we uncover 15 phenotypic and ontogenetic features that distinguish pre- and postganglionic neurons of the cranial parasympathetic outflow from those of the thoracolumbar sympathetic outflow in mice. By every single one, the sacral outflow is indistinguishable from the thoracolumbar outflow. Thus, the parasympathetic nervous system receives input from cranial nerves exclusively and the sympathetic nervous system from spinal nerves, thoracic to sacral inclusively. This simplified, bipartite architecture offers a new framework to understand pelvic neurophysiology as well as development and evolution of the autonomic nervous system.

The sacral autonomic outflow is sympathetic

The allocation of the sacral autonomic outflow to the parasympathetic division of the visceral nervous system—as the second tier of a “cranio-sacral outflow”—has an ancient origin, yet a simple history: It is rooted in the work of Gaskell (1), was formalized by Langley (2), and has been universally accepted ever since (as in [3]). The argument derived from several similarities of the sacral outflow with the cranial outflow: (i) anatomical—a target territory less diffuse than that of the thoracolumbar outflow, a separation from it by a gap at limb levels, and a lack of projections to the paravertebral sympathetic chain (1); (ii) physiological—an influence on some organs opposite to that of the thoracolumbar outflow (4); and (iii) pharmacological— an overall sensitivity to muscarinic antagonists (2). However, analysis of cellular phenotype was lacking. Here, we define differential genetic signatures and dependencies for parasympathetic and sympathetic neurons, both pre- and postganglionic. When we reexamine the sacral autonomic outflow of mice in this light, we find that it is better characterized as sympathetic than parasympathetic.

Cranial parasympathetic preganglionic neurons are born in the “pMNv” progenitor domain of the hindbrain (5) that expresses the homeogene Phox2b and produces, in addition, branchiomotor neurons (6). The postmitotic precursors migrate dorsally (7) to form nuclei (such as the dorsal motor nucleus of the vagus nerve) and project through dorsolateral exit points (7) in several branches of the cranial nerves to innervate parasympathetic and enteric ganglia. In contrast, thoracic and upper lumbar (hereafter “thoracic”) preganglionic neurons, which are sympathetic, are thought to have a common origin with somatic motoneurons (8, 9). By implication, they would be born in the pMN progenitor domain (just dorsal to p3)—thus from progenitors that express the basic helix-loop-helix (bHLH) transcription factor Olig2 (10). The sympathetic preganglionic precursors then segregate from somatic motoneurons to form the intermediolateral column in mammals (11), project in the ventral roots of spinal nerves together with axons of somatic motoneurons, and, via the white rami communicantes, synapse onto neurons of the paravertebral and prevertebral sympathetic ganglia.

We sought to compare the genetic makeup and dependencies of lower lumbar and sacral (hereafter “sacral”) preganglionic neurons with that of cranial (parasympathetic) and thoracic (sympathetic) ones. As representative of cranial preganglionic neurons, we focused on the dorsal motor nucleus of the vagus nerve, a cluster of
neurons already well delineated at 13.5 days of embryonic development (E13.5), that expresses the vesicular acetylcholine transporter (VAChT) (Fig. 1B). Thoracic and sacral preganglionic neurons, which both form a mediolateral column in the spinal cord, did not express VAChT at this stage despite their eventual cholinergic nature. To localize them, we thus used their common marker nitric oxide synthase (NOS) (12) (Fig. 1, A and B), which was absent from the dorsal motor nucleus of the vagus nerve at E13.5 (Fig. 1B) or later (Fig. S1). Thus, NOS expression characterizes thoracic and sacral, but not cranial, preganglionic neurons.

In contrast to cranial (parasympathetic) preganglionic neurons, thoracic (sympathetic) ones

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**Fig. 1. Sacral preganglionic neurons develop like sympathetic, not parasympathetic, ones.** (A) Longitudinal thick section of the spinal cord reacted for a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity indicative of NOS expression, revealing the thoracolumbar and sacral visceromotor columns (arrowheads) separated by a gap. (B to K) Transverse sections at E13.5 through the right half of the medulla (left column in both panels), thoracolumbar spinal cord (middle), and sacral spinal cord (right), stained with the indicated antibodies and probes, or for NOS expression, in the genetic backgrounds indicated on the right. (B) The dorsal motor nucleus of the vagus nerve (nX) expresses VAChT but not NOS, whereas the thoracic and sacral preganglionic neurons (arrowheads) express NOS but not yet VAChT. The ventrally located somatic motoneurons, including the hypoglossal nucleus (nXII) in the hindbrain, express VAChT. (C) Phox2b (C) and Phox2a (D) are expressed in nX but in neither thoracic nor sacral preganglionic neurons (arrowheads). Lower panels in (C) and (D): higher magnifications of the preganglionic neurons. (E) Neurons of nX but neither thoracic nor sacral preganglionic ones (labeled by an antibody to Islet1/2, white arrowheads) derive from Phox2b+ precursors, permanently labeled in a Phox2b::Cre;Rosa<sup>tm1T</sup> background. (F) nX is missing in Phox2b knockouts (red arrowhead), but thoracic and sacral preganglionic neurons are spared (black arrowheads). (G) nX is spared in Olig2 knockouts (black arrowhead), but thoracic and sacral preganglionic neurons are missing (red arrowheads). nXII is also missing, as expected of a somatic motor nucleus (red arrowhead). [(H) to (J)] Tbx20, Tbx2, and Tbx3 are expressed in all or a subset of nX neurons (arrowheads in panels of the left column) but in no thoracic or sacral preganglionic neuron (arrowheads in panels of the middle and right columns). (K) Foxp1 is not expressed in the nX (arrowhead in left column) but is a marker of both thoracic and sacral preganglionic neurons (arrowheads in middle and right columns). nTS, nucleus of the solitary tract. Scale bars: 1 mm (A), 100 μm [(B) to (K)].
Fig. 2. All pelvic ganglionic cells have a sympathetic, not parasympathetic, transcriptional signature. Sagittal sections through parasympathetic ganglia (columns headed “Parasympathetic”), the lumbar paravertebral sympathetic chain (columns headed “Sympathetic”), and the pelvic ganglion (columns headed “Pelvic”) at E13.5, stained by immunohistochemistry for Phox2b, a determinant of all autonomic ganglia (33), and in situ hybridization for the indicated probes. GG, geniculate ganglion (a cranial sensory ganglion); O, otic ganglion; S, sphenopalatine ganglion; SM, submandibular ganglion (all parasympathetic ganglia).

Fig. 3. The pelvic ganglion forms independently of its nerve, like sympathetic and unlike parasympathetic ones. (A and C). Whole-mount immunofluorescence with the indicated antibodies on E11.5 embryos either heterozygous (A) or homozygous (C) for an Olig2 null mutation. The nascent pelvic nerves [yellow arrowhead in (A)] seem to derive mostly from the L6 nerve at that stage. The Olig2 null mutation (C) spares two thin sensory pelvic projections. The pelvic ganglion (PG) lies ahead of most fibers in both heterozygous and mutant background. (B and D). View of the L6 nerve, covered with Sox10+ cells but no Phox2b+ cells (yellow arrowheads), unlike cranial nerves that give rise to parasympathetic ganglia at the same stage [Jacobson’s nerve in (E)]. (F and G) In situ hybridization for Phox2b and immunohistochemistry for neurofilament (NF) on heterozygous and homozygous Olig2 knockouts at E13.5, when parasympathetic ganglia have formed elsewhere in the body. Graph: the pelvic ganglion has the same volume whether its preganglionic nerve is present [black arrowhead in (F)] or not (6369 ± 1066 versus 6441 ± 919, $P = 0.96, n = 5$ embryos), gt, genital tubercle; L5 and L6, 5th and 6th lumbar roots; SI, 1st sacral root; SC, sympathetic chain.
Fig. 4. Revised anatomy of the autonomic nervous system. The efferent path of the autonomic nervous system is made up of a spinal sympathetic outflow (left, in red) and a cranial parasympathetic outflow (right, in blue). Sympathetic targets in the skin other than arteries are piloerector muscles and sweat glands. III, oculomotor nerve; VII, facial nerve; IX, glossopharyngeal nerve; X, vagus nerve; A.M., adrenal medulla; gg, ganglion; Pulm, pulmonary; SCG, superior cervical ganglion; Sph, sphenopalatine; Smb, submandibular. For a larger version, see fig. S11.

not only failed to express Phox2b or its paralogue Phox2a at E13.5 but also arose from Phox2b-negative progenitors and did not depend on Phox2b for their differentiation (Fig. 1, C to F, left and middle columns) but instead depended on Olig2 (Fig. 1G). Sacral preganglionic neurons shared all these features with thoracic ones (Fig. 1, C to G, middle and right columns). At E13.5, the T-box transcription factors Tbx20, Tbx2, and Tbx3 were expressed by cranial (parasympathetic) neurons but by neither thoracic (sympathetic) nor sacral preganglionic ones (Fig. 1, H to J, and fig. S2). The F-box transcription factor Foxp1, a determinant of thoracic preganglionic neurons (13), was expressed by sacral but not cranial preganglionic neurons (Fig. 1K). Differential expression of Phox2b, Tbx20, and Foxp1 between cranial and all spinal preganglionic neurons, thoracic and sacral, was still observed at E16.5 (fig. S3). In sum, the ontogeny and transcriptional signature of sacral preganglionic neurons was indistinguishable from that of thoracic ones and therefore sympathetic as well.

Thoracic and sacral preganglionic neurons share a settling site in the mediolateral region of the spinal cord and a ventral exit point for their axons, whereas cranial pregangliones have a less systematized topography and a dorsal axial exit point. These similarities of thoracic with sacral, and differences of both with cranial, are at odds with the notion of craniosacral outflow since its first description (7).

The targets of the sacral preganglionic neurons are in the pelvic plexus (figs. S4 and S5) and are considered, by definition, parasympathetic (14). Because a proportion of pelvic ganglionic neurons receive input from upper lumbar levels (half of them in rats (25)) and thus from sympathetic preganglionic neurons, the pelvic ganglion is considered mixed sympathetic and parasympathetic (16). This connectivity-based definition runs into a conundrum for cells that receive a dual lumbar/sacral input (17). The sympathetic identity of both thoracic and sacral preganglionic neurons that we have here makes the issue moot. Regardless, we looked for a cell-intrinsic criterion that would corroborate the sympathetic nature of all pelvic ganglionic cells in the form of genes differentially expressed in sympathetic versus parasympathetic ganglionic cells elsewhere in the autonomic nervous system. Neurotransmitter phenotypes do not map on the sympathetic/parasympathetic partition because cholinergic neurons in the pelvic ganglion comprise both “parasympathetic” and “sympathetic” ganglionic cells, as defined by connectivity (14), and bona fide sympathetic neurons of the paravertebral chain are cholinergic [reviewed in (18)]. However, we found that three transcription factors expressed and required in the sympathoadrenal lineage—Isl1 (19), Gata3 (20), and Hand1 (21)—were not expressed in parasympathetic ganglia such as the sphenopalatine, the submandibular, or the otic ganglia (Fig. 2 and fig. S6) (although Isl1 is expressed in ciliary ganglia (22) and Gata3 in cardiac ones (20), which thus diverge from the canonical parasympathetic molecular signature). Conversely, we found that the two paragamous homeobox genes Hmx2 and Hmx3 are specific markers of all parasympathetic versus sympathetic ganglia and adrenal medulla (Fig. 2 and figs. S6 and S7). All cells of the pelvic ganglion were Isl1+, Gata3+, Hand1+, Hmx3+, and Hmx2+ at E13.5 (Fig. 2) and at E16.5 (fig. S8), as were smaller scattered ganglia of the pelvic organs (fig. S8). Thus, all had a sympathetic transcriptional fingerprint. Similarly, the chicken ganglion of Remak, classically considered parasympathetic (23), displayed an Isl1+, Hand1+, Hmx3+ signature, and thus is sympathetic (fig. S9).

Finally, we tested the pelvic ganglion for the contrasted modes of development of sympathetic and parasympathetic ganglia. Parasympathetic ganglia, unlike sympathetic ones, arise through the migration of Sox10+/Phox2b+ Schwann cell precursors along their future preganglionic nerve toward the site of ganglion formation and do not form if these nerves are absent (24, 25). At E11.5, the lumbosacral plexus, which gives rise to the pelvic nerve, extended some fibers that reached the lateral and rostral edge of the pelvic ganglion anlagen, most of which was already situated well ahead of them (Fig. 3A and movie S1). These fibers were coated with Sox10 cells, none of which, though, expressed Phox2b (Fig. 3B), in contrast to the cranial nerves that produce parasympathetic ganglia at the same stage (Fig. 9E). Deletion of all motor fibers in Olig2−/− embryos spared only two thin, presumably sensory, projections from the lumbosacral plexus (Fig. 3C), also devoid of Phox2b+ cells (Fig. 3D and fig. S10). Despite this massive atrophy, the pelvic ganglion appeared intact (Fig. 3C, fig. S10, and movie S2). This was verified quantitatively at E13.5 (Fig. 3, F and G). Thus, even though 50% of its cells are postganglionic to the pelvic nerve, the pelvic ganglion forms before and independently of it, as befits a sympathetic ganglion but contrary to parasympathetic ones.

Thus, the sacral visceral nervous system is the caudal outpost of the sympathetic outflow (Fig. 4 and fig. S11), the autonomic nervous system being divided in a cranial and a spinal autonomic system, in line with certain evolutionary speculations (26). This new understanding of the anatomy accounts for many data that were at odds with the previous one. For example, although schematics generally represent the sacral pathway to the rectum as disynaptic—i.e., vagal-like—[e.g., (3)], it is in fact predominantly (27) if not exclusively (28) trisynaptic—i.e., sympathetic-like (29). Despite the dogma of lumbosacral antagonism on the bladder detrusor muscle, the lumbar inhibition is experimentally absent (4) or of dubious functional relevance (30). The synergy of the lumbar and sacral pathway for vasodilatation in external sexual organs [reviewed in (29)] shows a continuity of action—rather than antagonism, as the old model suggested—across the gap between the thoracolumbar and sacral outflows.

The sympathetic identity of all sacral and pelvic autonomic neurons, which our data unveil, provides a new framework for discoveries on pelvic neuroanatomy and physiology.

REFERENCES AND NOTES

Ambient temperature regulates many aspects of plant growth and development, but its sensors are unknown. Here, we demonstrate that the phytochrome B (phyB) photoreceptor participates in temperature perception through its temperature-dependent reversion from the active Pfr state to the inactive Pr state. Increased rates of thermal reversion upon exposing Arabidopsis seedlings to warm environments reduce both the abundance of the biologically active Pfr:Pfr dimer pool of phyB and the size of the associated nuclear bodies, even in daylight.

Mathematical analysis of stem growth for seedlings expressing wild-type phyB or thermally inactivated Pfr state—absorbing Pfr state that is biologically active (6, 7). Whereas Pr arises upon assembly with the bilin, formation of Pfr requires light, and its levels are strongly influenced by light up to irradiances much higher than expected if thermal reversion were slow (12). Second, it is now clear that thermal reversion occurs in two steps. Although the first step, from the Pfr:Pr homodimer (D2) to the Pfr:Pr heterodimer (D1), is slow (k3), the second step, from the Pfr:Pr heterodimer to the Pfr:Pr homodimer (D0), is almost two orders of magnitude faster (k2) (Fig. 1A) (11).

Physiologically relevant temperatures could change the magnitude of k2 and consequently affect Pfr and D2 levels, even under illumination (Fig. 1A). To test this hypothesis, we used in vitro and in vivo spectroscopy and analysis of phyB nuclear bodies by means of confocal microscopy. For the first of these approaches, we produced recombinant full-length phyB bearing its phytochromobilin chromophore. When irradiated under continuous red light, the in vitro absorbance at 725 nm reached lower values at higher temperatures—absorbing phyB exists in two photo-interconvertible forms: a red light—absorbing Pr state that is biologically inactive and a far-red light—absorbing Pfr state that is biologically active (6, 7). Whereas Pr arises upon assembly with the bilin, formation of Pfr requires light, and its levels are strongly influenced by light up to irradiances much higher than expected if thermal reversion were slow (12). Second, it is now clear that thermal reversion occurs in two steps. Although the first step, from the phyB:Pfr homodimer (D2) to the phyB:Pr heterodimer (D1), is slow (k3), the second step, from the phyB:Pr heterodimer to the phyB:Pr homodimer (D0), is almost two orders of magnitude faster (k2) (Fig. 1A) (11).

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The sacral autonomic outflow is sympathetic
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Editor's Summary

Sacral neurons reassigned

The autonomic nervous system regulates the function of internal organs such as the gut. The parasympathetic and sympathetic arms of this system tend to operate antagonistically. Espinosa-Medina et al. used anatomical and molecular analyses to reevaluate the assignment of neurons in the sacral autonomic nervous system (see the Perspective by Adameyko). Previously categorized as parasympathetic, these neurons are now identified as sympathetic. The results resolve a persistent confusion about how the two systems developed and open the avenue to more predictable outcomes in developing treatments targeted to the pelvic autonomic nervous system.

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Supplementary Materials for

The sacral autonomic outflow is sympathetic

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Other Supplementary Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/354/6314/893/DC1)

Movies S1 and S2
Materials and Methods

Histology.
-In situ hybridization and immunochemistry have been described in ref (32).
-Diaphorase staining on cryostat sections was performed as described in ref (33).
-Immunofluorescence on cryostat or vibratome sections was performed as previously described (25). Whole-processed embryos where fixed overnight in 4% paraformaldehyde (in PBS) and dissected spinal cords were fixed for 2 hours at room temperature. Antigen retrieval, by boiling for 10 minutes in sodium citrate (10mM) was needed for optimal labeling with the α-Islet antibody.

Wholemount immunofluorescent staining using the 3DISCO method was adapted from ref (34). All steps up to the imaging of the embryos were performed under nutation. Embryos at stage E11.5 were fixed overnight in 4% paraformaldehyde (in PBS), serially dehydrated in graded methanol (in PBS) up to 100% methanol and then bleached using Dent’s bleach overnight at 4°C. Following serial washes in 100% methanol, the embryos were incubated in Dent’s fixative overnight at 4°C. The embryos were then serially rehydrated in graded methanol (in PBS) up until re-immersion in PBS. Embryos were further subjected to incubation at 70°C to optimize antigen recognition by the anti-Phox2b antibody. Following washes in PBS-Tween (0.1%), tiny superficial perforations were made in the embryo with a minutien pin to facilitate antibody penetration. The embryos were then incubated with primary antibodies in blocking buffer (20% DMSO, 5% FCS in PBS) for 5 days at room temperature. Following washes in PBS-Tween (0.1%) at room temperature, secondary antibodies in blocking buffer were then applied for 4 days at room temperature. Finally, embryos were cleared following the 3DISCO protocol subsequent to washes in PBS-Tween (0.1%) at room temperature, Embryos were imaged using a SP8 confocal microscope (Leica). 3D reconstructions and videos were obtained using the IMARIS imaging software.

Antibodies
The following primary antibodies were used for immunochemistry and immunofluorescent staining:

α-2H3 (NF), Mouse, 1:500, Hybridoma Bank (#2H3)
α-bIII Tubulin (Tuj1), Mouse, 1:500, Covance (#MMS-435P)
α-dsRed, Rabbit, 1:500, Clontech (#632496)
α-Tomato, Goat, 1:1000, Sicgen (#AB0040-200)
α-Islet1:2, Mouse, 1:400 (40.2D6 and 39.4D5, Hybridoma Bank)
α-Phox2b, Rabbit, 1:500 (35)
α-Phox2b, Guinea Pig, 1:500 (36)
α-Phox2a, Rabbit, 1:500 (37)
α-Sox10, Goat, 1:250, Santa Cruz (#SC-17342)
α-FoxP1, Rabbit, Abcam, 1:200 (#AB-16645)

The following secondary antibodies were used:
α-rabbit Cy3, 1:500, Jackson Immunoresearch Laboratories (#711-165-152)
α-rabbit A488, 1:500, Jackson Immunoresearch Laboratories (#711-545-152)
α-goat Cy3, 1:500, Jackson Immunoresearch Laboratories (#705-166-147)
α-goat A647, 1:500, Jackson Immunoresearch Laboratories (#705-606-147)
α-rabbit Cy3, 1:500, Jackson Immunoresearch Laboratories (#711-165-152)
α-mouse Cy3, 1:500, Jackson Immunoresearch Laboratories (#715-165-150)
α-mouse A488, 1:500, Invitrogen (#A-21202)
α-mouse Cy5, 1:500, Jackson Immunoresearch Laboratories (#715-175-150)

Immunohistochemical reactions were processed with the Vectastain Elite ABC kits (PK-6101 and PK-6012; Vector Laboratories) as per manufacturer’s guidelines followed by colour development using DAB (3,3’-Diaminobenzidine).

**Probes**

For the Phox2b riboprobe, primers containing SP6 and T7 overhangs were used to amplify a 635 bp region (nucleotides 123 – 757) from a plasmid containing the full-length Phox2b cDNA sequence. The purified amplicon was then used as the template for antisense probe synthesis using T7 RNA polymerase.

Forward Primer: 5’-CCGTCTCCACATCCATCTTT-3’
Reverse Primer: 5’-TCAGTGCTCTGGCCTCTTT-3’

The other probes were: Gata3 (gift of JD Engel), Hand1 (Stratagene), Hmx2 (gift of E.E. Turner), Hmx3 (gift of S. Mansour), Islet1 (37), Tbx2 (gift of A. Kispert), Tbx3 (gift of V.M Christoffels), Tbx20 (38), VACHT (Source BioScience, UK, 40129421 (CK3-a14) IMAGE clone).

Transgenic Mouse Lines:

- Phox2b::Cre (39): BAC transgenic line expressing Cre under the control of the Phox2b promoter.
- Rosa<sup>lox-stop-lox-tdTomato</sup> (<em>Rosa<sup>adT</sup></em>) (40): Knock in line expressing the reporter gene tdTomato from the Rosa locus in a Cre-dependent manner.
- Phox2b<sup>LacZ+/+</sup> line (31): Knock in line expressing the reporter gene LacZ from the second exon of the Phox2b locus, which is disrupted and lead to a null phenotype in Phox2b<sup>LacZ/LacZ</sup> embryos.
- Olig2Cre line (41): Knock in of Cre in the Olig2 locus (Jackson Laboratories, Stock #25567).

All animal studies were done in accordance with the guidelines issued by the French Ministry of Agriculture and have been approved by the Direction Départementale des Services Vétérinaires de Paris.

**Image Analyses.**

To measure the size of the pelvic ganglion on cryosections from E13.5 Olig2<sup>+/−</sup> and Olig2<sup>−/−</sup> embryos hybridized for Phox2b and immunostained for neurofilament, we used the open source image analyses tool ilastik (42). Pixels were segmented by a Random Forest Classifier into signal (corresponding to the pelvic ganglion) and background (corresponding to surrounding tissues and nerve fibers). Segmentation on one section was...
optimized through an iterative training procedure based on color/intensity, edge and
texture, and subsequently applied to the batch processing of all sections passing through
one pelvic ganglion. Local neighborhoods for calculating edge and texture were defined
as 3 X 3 pixels and 5 X 5 pixels. Finally, scattered signal areas smaller than 0.2μm² were
removed on FIJI. The remaining signal area corresponded to the pelvic ganglion and was
measured on 5 to 6 consecutive sections, depending on ganglia. The volume of the
ganglion was deduced by multiplying the surface by the thickness of the sections (20μm).
Wild-type and mutant ganglia were compared by a paired two-tailed Student’s t-test.
**Figure S1**

**NOS is not expressed neither in branchiomotor neurons nor in hindbrain preganglionic neurons.** Transverse sections of the hindbrain at E17.5 stained for diaphorase activity and Phox2b immunohistochemistry and passing through: (A) the facial nucleus (nVII); (B) the nucleus ambiguus (nA); (C) the dorsal nucleus of the vagus nerve (nX); (D) the pons, showing NOS+ neurons of the raphe (blue arrowhead). No double Phox2b+/NOS+ neurons were found in the hindbrain.
Figure S2

Expression of Tbx3 in all branchial and visceral motoneurons of the hindbrain. Longitudinal section though an E11.5 medulla, stained by combined Phox2b immunohistochemistry and Tbx3 in situ hybridization. In addition to nX (Fig. 2), Tbx3 is expressed in salivatory motoneurons (nSal) and the nucleus ambiguus (nA). Expression is also found in a subset of migrating facial motoneuronal precursors (red arrowheads). nVII: facial motor nucleus.
Maintenance at E16.5 of a parasympathetic genetic signature by cranial preganglionics and of a sympathetic genetic signature by both thoracic and sacral preganglionics. Transverse sections at E16.5 through the right half of the medulla (left column), thoracolumbar spinal cord (middle column) and sacral spinal cord (right column), stained with the indicated antibodies and probes. Arrowheads point to the nX in the left column and to spinal preganglionics in the middle and right columns.
Figure S4

Anatomical location of sympathetic and parasympathetic ganglia in mouse embryos at E11.5 and E13.5. (a-c) Parasagittal sections through a whole mouse embryo at E11.5 (a) or E13.5 (b,c), stained by immunohistochemistry for Phox2b. (d-f) Parasagittal sections through the urogenital region of an E13.5 embryo, showing different aspects of the pelvic ganglion. (d) is a higher magnification of the area boxed in (b). Red arrow: an intramural ganglion of the bladder. gg: ganglion; dmnX: dorsal motor nucleus of the vagus nerve; nTS: nucleus of the solitary tract. Scale bar: a-c, 1mm; d-f, 0.5mm.
**Figure S5**

**Pelvic and accessory ganglia at E16.5.** Two parasagittal sections through the bladder and the pelvic ganglion at E16.5 stained by immunohistochemistry for Phox2b. The main ganglion appears split in a number of lobes. As previously described (43), small ganglia or isolated Phox2b+ neurons can be seen in the wall of the bladder (red arrowheads), along the urethra (black arrowhead) and along the ureter (blue arrowhead).
Figure S6

**Sympathetic genetic signature of the adrenal medulla.** Parasagittal sections through the adrenal medulla at E13.5 stained with the indicated probes or antibodies. The transcriptional signature is Phox2b+/Gata3+/Hand1+/Islet+/Hmx2-/Hmx3-, thus sympathetic.
Figure S7

Expression of *Hmx2* and *Hmx3* in cardiac and ciliary ganglia. Parasagittal sections in an E13.5 embryo stained for immunohistochemistry against Phox2b and Hmx3 (left) or Hmx2 (right) in situ hybridization, showing expression of all three genes in the ciliary ganglion (upper panels) and the cardiac ganglia (lower panels).
**Figure S8**

**Pelvic and bladder intramural ganglia retain a sympathetic signature at E16.5.** Sagittal sections through parasympathetic ganglia (left), the lumbar paravertebral sympathetic chain (middle) and the pelvic ganglion (right) and intramural ganglia of the bladder (arrowheads in the right panels) at E16.5, stained by immunohistochemistry for Phox2b, a determinant of all autonomic ganglia (31), and in situ hybridization for the indicated probes. O: otic ganglion; S: sphenopalatine ganglion; SM: submandibular ganglion (all parasympathetic ganglia). By this stage Hmx2 expression has been partially downregulated in parasympathetic ganglia. Note that some intramural ganglia of the bladder have been previously shown to contain noradrenalin (43), in line with their sympathetic nature demonstrated here.
The ganglion of Remak has a sympathetic genetic identity. Transverse sections through a chicken embryo at 5 days post fertilization, passing through the hindgut. The ganglion of Remak (arrowhead) coexpresses Phox2b with the sympathetic markers Islet (detected by an Islet1-2 antibody) and Hand1, but not the parasympathetic marker Hmx3, which is expressed at the same stage in the ciliary ganglion (cg). Islet and Hand1 are also expressed in the mesenchymal wall of the gut (m).
Figure S10

The pelvic ganglion forms in the absence of the pelvic nerve. Wholemount immunofluorescence with the indicated antibodies on an Olig2<sup>−/−</sup> littermate of the E11.5 embryo shown in Fig. 3. In this embryo, no nerve projection is seen at all towards the pelvic ganglion, which nevertheless is present and indistinguishable from its counterpart in heterozygotes (see Fig. 3). L5, L6 and S1: fifth and sixth lumbar and first sacral roots. PG: pelvic ganglion.
Figure S11. Revised anatomy of the autonomic nervous system. The efferent path of the autonomic nervous system is made up of a spinal sympathetic outflow (in red) and a cranial parasympathetic outflow (in blue). III: oculomotor nerve; VII: facial nerve; IX: glossopharyngeal nerve; X: vagus nerve; gg: ganglion.
**Movie S1**

**The pelvic ganglion at E11.5 in a wild type.** The pelvic nerve (in green) reaches the rostral dorsal and lateral edge of the pelvic ganglion (that expresses Phox2b, in red), whose cells lie for the most part distal and medial to them.

**Movie S2**

**The pelvic ganglion at E11.5 in an Olig2 null mutant.** When all motoneurons are deleted, a vestigial pelvic nerve, made up exclusively of sensory fibers, barely touches the pelvic ganglion (that expresses Phox2b, in red), which has the same appearance and size than in wild type embryos (see Movie S1).
References


4.3 Discussion

In this study we demonstrated that the sacral autonomic outflow cannot be differentiated from the sympathetic thoracolumbar division of the autonomic nervous system, challenging an old-century dogma that allocated it to the parasympathetic division. Thus, we propose that the autonomic nervous system is divided in a cranial parasympathetic and a spinal sympathetic nervous system. This new understanding helps resolve the anatomical inconsistencies that I discussed in the introduction.

a. Physiological considerations

What are the consequences of the new assignment of the sacral outflow to the sympathetic division?

Physiologically, the sacral outflow was considered to have an opposite effect on some organs to that of the thoracolumbar sympathetic outflow. Langley in his ‘Presidential address, Section I—Physiology’, Report of British Association for 1899, pp. 881–92, provides one reason (“I may give one reason”) to consider the “Bulbar and sacral nerves as one system”: opposing action of the lumbar and sacral nerves “on some blood vessels” without any further precision. However and in contrast to the classical example of the opposite role of cranial parasympathetic versus thoracic sympathetic neurons on the heart, evidence of an antagonism between the thoracolumbar and sacral outflows on most pelvic organs are few and far between. The classically cited lumbo-sacral antagonism on the bladder detrusor muscle and particularly a lumbar sympathetic inhibition has been found non existent experimentally (Langley, 1895; De Groat, 1972) and it is known that the blockage of the lumbar input in humans has no functional consequence (acknowledged in Folwer, Griffiths and De Groat, 2008). Similarly, the belief in a parasympathetic/sympathetic antagonism in the pelvis has led to overlook the evidence for a role of the lumbar sympathetic input in erection (thus in blood vessel dilatation), generally presented as being mediated exclusively by sacral parasympathetic nerves. Already in 1979, Sjöstrand and Klinge stated the existence of the sympathetic hypogastric erectile fibers, but since then, the majority of reviews keep the dogma of an antagonism alive. On the contrary, there is evidence of a synergy of the lumbar
and sacral pathways for vasodilation in external reproductory organs (Jänig, 2006) that argues against an antagonism between them.

A more general consequence of the reclassification of the sacral autonomic outflow to the sympathetic division is that two functions, hitherto classified as parasympathetic, are in fact sympathetically controlled: voiding (of both urine and feces) and sexual arousal. These functions are performed by an awake, alert animal, and are counterproductive during periods such as sleep, in which the parasympathetic branch triggers rest and digest responses. In retrospect, it is not surprising that voiding and sexual arousal are activated by the sympathetic branch of the autonomic nervous system, which triggers fight or flight responses.

b. Molecular determinants of sacral preganglionic neurons

In this study we have defined a molecular signature of the sacral autonomic outflow using differential cranial parasympathetic and thoracic sympathetic markers. One genetic determinant of thoracic sympathetic VMs is not included in the study and deserves special mention here.

One of the rare transcriptional determinants of thoracolumbar sympathetic neurons studied prior to our work was a Hox gene. Indeed, the segregation of spinal motor neurons into discrete columns innervating different targets is established by regional domains of Hox genes activity. Preganglionic motor column (PGC) formation and innervation of sympathetic ganglia was found controlled by the expression of HoxC9 at thoracic levels through its interactions with the accessory factor FoxP1 (Dasen et al., 2003; Dasen et al., 2008; Jung et al., 2014). HoxC9 KO mice lack PGC and all thoracic motor neurons adopt a lateral motor column (LMC) identity, and project to limb muscles (Jung et al., 2010). In wild type mice HoxC9 restricts the expression of forelimb specification factors, including Hox6 and Hox8 paralogs (Dasen et al., 2003; Vermot et al., 2005), to branchial levels whereas at lumbar levels it is itself repressed by Hoxc10 and Hoxd10 (Shah et al., 2004; Wu et al., 2008; Rousso et al., 2008). In these studies, sacral PGC formation was not examined and I have performed some preliminary experiments, with the idea that like thoracolumbar preganglionic neurons, sacral ones could depend on HoxC9. I have analysed HoxC9 KO embryos (imported from the laboratory of Dr. Deneen M. Wellik in Michigan) for the presence of thoracic and sacral PGC
neurons. As expected, thoracic PGC neurons were absent in the \textit{HoxC9} KO (unpublished results, Fig. 27, red arrow in B), but sacral PGC were present (Fig. 27, black arrow in D). In retrospect, this difference is not surprising if we consider that in mice, at least from our observations, sacral PGCs extend from lower lumbar segment L5 to sacral S1 (instead of S2-S4 in humans) and that at lumbar levels \textit{HoxC9} is not expressed, but repressed by \textit{Hox10} paralogs. Instead, other \textit{Hox9} could be playing the same role during sacral PGC formation. In this respect, \textit{Hoxd9} expression boundary is displaced posteriorly to the lumbo-sacral transition whereas its paralogs are exclusively associated to the thoracic series (Burke et al., 1995), and thus it would be interesting to explore if sacral PGC neurons are affected in \textit{Hoxd9} mutants.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Presence of sacral preganglionic motor neurons in \textit{HoxC9} KO mice. Transverse sections through the thoracic (A and B) and sacral (C and D) spinal cord at E13.5 reacted for NADPH diaphorase activity (indicative of NOS expression by preganglionic neurons, black arrowheads) and Islet1,2 immunohistochemistry in the genetic backgrounds indicated on the left. In \textit{HoxC9} KO embryos, thoracic preganglionic neurons were absent (red arrowhead in B) whereas sacral preganglionic neurons were spared (black arrowhead in D).}
\end{figure}
c. Migration and molecular identity of sacral ganglionic neurons

We demonstrated that sacral ganglionic neurons form pelvic ganglia independently of their future preganglionic nerves (Publication-Figure 3), unlike parasympathetic ganglia such as the sphenopalatine, submandibular, otic or cardiac ganglia that arise from nerve-associated Schwann cell precursors (SCPs) (Chapter 2-Publication). However, the molecular mechanisms responsible for sacral neural crest migration to coalesce into pelvic ganglia nearby the urogenital sinus are unknown. There is only one study that points to the role of ErbB3, in which pelvic ganglia are missing when analysed at postnatal day 1 (P1) (Dyachuck et al., 2014, Fig. S2-D, E). The authors present this piece of evidence as yet another example of parasympathetic ganglia derivation from nerve-associated, ErbB3-dependent, SCPs. However, we found that pelvic neurons have a sympathetic nature and do not require the nerve to form the ganglia. Along with its role in SCPs migration, ErbB3 and its ligand Neuregulin-1 are involved in early stages of sympathetic ganglia formation (Britsch et al., 1998; Saito et al., 2012 and Chapter 3 of this manuscript) and the absence of pelvic ganglia at P1 shown by Dyachuck et al. could reflect such a role. Alternatively, the lack of ErbB3 could cause the late atrophy of pelvic neurons. I recently observed that after conditional inactivation of ErbB3 in neural crest derivatives using a Wnt1::Cre; ErbB3 lox/lox genetic background analysed at E13.5, pelvic ganglia were present but appeared massively atrophic (unpublished results, Fig. 28). This suggests that ErbB3 is necessary for pelvic neuron maintenance, although we cannot rule out a migration defect. A more detailed analysis of the migration at earlier stages of development in this mutant background should be performed to distinguish between both possibilities.

The previous definition of a mixed sympathetic-parasympathetic pelvic ganglion was based on connectivity, i.e. on the nature of preganglionic neurons, that we now show are all sympathetic. Regardless, we also provided a cell-intrinsic criterion that confirmed the sympathetic nature of pelvic postganglionic neurons: they express the sympathoadrenal markers Islet1, Gata3 and Hand1 but not the parasympathetic markers Hmx2 and Hmx3. It is important to notice that the paralogous homeobox genes Hmx2 and Hmx3 were previously implicated in vertebrate inner ear and hypothalamus development (Wang et al., 2004; Feng and Xu, 2010), but their role during parasympathetic gangliogenesis is unknown and here we provide the first evidence that they represent differential markers of the parasympathetic lineage.
**Figure 28. Pelvic ganglion atrophy in the absence of ErbB3 signalling.** (A and B) Parasagittal sections through the urogenital region in the indicated genetic backgrounds at E13.5, stained by in situ hybridization for *Peripherin* and immunohistochemistry for Phox2b. Pelvic ganglionic neurons are present but massively atrophic in a *Wnt1::Cre;ErbB3*<sup>lox/lox</sup> embryo (red arrowhead in B) while compared to the wild-type (black arrowhead in A). ENS: enteric nervous system.

**d. Evolutionary considerations**

The classical subdivision of the autonomic nervous system by Langley (1989, 1921) into a thoraco-lumbar sympathetic, cranio-sacral parasympathetic and enteric portions stemmed mainly from anatomical studies in mammals, by far the best-known vertebrates. However, several studies in non-mammalian vertebrates found little evidence supporting that classification (Nilsson, 1983). For instance, Young (1933c, 1936) and Lutz (1931) claimed that based on the data in elasmobranch and teleost fishes, a physiological distinction between the sympathetic and the parasympathetic divisions of the autonomic nervous system could not be made. In anuran amphibians, Langley and Orbeli (1911) distinguished between ‘sympathetic’ and ‘parasympathetic’ spinal autonomic nerves based on a gap in the outflow at the level of the eighth spinal nerve. Later, however, Burnstock (1969) and Pick (1970) expressed their doubts about using this gap at spinal levels to distinguish a sacral parasympathetic system in amphibians. Their doubts stemmed from studies of the innervation of the toad urinary bladder, which did not show any clearcut physiological differences
between the thoraco-lumbar ‘sympathetic’ and sacral ‘parasympathetic’ excitatory fibers (Burnstock et al., 1963; Boyd et al., 1964). Moreover, they stated ‘In groups lower than amphibia, it seems highly likely that there are also gaps in the spinal autonomic outflow to the viscera, but this does not appear to be sufficient evidence in itself for defining a separate sacral parasympathetic system’ (Burnstock, 1969).

In line with these previous speculations our study reveals that also in mammals and birds, the sacral outflow cannot be distinguished from the thoraco-lumbar outflow, dividing the autonomic nervous system into a cranial parasympathetic and a spinal sympathetic autonomic system.

e. Perspectives

What are the consequences of this anatomical reassignment for future studies?

- Our discovery is an invitation to re-examine past physiological data, without the conceptual filter of the sympathetic/parasympathetic antagonism. As in the examples provided earlier, this might lead to realize that some facts have been overlooked and others have been overemphasized or exaggerated.

- The complexity of pelvic organ regulation must reside in the connectivity, within the sympathetic nervous system. Our study is an invitation to explore this connectivity with modern means, and can facilitate this task by providing transcriptional signatures of different neuronal classes, to use in transgenic approaches to map connectivity (for example with recombinant defective rabies viruses).

- During further studies of connectivity or neuronal diversity in the sacral autonomic nucleus or in the pelvic ganglion, parallels and comparisons will be more fruitfully made with the rest of the sympathetic system, not with the cranial parasympathetic system.

- Any attempt at cell replacement therapies requires knowledge of the type of cells to be replaced. If these therapies are one day applied to the pelvic ganglion, often damaged after accidents or surgery, it is essential that they involve sympathetic, not parasympathetic neurons.
Conclusion

In the present work, I uncovered different developmental strategies used by autonomic precursors to build functional circuits, and found some unifying principles among the divisions of the autonomic nervous system. Postganglionic parasympathetic neurons were shown to arise from Schwann cell precursors (SCPs) that migrate along their future preganglionic partners, projecting in cranial nerves, to occupy scattered positions close to their target viscera (Espinosa-Medina et al., 2014), while postganglionic sympathetic neurons, from cervical to sacral levels migrate independently of their preganglionic spinal counterparts and form prevertebral, paravertebral and pelvic ganglia (studies by other authors and Espinosa-Medina et al., 2016). The enteric nervous system, which receives input from both parasympathetic and sympathetic nerves, was shown to share the developmental strategies present in both systems. These conclusions raise new questions:

What factors favour the restriction of SCPs to a parasympathetic fate rather than a sympathetic fate and which signals elicit the formation of the ganglia? Can parasympathetic precursors differentiate into other non-neuroglial crest derivatives or do they represent a partially restricted population of SCPs? What signals make them stop their migration along nerves?

Do “vagal SCPs” or “cervical sympathetic-like” enteric precursors generate different subtypes of mature enteric neurons and what is the correlation to extrinsic circuit formation?

What organs and molecules attract the pelvic ganglion precursors to its final location (since there is no aorta at that level)? What factors elicit the differentiation of some pelvic ganglionic neurons into cholinergic and others into noradrenergic neurons? Is there a further diversity of pelvic ganglion neurons?

Hopefully, these and many other questions derived from the work presented here would be answered in the future, but as S.R.Cajal said: ‘While awaiting the work of the future, let us be calm and confident in the future of our work [...]. If future science reserves big surprises and wonderful conquests for us, it must be supposed that she will complete and develop our knowledge indefinitely, while still starting from the present facts’. 
Annex: Neuroanatomy of cranial nerves

Cranial nerve III: Oculomotor nerve

Cranial nerve VII: Facial nerve

nTS: nucleus of the solitary tract; SS: superior salivatory; nV: nucleus of the V cranial nerve; GSPN: greater superficial petrosal nerve; Spg: sphenopalatine ganglion; Smg: submandibular ganglia.
Cranial nerve IX: Glossopharyngeal nerve

Cranial nerve X: Vagus nerve

nTS: nucleus of the solitary tract; IS: inferior salivatory; nV: nucleus of the V cranial nerve; dmX: dorsal motor nucleus of the X cranial nerve.


Estudio del desarrollo de los sistemas nerviosos parasimpático, entérico y sacro

Las células de la cresta neural migran extensamente y forman el sistema nervioso autónomo, que incluye los ganglios parasimpáticos, simpáticos y entéricos, encargados de regular la homeostasis. En este trabajo, investigo las migraciones, interacciones neuronales y dependencias moleculares durante la formación de los circuitos nerviosos autónomos. Demuestro que los precursores de los ganglios parasimpáticos derivan de los precursores de las células de Schwann (SCPs), que invaden los nervios preganglionares hasta su destino, próximo a sus órganos diana (Espinosa-Medina et al. 2014). De esta forma, los SCPs producen no sólo células gliales, melanocitos, fibroblastos y células dentarias mesenquimatosas, como ya se sabía, sino además, neuronas. Por otra parte, muestro un paralelismo entre el mecanismo de migración de los precursores parasimpáticos y el de una población de precursores del sistema nervioso esofágico, que migran a lo largo del nervio Vago. Muestro que el resto del sistema nervioso esofágico y sistema nervioso entérico propiamente dicho derivan de la cresta neural cervical simpática. Finalmente, propongo una revisión del sistema nervioso sacro, que regula las funciones urinaria, digestiva y reproductora y que ha sido considerado como parasimpático desde hace más de un siglo, sin fundamento molecular. Presento una firma molecular que permite distinguir las neuronas parasimpáticas craneales y las neuronas simpáticas toraco-lumbares y demuestro que el plexo sacro es “simpático”. En consecuencia, el sistema nervioso autónomo comprende tres divisiones bien diferenciadas entre sí tanto por su origen embrionario como por su anatomía adulta: una parasimpática de origen y conectividad exclusivamente craneal, una simpática de origen y conectividad espinal desde la zona cervical hasta el sacro (Espinosa-Medina et al. 2016), y una división entérica en la que tanto su origen como su conectividad la sitúan a caballo entre los sistemas simpático y parasimpático.

Palabras clave: Sistema nervioso autónomo; cresta neural; parasimpático; entérico; sacro.
Résumé:

**Sur le développement des systèmes nerveux parasympathique, entérique et sacré**

Les cellules de la crête neurale migrent extensivement et forment le système nerveux autonome comprenant les ganglions parasympathiques, sympathiques et entériques, qui maintiennent l'homéostasie. Dans cette étude, j’explore les migrations, interactions neuronales et dépendances moléculaires lors de la formation des circuits nerveux autonomes. Je démontre que les précurseurs des ganglions parasympathiques dérivent des précurseurs des cellules de Schwann (SCPs) qui envahissent les nerfs préganglionaires jusqu’à leur destination, proche des organes cibles (Espinosa-Medina et al., 2014). Ainsi, les SCPs produisent non seulement de la glie, des mélanocytes, des fibroblasts et des cellules dentaires mésenchymateuses, comme on le savait déjà, mais aussi des neurones. D’autre part, je montre un parallélisme entre le mécanisme de migration des précurseurs parasympathiques et celui d’une population de précurseurs du système nerveux œsophagien, qui migrent le long le nerve vague. Je montre que le reste du système œsophagien et le système nerveux entérique proprement dit dérivent de la crête neurale cervicale sympathique. Enfin, je propose un réexamen du système nerveux sacré, qui régule les fonctions urinaire, digestive et reproductrice et qui est considéré comme parasympathique depuis plus d’un siècle, sans argument moléculaire. Je présente une signature moléculaire pour distinguer les neurones parasympathiques crâniens et les neurones sympathiques thoraco-lombaires et démontre que le système nerveux sacré est en fait sympathique. En conséquence, le système nerveux autonome est composé de trois divisions contrastées par leur origine embryonnaire aussi que leur anatomie adulte: une parasympathique d’origine et de connectivité exclusivement crâniennes, une sympathique d’origine et de connectivité spinale, allant de l’étage cervical au sacré (Espinosa-Medina et al., 2016) et une division entérique que son origine aussi bien que sa connectivité placent à l’interface des systèmes sympathique et parasympathique.

**Mots clés:** Système nerveux autonome; crête neurale; parasympathique ; entérique ; sacré.
Summary:

On the development of the parasympathetic, enteric and sacral nervous systems

Neural crest cells migrate extensively to form the autonomic nervous system including sympathetic, parasympathetic and enteric ganglia essential for regulating bodily homeostasis. In the present work, I explore the migratory mechanisms and neuronal interactions during autonomic circuit assembly, as well as their molecular dependencies. I show that parasympathetic ganglia derive from Schwann cell precursors (SCPs) and migrate along their preganglionic nerves to locate close to their target tissues (Espinosa-Medina et al., 2014). SCPs were previously known to give rise to melanocytes, endoneurial fibroblasts and dental mesenchymal cells as well as Schwann cells in vivo, and this study extends their fate to neurons. In line with this work, I show that vagal-associated SCPs give rise to part of the oesophageal nervous system, whereas cervical sympathetic-like crest cells colonize all the gastrointestinal tract, demonstrating a dual origin and different migration mechanisms for enteric neurons. Finally, I revise the identity of the sacral autonomic outflow, whose allocation to the parasympathetic nervous system has been accepted for a century. Sacral autonomic neurons control rectal, bladder, and genital functions and analysis of their cellular phenotype was lacking. Here I present a differential molecular signature for cranial parasympathetic versus thoraco-lumbar sympathetic neurons and show that, in this light, the sacral autonomic outflow is sympathetic. Accordingly, the parasympathetic nervous system receives input from cranial nerves exclusively and the sympathetic nervous system from spinal nerves, thoracic to sacral inclusively (Espinosa-Medina et al., 2016). Interestingly the enteric nervous system, which receives input from both sympathetic and parasympathetic nerves, shares with each system aspects of its ontogeny.

Keywords: Autonomic nervous system; neural crest; parasympathetic; enteric; sacral outflow.