

# The sacral autonomic outflow is sympathetic

**Isabel Espinosa-Medina<sup>1,†</sup>, Orthis Saha<sup>1,†</sup>, Franck Boismoreau<sup>1</sup>, Zoubida Chettouh<sup>1</sup>, Francesca Rossi<sup>1</sup>, William D. Richardson<sup>2</sup> and Jean-François Brunet<sup>1,\*</sup>**

<sup>1</sup> Institut de Biologie de l'ENS (IBENS), INSERM, CNRS, École Normale Supérieure, PSL Research University, Paris, 75005 France.

<sup>2</sup> Wolfson Institute for Biomedical Research, University College London, London UK.

†These authors contributed equally to this work

\*Correspondence to [jfbrunet@biologie.ens.fr](mailto:jfbrunet@biologie.ens.fr)

## Abstract

In the autonomic nervous system of mammals and birds, sacral preganglionic neurons are considered parasympathetic, as are their targets in the pelvic ganglia that prominently control rectal, bladder and genital functions. The allocation of the sacral autonomic outflow to the parasympathetic nervous system —i.e. as the second tier of a “cranio-sacral outflow”— has an ancient history: rooted in the work of Gaskell<sup>1</sup>, formalized by Langley<sup>2</sup> and universally accepted ever since (e.g. <sup>3</sup>). The rationale lied in several perceived similarities between the sacral and cranial outflows: anatomical —separation from the thoracolumbar, sympathetic outflow by a gap at limb levels<sup>1</sup>, a target territory less diffuse than that of the latter and a lack of projections to the paravertebral sympathetic chain<sup>1</sup>; physiological —an influence on some organs opposite to that of the thoracolumbar outflow<sup>4</sup>; and pharmacological —an overall sensitivity to muscarinic antagonists<sup>2</sup>. However, cell-phenotypic criteria have been lacking and were never sought. Here we uncover fifteen phenotypic and ontogenetic features that distinguish pre- and postganglionic neurons of the cranial parasympathetic outflow from those of the thoracolumbar sympathetic outflow. By every single one, the sacral outflow is indistinguishable from the latter. Thus the parasympathetic nervous system is associated with cranial nerves exclusively and the sympathetic with spinal nerves, from thoracic to sacral levels inclusively. This simplified bipartite architecture of the autonomic nervous system offers a new framework to understand its development and evolution, as well as pelvic neurophysiology.

## Main text

Cranial parasympathetic preganglionic neurons (or visceromotor (VM) neurons) are born in the pMNv domain progenitor domain of the hindbrain<sup>5</sup> that expresses the homeogene *Phox2b* and produces, in addition to VM neurons, branchiomotor (BM) ones<sup>6</sup>. The postmitotic precursors migrate dorsally<sup>7</sup> to form nuclei —such as the dorsal motor nucleus of the vagus nerve (dmnX)— or looser neuronal groups —such as the salivatory nuclei or the diffuse part of the nucleus ambiguus. They project through dorsolateral exit points<sup>7</sup> and course in several branches of the cranial nerves to innervate parasympathetic and enteric ganglionic neurons. In contrast, thoracic and upper lumbar (hereafter “thoracic” for short) VM neurons, which are sympathetic preganglionic neurons, are thought to have a common origin with somatic motoneurons<sup>8,9</sup>. By implication, they would be born in the pMN domain (just dorsal to p3) —thus from progenitors that express *Olig2*<sup>10</sup>, although this has never been directly tested. They then segregate from somatic motoneurons by migrating dorsally to form the intermediolateral column in mammals<sup>11</sup> and the column of Terni in birds<sup>12</sup>. They project in the ventral roots of spinal nerves together with axons of somatic motoneurons, then veer ventralwards to form the white *rami communicantes* and synapse onto the ganglionic cells of the paravertebral ganglia and prevertebral sympathetic plexi. Thus, available data suggest that parasympathetic and sympathetic preganglionic neurons have contrasted ontogenies and are genetically related to branchiomotor and somatic motoneurons, respectively.

We sought to compare the genetic make up and dependencies of lower lumbar and sacral (hereafter “sacral” for short) VM neurons with that of cranial (parasympathetic) and thoracic (sympathetic) ones. As representative of hindbrain VM neurons we focused on the dorsal motor nucleus of the vagus nerve (nX), a cluster of neurons already well delineated at E13.5, ventromedial to the nucleus of the solitary tract and dorsal to the hypoglossal nucleus, that expresses the vesicular acetylcholine transporter (*VACHT*) (Fig. 1a). Thoracic and sacral VM neurons, that both form a mediolateral column in the spinal cord did not express *VACHT* at this stage despite their eventual cholinergic nature, shared with all motoneurons. We thus used their common marker Nitric Oxide Synthase (NOS)<sup>13</sup>. Of note, NOS was absent from the dmnX at E13.5 (Fig. 1b), or later (Extended data Fig.1) — making NOS a differential marker of both, thoracic and sacral, versus cranial VM neurons.

We first established that, in contrast to cranial (parasympathetic) VM neurons, thoracic (sympathetic) ones not only failed to express *Phox2b* or its paralogue *Phox2a* at E13.5, but arose from *Phox2b*-negative progenitors and did not depend on *Phox2b* for their differentiation (Fig.1c-f). Conversely, and also in contrast to hindbrain VM neurons, they depended on *Olig2*<sup>+</sup> for their differentiation (Fig. 1g). At sacral levels, VM neurons did not express *Phox2b* or *Phox2a* at E13.5, did not arise from *Phox2b*-expressing progenitors and did not depend on *Phox2b*, but depended instead on *Olig2* (Fig.1b-g). Moreover, at E13.5, the T-box transcription factors *Tbx20*, *Tbx2* and *Tbx3* were expressed by cranial (parasympathetic) but neither thoracic (sympathetic) nor sacral VM neurons (Fig. 2a and Extended Data Fig. 2). Conversely, we found the F-box transcription factor *Foxp1*, a marker and determinant of thoracic VM neurons<sup>14</sup> to be also expressed by sacral but not cranial VM neurons (Fig. 2b). In sum, the ontogeny and transcriptional signature of sacral VM neurons was indistinguishable from that of thoracic VM neurons by the criteria that differentiate them from cranial VM neurons — thus was of a sympathetic nature.

Of note, thoracic and sacral VM neurons, in addition to their location in the mediolateral region of the spinal cord, share a ventral exit point for their axons, whereas cranial VM neurons have a dorsolateral one, another common trait and difference from cranial VM neurons, somehow not mentioned by Gaskell<sup>1</sup> or taken into consideration since.

The peripheral targets of the sacral VM neurons are located in the pelvic plexus—which, in some species, such as the mouse, is condensed into a bona fide ganglion—and are considered, by definition, parasympathetic<sup>25</sup>. Since a proportion of pelvic ganglionic neurons receive input from upper lumbar levels (half of them in rats<sup>26</sup>) thus from sympathetic VMs, the pelvic ganglion is considered mixed, sympathetic and parasympathetic<sup>27</sup>. This connectivity-based definition runs into a conundrum for cells that receive a dual lumbar/sacral input<sup>18</sup>. The sympathetic identity of both thoracolumbar and sacral VM neurons that we unveil here makes the issue moot. Regardless, we looked for a cell-intrinsic criterion that would corroborate the sympathetic nature of all pelvic ganglionic neurons, 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 since cholinergic neurons in the pelvic ganglion comprise both “parasympathetic” and “sympathetic” ganglionic cells as defined by the criterion of connectivity<sup>25</sup> and bona fide sympathetic neurons of the paravertebral chain are cholinergic (reviewed in ref<sup>29</sup>). However, we found that three transcription factors expressed and required in sympathoblasts: *Islet1*<sup>20</sup>, *Gata3*<sup>21</sup> and *Hand1*<sup>22</sup>, were not expressed in parasympathetic ganglia such as the sphenopalatine, the submandibular or the otic ganglia (Fig. 3). Conversely, we found that the two paralogous homeobox genes *Hmx2* and *Hmx3* are specific markers of parasympathetic versus sympathetic ganglia (Fig. 3 and Extended data Fig. 3). All cells of the pelvic ganglion were *Islet1*<sup>+</sup>, *Gata3*<sup>+</sup>, *Hand1*<sup>+</sup>, *Hmx3*<sup>−</sup> and *Hmx2*<sup>−</sup> (Fig. 3), thus had a sympathetic molecular fingerprint. Similarly, the chicken ganglion of Remak, which runs in the mesentery dorsal to the gut and is continuous with the pelvic ganglion with which it shares a sacral input, and is classically considered parasympathetic<sup>23</sup>, displayed an *Islet1*<sup>+</sup>, *Hand1*<sup>+</sup>, *Hmx3*<sup>−</sup> signature, thus sympathetic (Extended data Fig. 4).

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<sup>+</sup>/Phox2b<sup>+</sup> Schwann cell precursors along their future preganglionic nerve to the site of ganglion formation and do not form if these nerves are absent<sup>24,25</sup>. At E11.5, the lumbosacral plexus, that gives rise to the pelvic nerve, extended some fibres that reached the external and caudal edge of the pelvic ganglion anlagen, most of which lied well ahead of them (Fig. 4a). These fibres were coated with Sox10<sup>+</sup> cells, none of which, though, expressed Phox2b (Fig. 4b), in contrast to the cranial nerves that produce parasympathetic ganglia at the same stage (Fig. 4e). Deletion of all motor fibres in *Olig2*<sup>Cre/cre</sup> embryos spared only two thin, presumably sensory, projections from the lumbosacral plexus (Fig. 4c), also devoid of Phox2b<sup>+</sup> cells (Fig. 4d). Occasionally this residual sensory projection was not even seen (Extended data Fig. 5). Despite this massive atrophy, the pelvic ganglion appeared intact (Fig. 4c and Extended data Fig. 5 and Mov.1,2). This was verified quantitatively at E13.5 (Fig. 4f,g,h). Thus, even though 50% of its cells are post-ganglionic to the pelvic nerve, the pelvic ganglion forms before and independently of it, as befits a sympathetic ganglion—but contrary to parasympathetic ones.

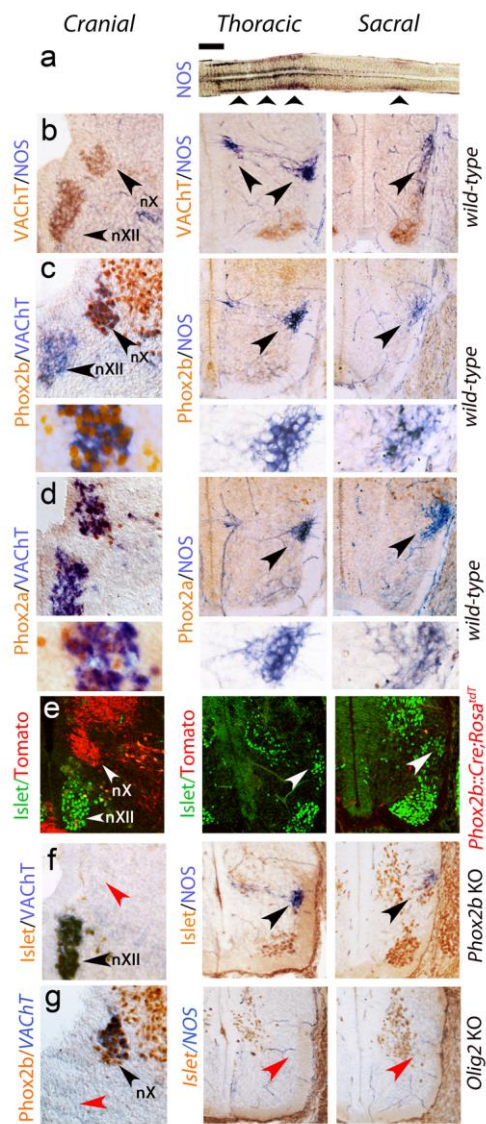
Altogether, our molecular and developmental data reveal the sacral visceral nervous system to be the caudal outpost of the sympathetic outflow. Comparative studies have found a sacral parasympathetic outflow hard to delineate in fish and amphibians, suggesting that the ancestral pattern was a dichotomy between a cranial and a spinal autonomic system, the latter encompassing both, Langley’s sympathetic and his “sacral parasympathetic” outflows<sup>26</sup>. Our data vindicates this speculation and show that this pattern (Extended data Fig. 6, schematic to be added) is unchanged in mammals and probably birds.

In retrospect, the mis-allocation of the sacral autonomic outflow to the parasympathetic division of the autonomic nervous system sat uncomfortably with many anatomic and physiological data, better accommodated by the revision we propose. To take but four examples: first, although schematics (e.g.<sup>3</sup>) generally represent the sacral pathway to the distal colon and rectum as disynaptic—i.e. vagal-like—, it is in fact predominantly<sup>27</sup> if not exclusively<sup>28</sup> trisynaptic—i.e. sympathetic-like<sup>29</sup>. Second, the dual lumbar-sacral input to the pelvic ganglion is shared by the inferior mesenteric ganglion<sup>30</sup>, which is nevertheless considered exclusively sympathetic. Third, the lumbar inhibition and sacral excitation of the bladder detrusor muscle have remained, to this day, a paradigm of sympathetic/parasympathetic antagonism, even though most reports find the former absent<sup>4</sup> or of dubious functional relevance<sup>31</sup>. Finally, numerous observations in animal models and humans point to a synergy of the lumbar and sacral pathway for vasodilatation in external sexual organs (reviewed in ref<sup>29</sup>), which suggests a continuity of action across the gap between the thoracolumbar and sacral outflows.

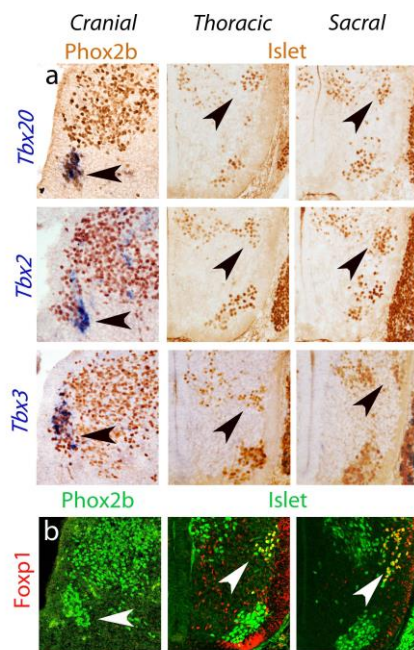
If there are bona fide differences in action of the thoracic and sacral outflow on common pelvic targets, they will have to be understood outside the framework of the sympathetic/parasympathetic duality, which does not exist at that level. The sympathetic identity of all sacral and pelvic autonomic neurons, which our data unveil, is bound to provide a more coherent framework than previously available for past and future discoveries on pelvic neuroanatomy and physiology, and is relevant to any prospect of cell replacement therapy of urogenital or rectal neurological dysfunction.

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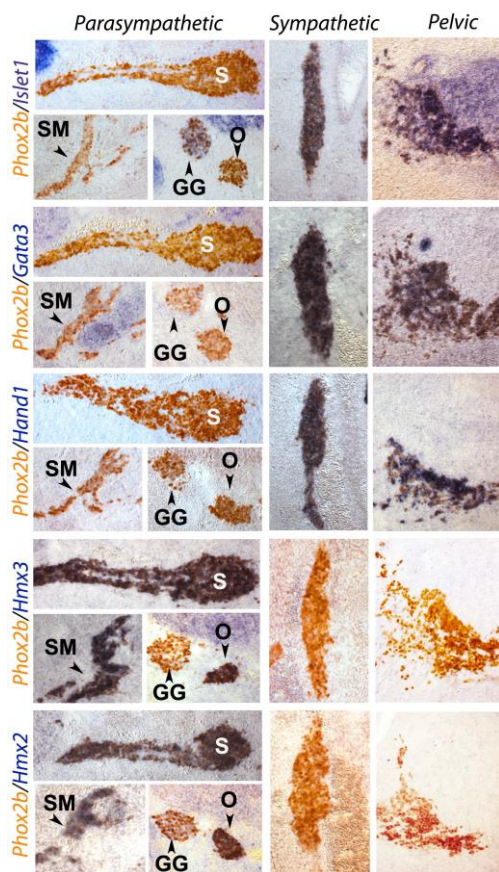
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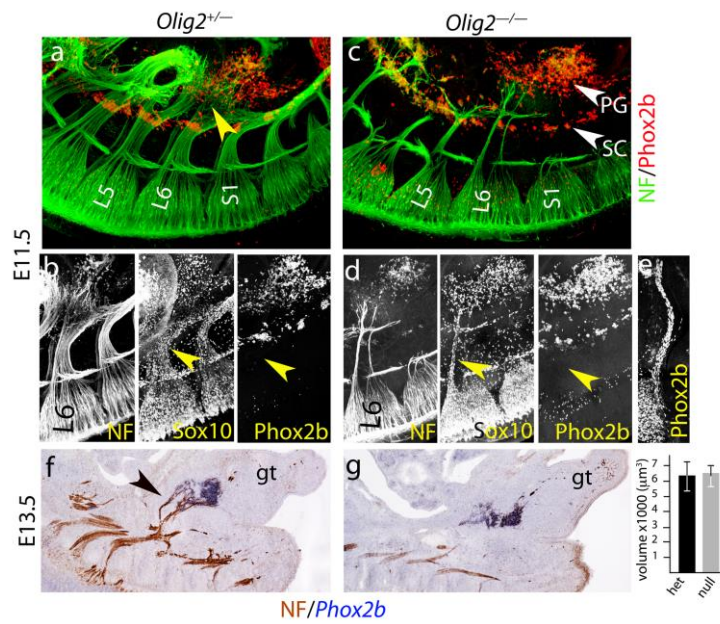
**Figure 1 Sacral preganglionic neurons develop like sympathetic, not parasympathetic ones.** **a.** Longitudinal thick section of the spinal cord reacted for NADPH diaphorase activity indicative of NOS expression, revealing the thoracolumbar and sacral visceromotor columns (arrowheads) separated by a gap. **(b-g)** Transverse sections at E13.5 through the medulla (left), 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.** At E13.5 the dorsal motor nucleus of the vagus nerve (nX) expresses *VACHT* but not NOS, while the thoracic and sacral preganglionic neurons (arrowheads) express NOS but not yet *VACHT*. The ventrally located somatic motoneurons, including nXII in the hindbrain, express *VACHT*. **c,d.** *Phox2b* (c) and *Phox2a* (d) are expressed in nX at E13.5, but in neither thoracic nor sacral preganglionic neurons (arrowheads). Lower panels in **b** and **c**: higher magnifications of the preganglionic neurons. **(e)** Neurons of nX but neither thoracic nor sacral preganglionic ones (labelled by an anti-*Islet1/2* antibody, white arrowheads) derive from *Phox2b*<sup>+</sup> precursors, permanently labeled in a *Phox2b::Cre;Rosa<sup>tdT</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 (ref arrowheads). The hypoglossal nucleus (nXII), is also missing, as expected of a somatic motor nucleus (asterisk). nTS: nucleus of the solitary tract. Scale bars, 1mm (**a**), 100- $\mu$ m (**b-g**).



**Figure 2 Sacral preganglionic neurons have a sympathetic, not parasympathetic, transcriptional signature.** Transverse sections of E13.5 wild type embryos, passing through the medulla (left), thoracolumbar spinal cord (middle) and sacral spinal cord (right), stained with the indicated antibodies and probes. **a.** *Tbx20*, *Tbx2* and *Tbx3* are expressed in all or a subset of nX neurons, but in no thoracic or sacral preganglionic neuron. **b.** *Foxp1* is not expressed in the nX, but is a marker of both thoracic and sacral preganglionic neurons.



**Figure 3. All pelvic ganglionic cells have a sympathetic, not parasympathetic, transcriptional signature.** Sagittal sections through parasympathetic ganglia (left), the paravertebral sympathetic chain (middle) and the pelvic ganglion (right) at E13.5, stained by immunohistochemistry for Phox2b, a determinant of all autonomic ganglia<sup>32</sup>, 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).



**Figure 4. The pelvic ganglion forms independently of its nerve, like sympathetic and unlike parasympathetic ones. a,b.** Wholemount immunofluorescence with the indicated antibodies on E11.5 embryos either heterozygous (a) or homozygous (b) 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 (b) spares two thin sensory pelvic projections. The pelvic ganglion lies ahead of most fibers in both heterozygous and mutant background. c,d. View of the L6 nerve, covered with *Sox10*<sup>+</sup> cells but no *Phox2b*<sup>+</sup> cells (yellow arrowheads), unlike cranial nerves that give rise to parasympathetic ganglia, at the same stage (Jacobson's nerve in e). f,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. h, the pelvic ganglion has the same volume whether its preganglionic nerve is present or not ( $6369 \mu\text{m}^3 \pm 1066$  versus  $6441 \mu\text{m}^3 \pm 919$ ,  $p=0.96$ ,  $n=5$ ). gt, genital tubercle; PG: pelvic ganglion; SP, sympathetic chain.

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## Contributions

I. E.-M., F.B. and F.R. analyzed the central nervous system, O.S. and Z.C., analyzed the peripheral nervous system. J.-F.B. designed the study and supervised the project; W.D.R. provided the *Olig2* KO line; J.-F.B. wrote the manuscript. All authors commented on the manuscript.

## Competing financial interests

The authors declare no competing financial interests.