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Margaux Sivori, Bowen Dempsey, Zoubida Chettouh, Franck Boismoreau, Maïlys Ayerdi, Annaliese Nucharee Eymael, Sylvain Baulande, Sonia Lameiras, Fanny Culpier, Olivier Delattre, Hermann Rohrer, Olivier Mirabeau, Jean-François Brunet 

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Abstract

The pelvic organs (bladder, rectum and sex organs) have been represented for a century as receiving autonomic innervation from two pathways — lumbar sympathetic and sacral parasympathetic — by way of a shared relay, the pelvic ganglion, conceived as an assemblage of sympathetic and parasympathetic neurons. Using single cell RNA sequencing, we find that the mouse pelvic ganglion is made of four classes of neurons, distinct from both sympathetic and parasympathetic ones, albeit with a kinship to the former, but not the latter, through a complex genetic signature. We also show that spinal lumbar preganglionic neurons synapse in the pelvic ganglion onto equal numbers of noradrenergic and cholinergic cells, both of which therefore serve as sympathetic relays. Thus, the pelvic viscera receive no innervation from parasympathetic or typical sympathetic neurons, but instead from a divergent tail end of the sympathetic chains, in charge of its idiosyncratic functions.

eLife assessment

This **useful** study compares gene expression patterns among different autonomic ganglia and will be of interest to developmental neuroscientists and neurophysiologists. The study expands the database of genes expressed by subpopulations of autonomic neurons in ganglia, a key step in decoding their developmental origins and physiological functions. The evidence supporting the alternative view that the pelvic ganglionic neurons are actually modified sympathetic neurons is **incomplete** and may cause confusion, given the enrichment of cholinergic neurons, as well as the large number of molecular and functional differences known to be present between cranial and sacral neurons.

Introduction

The pelvic ganglion is a collection of autonomic neurons, close to the walls of the bladder, organized as a loose ganglionated plexus (as in humans) or a bona fide ganglion (as in mouse). It receives input from preganglionic neurons of the thoracolumbar intermediate lateral motor column, a sympathetic pathway that travels through the hypogastric nerve. A second input is from the so-called “sacral parasympathetic nucleus” that projects through the pelvic nerve. The assignment of these two inputs to different divisions of the autonomic nervous system by John Langley (1 [↗](#), 2 [↗](#)), largely based on physiological observation on external genitals which were never generally accepted (reviewed in (3 [↗](#))), has led, in turn, to propose that the pelvic ganglion, targeted by both pathways, is of a mixed sympathetic/parasympathetic nature (4 [↗](#)). Of note, this unique case of anatomic promiscuity between the two types of neurons poses a challenge for the schematic representation of the autonomic nervous system, so that the pelvic ganglion is most often omitted from such representations (3 [↗](#)). A duality of the pelvic ganglion was also suggested by the coexistence of noradrenergic and cholinergic neurons, which elsewhere in the autonomic nervous system form, respectively, the vast majority of sympathetic ganglionic cells and the totality of parasympathetic ones. Here we directly explore the composition of the mouse pelvic ganglion in cell types, using single-cell transcriptomics, and compare it to sympathetic and parasympathetic ganglia.

Results

We isolated cells from several autonomic ganglia of postnatal day 5 mice: the stellate ganglion and the lumbar chain (both belonging to the paravertebral sympathetic chain), the coeliac+mesenteric ganglia (belonging to the prevertebral ganglia), the sphenopalatine ganglion (parasympathetic) and the pelvic ganglion, and processed them for single-cell RNA sequencing (cf Material and Methods). Neuronal cells segregated in three large ensembles (**Fig. 1A** [↗](#); **Fig. S1** [↗](#)): one that contained all sympathetic neurons, one that contained all parasympathetic neurons, and the third that contained most pelvic neurons — except one subset that segregated close to the sympathetic cluster. Thus, no pelvic neuron segregates with parasympathetic neurons, but the great majority of them do not segregate with sympathetic neurons either.

The separation, on the UMAP, of most pelvic from all other ganglionic cells contrasts with the suite of 5 developmental transcription factors that we previously reported as differentially expressed between the sympathetic and pelvic ganglia on one hand, and parasympathetic ganglia on the other (5 [↗](#)). To explore this conundrum, we searched for more genes that would help place the pelvic ganglion relative to the sympatho-parasympathetic dichotomy: additional genes that would put the pelvic ganglion in the sympathetic category (as per our previous findings), genes that would put it in a class by itself (as the UMAP suggests), or genes that would split pelvic neurons into parasympathetic-like and sympathetic-like clusters (as the current dogma implies).

In an unbiased approach, we sought genes expressed in a higher proportion of cells in any set of ganglia compared to its complementary set (see Material and Methods). To do justice to the classical notion that the pelvic ganglion is heterogeneous, i.e. mixed sympatho/parasympathetic, we treated the clusters of pelvic ganglionic cells, 4 in our conservative estimate (P1-4) (**Fig. 1A** [↗](#); **Fig. S1** [↗](#)) as 4 ganglia, alongside the 4 other ganglia (sphenopalatine, stellate, coeliac and lumbar) — i.e. we made $(2^8-2) = 254$ comparisons and analyzed the 100 “top” genes, i.e. with the highest discrimination score, irrespective of the comparison scored (Table S1, S2, S3).

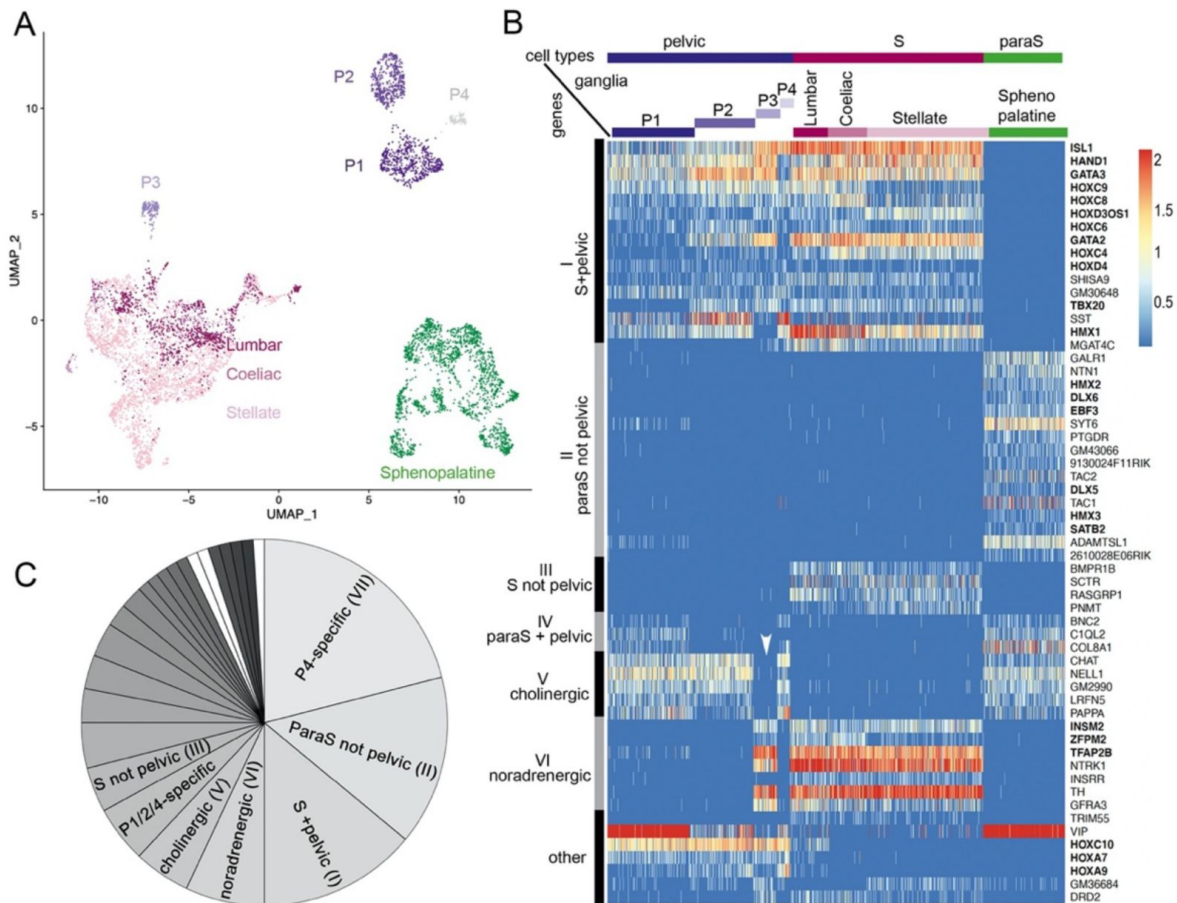


Figure 1.

The pelvic ganglion does not contain parasympathetic neurons and is made of sympathetic-like neurons.

(A) UMAP of cells isolated from 3 sympathetic ganglia (lumbar, stellate and celiac), a parasympathetic ganglion (sphenopalatine) and the pelvic ganglion dissected from postnatal day 5 mice. The pelvic ganglion is sharply divided into 4 clusters (P1-4), none of which co-segregates with sympathetic or parasympathetic neurons. (B) Heatmap of the highest scoring 100 genes in an all-versus-all comparison of their dichotomized expression pattern among the 4 ganglia and 4 pelvic clusters (see Material and Methods), excluding genes specific to the pelvic ganglion (shown in [Fig. S2](#)), and keeping only the top-scoring comparison for genes that appear twice. For overall legibility of the figure, the three largest cell groups (lumbar, stellate and sphenopalatine) are subsampled and genes are ordered by expression pattern (designated on the left), rather than score. “cholinergic” and “noradrenergic” genes are those that are coregulated with ChAT or Th, regardless of known function. “Other” refers to various groupings that split sympathetic ganglia and are thus not informative as to a sympathetic or parasympathetic identity. Transcription factors are indicated in bold face. White arrowhead: pelvic P3 cluster; S, sympathetic; ParaS, parasympathetic. (C) Pie chart of the top 100 genes, counted by expression pattern in the all-versus-all comparison. Genes specific for the P4 cluster dominate (see heatmap in [Fig. S1](#)), followed by those which are “parasympathetic-not-pelvic” and “sympathetic-and-pelvic” (seen in 1B). The three genes marked in white are the only ones that are compatible with the current dogma of a mixed sympathetic/parasympathetic pelvic ganglion, by being expressed in the sphenopalatine ganglion and a subset of pelvic clusters (other than the full complement of cholinergic ones).

The vast majority of the top 100 genes fell into 7 of the 254 possible dichotomized expression patterns, visualized on a heatmap (patterns I-VI, **Fig. 1B,C**; pattern VII, **Fig. S2**) where cells are grouped by ganglion, and genes by expression pattern (i.e. disregarding their score). These 7 patterns can be consolidated into 3, among which only the first is informative about a sympathetic or parasympathetic identity of pelvic neurons:

1. Patterns I-IV comprise 39 genes with an opposite status in sympathetic and parasympathetic cells, among which 20 (I+III) are sympathetic-specific and 19 (II+IV) parasympathetic-specific (**Fig. 1B,C**). Those which are also expressed in pelvic clusters (I and IV) argue for their sympathetic (conversely parasympathetic) identity, and against the opposite identity; those which are not expressed in pelvic clusters (II and III), argue solely against such an identity. Overall, 32 genes (I+II) argue against a parasympathetic identity of all pelvic clusters, among which 16 genes (I) argue for their sympathetic identity; 7 genes (III+IV) argue against a sympathetic identity of all clusters, among which 3 genes (IV) argue for a parasympathetic identity of P1 or P4 (which are otherwise not parasympathetic by the criterion of 31 and 32 genes, respectively). We verified expression of 7 sympathetic and 7 parasympathetic markers by in situ hybridization on the pelvic ganglion at E16.5 (**Fig. 2**).
2. Patterns V+VI comprise 12 genes that correlate with neurotransmitter phenotype (cholinergic or noradrenergic) by being expressed in the sphenopalatine ganglion and P1, P2 and P4 (5 genes (V), including ChAT) or, conversely, in all sympathetic ganglia and P3 (7 genes (VI), including Th) (**Fig. 1B,C**). These genes point to noradrenergic and cholinergic “synexpression groups” (6) broader than the defining biosynthetic enzymes and transporters. Neurotransmitter phenotype has been suggested to largely coincide with origin of input, lumbar or sacral (7) which currently defines, respectively, sympathetic versus parasympathetic identity (8). However, we find that the lumbar sympathetic pathway targets both cholinergic and noradrenergic pelvic ganglionic cells (in a proportion than reflects their relative abundance), by anterograde tracing (**Fig. 3**). Thus, noradrenergic and cholinergic genes are excluded from a debate on the sympathetic or parasympathetic identity of pelvic neurons.
3. Pattern VII involves 42 genes that place all, or some pelvic clusters in a class by themselves (**Fig. S2**), thus neither sympathetic nor parasympathetic (as does the whole transcriptome, as evidenced by the UMAP (**Fig. 1A**)) by being expressed, or not expressed, exclusively in them. The cholinergic cluster P4 was particularly rich in genes with such idiosyncratic expression states (20 genes). **Fig. S3** provides the 5 top genes of each pelvic cluster.

In the aggregate, the pelvic ganglion is best described as a divergent sympathetic ganglion, devoid of parasympathetic neurons. The fact that few of the top 100 genes (4 genes, III) marked sympathetic neurons to the exclusion of pelvic ones (**Fig. 1B**), while many (40 genes, VII) did the opposite (**Fig. S2**), argues that the pelvic identity is an evolutionary elaboration of a more generic, presumably more ancient, sympathetic one.

A third of the top 100 genes (32 genes) are transcription factors: 6 define a parasympathetic/non{sympathetic+pelvic} identity (Hmx2, Hmx3, Dlx5, Dlx6, Ebf3, Satb2); 12 define a {pelvic+sympathetic}/non-parasympathetic identity (Isl1, Gata2, Gata3, Hand1, Hmx1, Tbx20 plus 6 Hox genes); 3 correlate with the noradrenergic phenotype (Tfap2b, Insm2 and Zfp2); 8 (including 6 Hox genes) are specific to the pelvic ganglion or some of its clusters (**Fig. S2**) and 3 Hox genes are specific to the pelvic+lumbar ganglia (**Fig. 1B**). Nothing is known of the function of the 6 parasympathetic transcription factors, but most of the non-Hox {sympathetic+pelvic} ones are implicated in sympathetic differentiation: Islet1 (9), Hand1 (10), Gata2 (11), Gata3 (12) and Hmx1 (13).

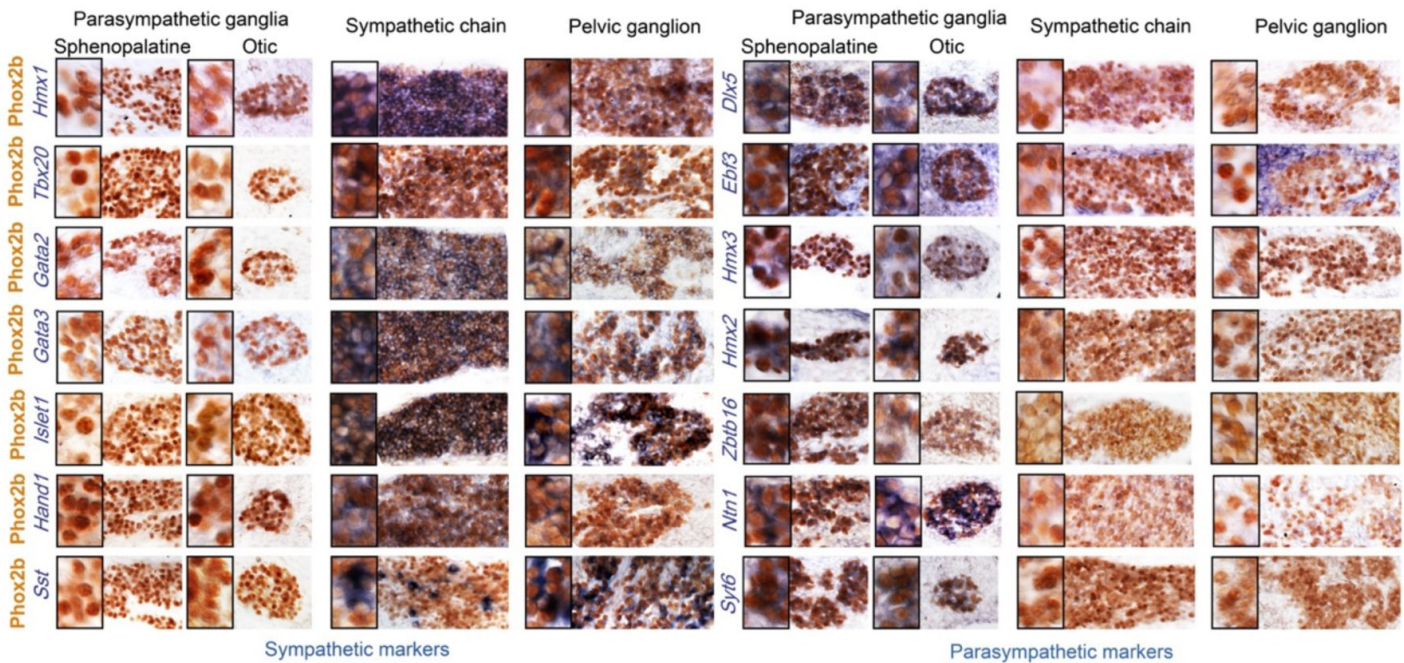


Figure 2.

Pelvic ganglion cells express sympathetic but not parasympathetic markers.

Combined immunohistochemistry for Phox2b and in situ hybridization for 7 sympathetic markers including 6 transcription factors (left panels) or 7 parasympathetic markers including 5 transcription factors (right panels), in two parasympathetic ganglia (sphenopalatine and otic), the lumbar sympathetic chain, and the pelvic ganglion, at low and high magnifications (inset on the left) in E16.5 embryos. *Ebf3* is expressed in both, the parasympathetic ganglia and the mesenchyme surrounding all ganglia. *Sst* is expressed in a salt and pepper fashion. *Zbtb16*, a zinc-finger transcriptional repressor, appeared after the 100 highest scorer gene of our screen, but was spotted as expressed in the sphenopalatine in Genepaint. Some transcription factors detected by the RNA-Seq screen at P5 (*Satb2*, *Dlx6*) were expressed below the detection limit by in situ hybridization at E16.5.

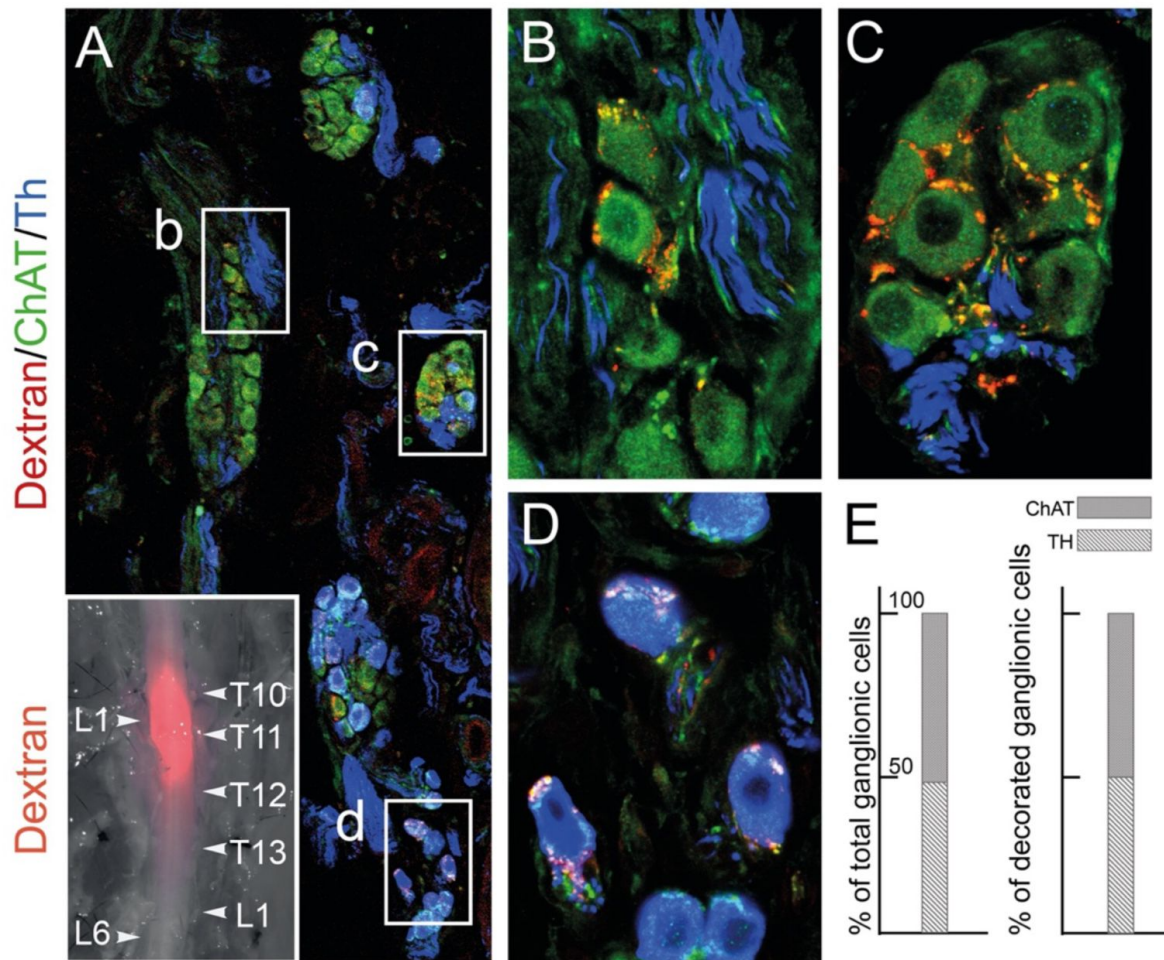


Figure 3

The lumbar outflow targets both cholinergic and noradrenergic pelvic ganglionic cells.

(A-E) Section (A, low magnification, B-D high magnifications of selected regions) through the pelvic ganglion of an adult male mouse stereotactically injected with Dextran at the L1 level of the lumbar spinal cord (inset) and showing dextran filled boutons decorating both ChAT+ (B-C) and Th+ cells (D). Whether they are filled by Dextran or not, cholinergic boutons (green), presumably from spinal preganglionics (lumbar or sacral), are present on most cells. In the inset, levels of the vertebral column are indicated with the right, levels of the spinal cord on the left. (E) Quantification of Th and ChAT cells among total or bouton-decorated ganglionic cells. ChAT+ cells represent 51% of total cells and 50% of decorated cells (for a total of 3186 counted cells, among which 529 decorated cells, on 48 sections in 4 mice).

Expression of a shared core of 12 transcription factors by pelvic and sympathetic ganglia, yet of divergent transcriptomes overall, logically calls for an additional layer of transcriptional control to modify the output of the 12 sympathetic transcription factors. Obvious candidates are the 6 Hox genes restricted to the pelvic ganglion (Hoxd9, Hoxa10, Hoxa5, Hoxd10, Hoxc11 and Hoxb3) (**Fig. S2**) and 6 more beyond the 100 top genes, mostly Hoxb paralogues (**Fig. S4**). Given that the parasympathetic and sympathetic ganglia are deployed according to a rostro-caudal pattern (parasympathetic ganglia in register with cranial nerves, sympathetic ganglia with thoraco-lumbar nerves, and the pelvic ganglion with lumbar and sacral nerves (**Fig. 4**), the entire taxonomy of autonomic ganglia could be a developmental readout of Hox genes (whose multiplicity makes this conjecture hard to test). A role for Hox genes in determining types of autonomic neurons would be reminiscent of their specification of other neuronal subtypes in *Drosophila* (14), *C. elegans* (15) and *Mus* (16).

Discussion

The notion that the pelvic ganglion is a caudal elaboration of the sympathetic ganglionic chains echoes the situation of its preganglionic neurons, in the lumbar and sacral spinal cord. We showed that lumbar and sacral preganglionics are indistinguishable by several criteria, including the expression state of 6 transcription factors (Phox2a, Phox2b, Tbx3, Tbx2, Tbx20, FoxP1) and their dependency on the bHLH gene *Olig2*, in addition to their well-known location (the intermediate lateral column) and ventral exit point of their axon (5). Recent surveys of spinal preganglionic neurons by single-cell transcriptomics (17, 18) discovered many subtypes, most of them evenly distributed from thoracic to sacral levels, and a few enriched at, or specific to the sacral level, confirming that the sacral preganglionic neurons are sympathetic, yet represent a caudal modification of the thoracolumbar intermediate lateral column.

In conclusion, the genetic signatures of neither pre-nor post-ganglionic neurons of the sacral autonomic outflow support its century-old assignment to the parasympathetic division of the visceral nervous system. Importantly, we have argued (3) that the supposed parasympathetic identity was unnecessary at best to understand the physiology of the pelvis, at worst incongruent with it, at least concerning sex organs and the bladder. The antagonism between an anti-erectile lumbar and a pro-erectile sacral autonomic pathways on the blood vessels of the external genitals — the key argument that Langley advanced for making the sacral pathway parasympathetic, even before he coined the term (2) — was repeatedly challenged by the evidence of a lumbo-sacral pro-erectile synergy (reviewed in (3)). And experimental support for a lumbo-sacral antagonism on the bladder is scant, despite common perception from textbooks and reviews (reviewed in (3)). It is thus remarkable that the cell type-based anatomy that we uncover in no way contradicts the physiology. There is no need to suppose that some sympathetic-like neurons in the pelvic region display a type of physiology common with that of parasympathetic ones in the head or thorax, which would deserve a common name. The classical parasympathetic label is thus best dropped and replaced by the genetic (i.e. embryological and evolutionary) notion of a sympathetic one, modified to suit the unique demands of pelvic physiology. The division of the autonomic nervous system that encompasses the pelvic ganglion and both its lumbar and sacral afferents (which could be termed “pelvo-sympathetic” and includes whatever antagonistic pathways operate in the region — e.g. on genital blood vessels) is now ripe for finer grain analysis, anatomical and physiological, at the level of neuron types, defined by their gene expression patterns.

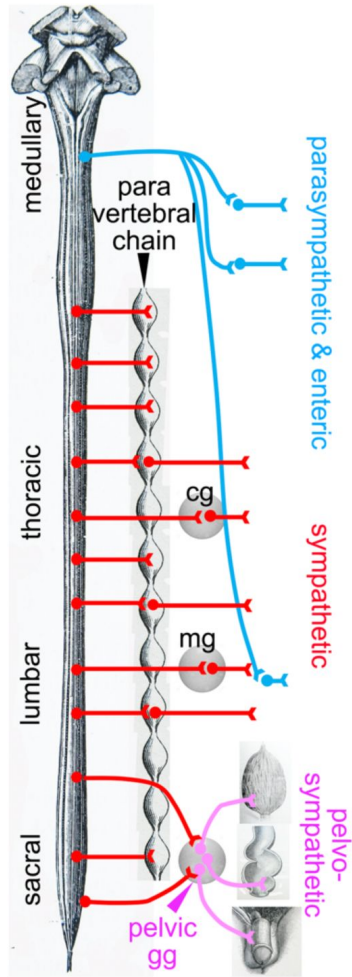


Figure 4

Deployment of the divisions of the autonomic nervous system on the rostro-caudal axis.

Cg, celiac ganglion; mg, mesenteric ganglion; pelvic gg, pelvic ganglion. Only the target organs of the pelvo-sympathetic pathway are represented. The pelvic ganglion is shown with its lumbar input (through the hypogastric nerve), and sacral input (through the pelvic nerve).

Materials and Methods

Mice

Phox2b::Cre (19): a BAC transgenic line expressing Cre under the control of the Phox2b promoter. Rosa^{lox-stop-lox-tdTomato} (Rosa^{tdT})(20): Knock in line expressing the reporter gene tdTomato from the Rosa locus in a Cre-dependent manner.

Obtainment of ganglionic cells

Sphenopalatine, stellate, coeliac, lumbar and pelvic ganglia were dissected from Phox2bCre;Rosa^{tdT} P5 pups representing both sexes and placed in artificial cerebrospinal fluid (aCSF) oxygenated with carbogen (4°C). Fat tissue was carefully removed and nerves emanating from the ganglia were cut. Ganglia were transferred into a 1.5ml Eppendorf tube containing 1ml PBS-Glucose (1mg/ml Glucose), 20 µl Papain solution (Worthington LS003126; 25.4 Units/mg) and 20 µl DNase (2mg/ml in PBS) and incubated at 37°C for 15min. The ganglia were collected by centrifugation (300g, 1min), the supernatant replaced by 1ml PBS-Glucose supplemented with 50 µl Collagenase/Dispase (Worthington CLS-1 345U/mg; Dispase II Roche 1.2U/mg; 80mg Collagenase and 92mg Dispase II dissolved in 1ml PBS-Glucose) and 20 µl DNase solution and the ganglia incubated at 37°C for 8min. After collecting the ganglia by centrifugation for 3min at 300g they were dissociated in 1ml PBS-Glucose supplemented with 0.04% bovine serum albumin (BSA) and DNase (20µl) by trituration, using a fire-polished, siliconized Pasteur pipette. The cell suspension was then filtered through a 40µm cell strainer. To eliminate cell debris, the cell suspension was centrifuged through a density step gradient by overlaying the cell suspension onto 1ml OptiPrep solution (80 µl OptiPrep (Sigma), 900µl PBS supplemented with 0.04%BSA, 20µl DNase) for 15 min, 100g at 5°C. After removal of the supernatant from the soft cell pellet, the cells were suspended in 100µl PBS-Glucose/0.04%BSA and collected again in 500µl Eppendorf tube for 15 min, 100g at 5°C. The supernatant was carefully removed (under control by fluorescence microscope). After addition of 40 µl PBS-Glucose/0.04%BSA the cell density was adjusted to 1000 cells/ml and transferred to the 10xGenomics platform.

Library construction and Sequencing

Single cell RNA-Seq was performed in two separate experimental rounds, one for the stellate, sphenopalatine and pelvic ganglia (pelvic_1) performed at the École normale supérieure GenomiqueENS core facility (Paris, France), and one for the celiac, lumbar and pelvic ganglia (pelvic_2) performed at the ICGex NGS platform of the Institut Curie (Paris, France). Cellular suspensions (10000 cells for the first round, 5300 cells for the second) were loaded on a 10X Chromium instrument (10X Genomics) to generate single-cell GEMs (5000 for the first round, 3000 for the second). Single-cell RNA-Seq libraries were prepared using Chromium Single Cell 3' Reagent Kit (v2 for the first round, v3 for the second) (10X Genomics) according to manufacturer's protocol based on the 10X GEMCode proprietary technology. Briefly, the initial step consisted in performing an emulsion where individual cells were isolated into droplets together with gel beads coated with unique primers bearing 10X cell barcodes, UMI (unique molecular identifiers) and poly(dT) sequences. Reverse transcription reactions were applied to generate barcoded full-length cDNA followed by disruption of the emulsions using the recovery agent and the cDNA was cleaned up with DynaBeads MyOne Silane Beads (Thermo Fisher Scientific). Bulk cDNA was amplified using a GeneAmp PCR System 9700 with 96-Well Gold Sample Block Module (Applied Biosystems) (98°C for 3min; 12 cycles: 98°C for 15s, 63°C for 20s, and 72°C for 1min; 72°C for 1min; held at 4°C). The amplified cDNA product was cleaned up with the SPRI select Reagent Kit (Beckman Coulter). Indexed sequencing libraries were constructed using the reagents from the Chromium Single Cell 3' Reagent Kit v3, in several steps: (1) fragmentation, end repair and A-tailing; (2) size selection with SPRI select; (3) adaptor ligation; (4) post ligation cleanup with SPRI select; (5) sample index PCR and cleanup with SPRI select beads (with 12 to 14 PCR cycles depending on the samples). Individual library quantification and quality assessment were performed using

Qubit fluorometric assay (Invitrogen) with dsDNA HS (High Sensitivity) Assay Kit and Bioanalyzer Agilent 2100 using a High Sensitivity DNA chip (Agilent Genomics). Indexed libraries were then equimolarly pooled and quantified by qPCR using the KAPA library quantification kit (Roche). Sequencing was performed on a NextSeq 500 device (Illumina) for the first round and a NovaSeq 6000 (Illumina) for the second, targeting around 400M clusters per sample and using paired-end (26/57bp for the first round, 28×91bp for the second).

Bioinformatic analysis

For each of the 6 samples (pelvic_1, stellate, sphenopalatine, pelvic_2, coeliac and lumbar) we performed demultiplexing, barcode processing, and gene counting using the Cell Ranger software (v. 6.0.1). The ‘filtered_feature_bc_matrix’ files (uploaded at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE232789>) were used as our starting points for defining cells. For each dataset, only droplets that expressed more than 1500 genes, less than 11000 genes and a percentage of mitochondrial genes below 15% were retained. This resulted in the selection of 4404 pelvic_1 cells, 7225 stellate cells, 4630 sphenopalatine cells, 1643 pelvic_2 cells, 1428 coeliac and 2120 lumbar cells.

We used Seurat version 3 ([21](#)) to read, manipulate, assemble, and normalize ([22](#)) the datasets. Specifically, we concatenated cells from all 6 datasets and normalized the data using the sctransform (SCT) method for which we fitted a Gamma-Poisson Generalized Linear Model (“glmGamPoi” option). Using the Seurat framework, we then performed a PCA on the normalized dataset.

Next, we integrated all 6 datasets using Batchelor ([23](#)) (a strategy for batch correction based on the detection of mutual nearest neighbors), and we visualized all cells, including neurons, in 2 dimensions using the Uniform Manifold Approximation and Projection method ([24](#)) on the first 50 components from the PCA.

We selected neurons based on their mean expression of a set of neuronal marker genes (Stmn2, Stmn3, Gap43 and Tubb3). Specifically, cells were classified as neurons if their mean marker SCT-normalized gene expression exceeded a threshold of 3, that best separated the bimodal distribution of the higher and lower values of the mean expression of neuronal markers. Likewise, we excluded glial-containing doublets using the following markers: Plp1, Ttyh1, Fabp7, Cryab and Mal. The number of neurons thus selected was 862 for Pelvic_1, 2689 for the stellate, 1857 for the sphenopalatine, 361 for Pelvic_2, 236 for coeliac and 925 for the lumbar chain.

We then clustered pelvic neurons based on the first two components from the UMAP generated from the 50 batch-corrected components, using the graph-based clustering method Louvain (Blondel et al 2008) implemented in the Seurat framework, with a resolution of 0.3. This procedure defined 24 clusters and split the pelvic ganglion into four clusters 1, 7, 15 and 19 (**Fig. S1B**), that we renamed P1, P2, P3, P4 (**Fig. 1A**). The efficiency of batch correction is attested by the equal contribution from both batches of pelvic ganglia to all four defined pelvic clusters (P1-4) (**Fig. S1A**), and the comparable expression level of the top 100 genes across both pelvic batches in the violin plots (Table S2).

To search in an unbiased manner for gene expression similarities between pelvic neurons and either sympathetic or parasympathetic ones, we systematically compared the expression of every gene of the dataset across all possible splits among ganglia, i.e. between every subset of ganglia (“subset_1”), and its complementary subset (“subset_2”). Because we treated each of the 4 pelvic clusters as a ganglion, there were $2^4=16$ such splits (2 of them, defined by “all_4_groups vs none” and “none vs all_4_groups”, being meaningless). For every split and every gene, we devised a score that would best reflect how higher the proportion is of cells expressing the gene (i.e. one read of the gene or more) in subset_1 than in subset_2. We used a metric based on the product of the proportions of cells expressing the gene in each ganglion of subset_1 or subset_2 (rather than

throughout subset_1 or subset_2), to give an equal weight to each ganglion (irrespective of its size), and to penalize splits that contain outliers (i.e. ganglia that contain much fewer cells positive for that gene than other ganglia in subset_1, or ganglia that contain much more cells positive for that gene than other ganglia in subset_2).

Specifically, for a given split and a given gene, the score (here defined by the variable *score*, in the R programming language) is defined as:

$$score = \sum_{i \in S_1} \frac{1}{n_{S_1}} \cdot \log_2 \left(-1 + eps_{pos} + \frac{numCellsExpr(i)}{(n_{cells}(i))} \right) - \sum_{j \in S_2} \frac{1}{n_{S_2}} \cdot \log_2 \left(eps_{nega} + \frac{numCellsExpr(j)}{(n_{cells}(j))} \right)$$

where:

- S_1 and S_2 are the ganglion subsets subset_1 and subset_2 as described above.
- n_{S_1} and n_{S_2} are the number of ganglion clusters that belong to subset_1 and subset_2.
- $\frac{numCellsExpr(i)}{(n_{cells}(i))}$ and $\frac{numCellsExpr(j)}{(n_{cells}(j))}$ are the proportion of cells from ganglion or pelvic cluster *i* and *j* (*i* belonging to subset_1 and *j* to subset_2) which express the given gene.
- $eps_{pos}=0.9$ and $eps_{nega}=0.02$ are parameters controlling a tradeoff between favoring genes expressed in subset_1 and penalizing genes expressed in subset_2.
- Note that when $eps_{pos}=0.9$ (the parameter value used here) the quantity $\left(-1 + eps_{pos} + \frac{numCellsExpr(i)}{(n_{cells}(i))} \right)$, can be negative, in which case the expression becomes equal to $-\infty$ and the gene gets the lowest possible score for that given split. This means that with these settings all dichotomies involving a ganglion or pelvic cluster in which less than 10% of cells express the gene ($p=0.1$) will get the minimal possible score.

In vivo gene expression

Tissue preparation

Embryo sections. E16.5 WT embryos were freshly dissected, washed with 1X PBS and fixed overnight in 4% PFA at 4°C. Tissues were washed a few times in 1X PBS and treated in 15% sucrose to cryopreserve the tissues for frozen tissue sections. Tissues were then embedded in the Optimal Cutting Temperature (OCT) compound and snap frozen. Tissues were cut at 14 μm with cryostat. Tissue sections were stored at -80°C until use for staining.

In situ hybridization

Frozen tissue sections were washed in 5X SSC buffer 15min at RT and treated in the pre-hybridization solution (50% Formamide, 5X SSC buffer and 40 μg/mL Herring sperm DNA in H₂O) for 1h at 60°C. Then, slides were put in the hybridization solution (50% Formamide, 5X SSC buffer, 5X Denhardt's, 500 μg/mL Herring sperm DNA, 250 μg/mL Yeast RNA and 1mM DTT in H₂O) containing the probe (100 ng/mL), at least 2 overnights at 60°C. Slides were washed two times in 5X SSC buffer (5min) and two times in 0.2X SSC buffer (30min) at 70°C, and then, three times in TBS at RT (10min). Then, tissues were put in the blocking solution (TBS + 10% FCS) for 1h in the dark, at RT and in humid atmosphere (250 μL/slide) and incubated 1h with the primary antibody (anti-DIG) diluted 1/200 in blocking solution (250 μL/slide). Then, slides were washed again three times in TBS

(10min) and treated 5min in the AP buffer solution (100 nM Tris pH9.5, 50nM MgCl₂ and 100 nM NaCl in H₂O). The revelation was made by the NBT-BCIP solution (Sigma) in the dark (250 µL/slide). The reaction was stopped in PBS-Tween (PBST).

Immunohistochemistry

After in situ hybridization, frozen tissues were fixed 15 min in 4% PFA at RT and washed in PBST. Then, tissues were incubated in PBST + 10% FCS in the dark for 1h (500 µL/slide, without coverslip) and incubated overnight with the primary antibody in the same solution at 4°C (250 µL/slide). Slides were washed in PBST three times (10min) and incubated for 2h at RT with the secondary antibody in the same solution again. Tissues were washed three times in PBST and then incubated at RT with avidin/biotin solution diluted 1/100 in 1X PBS. Then, tissues were washed three times and the revelation was made in DAB solution containing 3,3'-Diaminobenzidine (DAB) and urea in H₂O (Sigma). The reaction was stopped in PBST, and slides were washed in PBST and in ultra-pure H₂O. Slides were mounted in Aquatex mounting medium (Merck).

Imaging

Tissues processed by an in situ hybridization and immunohistochemistry were photographed on a Leica bright field microscope with a 40X oil immersion objective. Images were then treated by Photoshop v. 24.1.0.

Anterograde tracing

Tracer injections

Surgeries were conducted under aseptic conditions using a small animal digital stereotaxic instrument (David Kopf Instruments). Male, C57bl mice (2-4 months old) were anesthetized with isoflurane (3.5% at 1 l/min for induction and 2–3% at 0.3 l/min for maintenance). Carprofen (0.5 mg/kg) was administered subcutaneously for analgesia before surgery. A feed-back-controlled heating pad was used to maintain the animal temperature at 36 °C. Anesthetized animals were shaved along the spine and placed in a stereotaxic frame. The skin overlying the spine was sterilized with alternating scrubs of Vetadine and 80% (W/V) ethanol and a 100 µl injection of lidocaine (2%) was made subcutaneously along the spine before an incision was made from the iliac crest to the T9 vertebral spinous process. The T10 and T11 spinous processes were identified by counting rostrally from the L6 spinous process (identified relative to the iliac crest). Two small rostro-caudal incisions were made on each side of the vertebral column through the superficial-most layer of muscle. Parallel clamps were then placed within these incisions until firm contact was made with the transverse process of the T11 vertebra. The T11 vertebra was then raised upwards via the clamps until no respiratory movement was observed. The muscles connecting the T10 spinous process to transverse processes of caudal vertebra were dissected to expose the T10/T11 intervertebral space. A lateral incision was made across the dura to expose the underlying L1 spinal segment and 150nL injections of 4% lysine fixable, tetramethylrhodamine or Alexa-488 conjugated dextran, 3000MW (Thermofisher) were made bilaterally, into the intermediolateral nucleus (IML) and intermedio-medial nucleus (IMM) (0.600mm lateral, 0.600mm deep and 0.150mm lateral and 0.700mm deep) using a narrow-tapered glass pipette and a Nanoject III injector (Drummond Scientific). Injections were made at 5nL per second and left in place for 5 minutes following injection to prevent dextran leakage from the injection site. The pipette was then retracted, and the intervertebral space bathed with sterile physiological saline. A small piece of sterile gel foam hemostat (Pfizer) was then placed within the intervertebral space and the incision overlying the spine was sutured closed.

Histology

7-14 days after injection the mice were intracardially perfused with cold PBS until exsanguinated and subsequently fixed by perfusion with cold 4% PFA until the carcass became stiff. The bladder, prostate and pelvic ganglia were immediately dissected as an intact bloc and post fixed in 4% PFA overnight at 4 degrees. The intact spinal column was also dissected out and the dorsal surface of the spinal cord exposed before post fixation overnight in 4% PFA at 4 degrees. The fixed tissues were then rinsed in PBS 3× 30 minutes the following day. The “bladder block” was then cryopreserved in 15% sucrose (W/V) in PBS until non buoyant. The dorsal aspect of the injection site was imaged in situ using and fluorecence stereoscope. The spinal cord was then dissected out and cryopreserved as described above.

The bladder block was sectioned on a cryostat in a sagittal orientation to capture serial sections of the entire pelvic ganglion. Sections were cut at a thickness of 30µm and collected as a 1 in 4 series on Superfrost Plus glass slides. The spinal cord was sectioned coronally at a thickness of 60µm as a 1 in 2 series. On slide immunohistochemistry was performed against tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) for the bladder block sections, primary incubation: 4-6 hours at room temperature, secondary incubation: 2 hours at room temperature, with 3×5 minute washes in PBS after each incubation. Sections were then mounted with dako fluorecence mounting medium and cover slipped.

Imaging

Sections were imaged on a Leica Stellaris 5 confocal microscope (Leica microsystems). Images of the pelvic ganglia were captured as Z-stacks with 1µm interslice distances, with a 20x objective at 2000mp resolution.

Counting

All ganglionic cells, and cells surrounded by dextran labelled varicosities were identified as either ChAT or TH positive and counted, on a total of 4 animals, 48 sections and 3186 cells.

Probes

Primers were designed to amplify by PCR probe templates for the following genes:

Probe	Forward primer	Reverse primer
Dlx5	5' - GACGCAAACACAGGTGAAAATCTGG-3'	5'-GGGCGGGGCTCTCTGAAATG-3'
Gata2	5'- TTGTGTTCCTTGGGGTCCTTC-3'	5'- GCTTCTGTGGCAACGTACAA-3'
Hmx1	5'- CGTTCGCCACTATCCAAACGGG-3'	5'-TGTCAGGACTTAGACCACCTCCG-3'
Ntn1	5'-CTTCCTCACCGACCTCAATAAC-3'	5' - GCGATTTAGGTGACACTATAGTTGT GCCTACAGTCACACACC-3'
Syt6	5'-GTGGTCTTCTTGTCCCCTGT-3'	5'-CATGTGCTTACAGGGTGTGG-3'
Zbtb16	5'-ATGAAAACATACGGGTGTGAA-3'	5'-CCAAGGCCAAGTAACTATCAGG-3'

The PCR fragment were ligated into a pGEM-T Easy Vector System (Promega), transformed into chemically competent cells and sequenced. The other plasmid templates were Ebf3 (gift of S. Garel), Gata3 (gift of JD Engel), Hand1 (gift of P. Cserjesi), Hmx2 (gift of E.E. Turner), Hmx3 (gift of S. Mansour), Islet1 ([25](#)) and Tbx20 ([26](#)), Sst (Clone Image ID #4981984).

Plasmids were digested by restriction enzymes and purified using a DNA clean & concentrator kit (Zymo research). Antisense probes were synthesized with RNA polymerases and a DIG RNA labeling mix, and purified by the ProbeQuant G-50 micro columns kit (GE Healthcare). Probes were stored at -20°C.

Antibodies

Primary antibodies were α -Phox2b rabbit (1:500 or 1:1000, Pattyn et al., 1997). α -Th (Invitrogen: OPA1-04050, 1:1000) and α -choline acetyltransferase (ChAT) (Thermofisher: PA1-9027, 1:100).

Secondary antibodies were goat α -rabbit (PK-4005, Vector Laboratories), donkey anti-goat 647 (A-21447, Thermofisher), donkey anti-rabbit 488 (A-21206, Thermofisher) and donkey α -rabbit Cy3 (711-165-152, Jackson).

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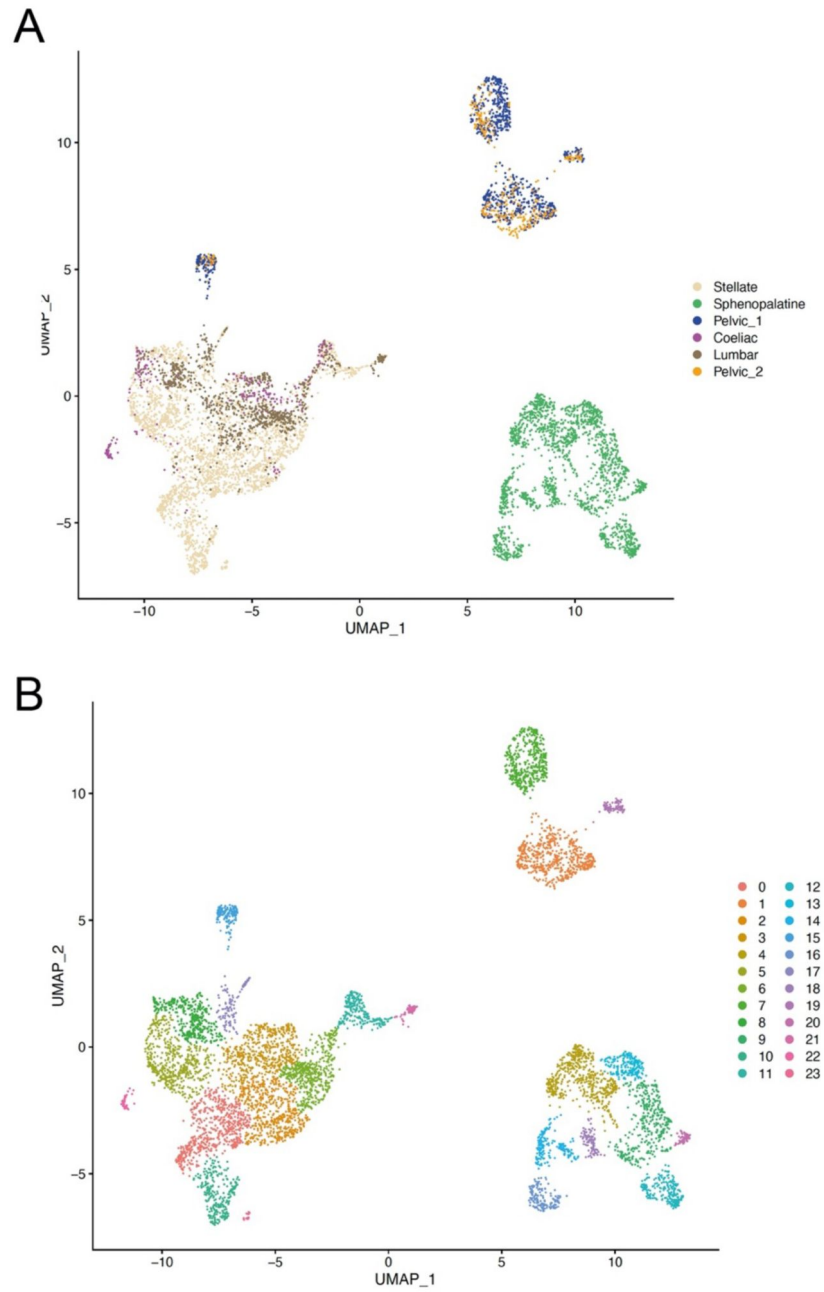


Fig. S1

Uniform Manifold Approximation and Projection (UMAP) of all ganglionic neurons.

(A) UMAP of neurons where the sample origin of cells is color-coded to show that both samples of the pelvic ganglion contribute to each of the P1-4 pelvic clusters. (B) UMAP of neurons where the clusters as defined by Seurat are color-coded. Clusters 1, 7, 15 and 19 correspond to ganglion clusters P1, P2, P3, P4 in the text.

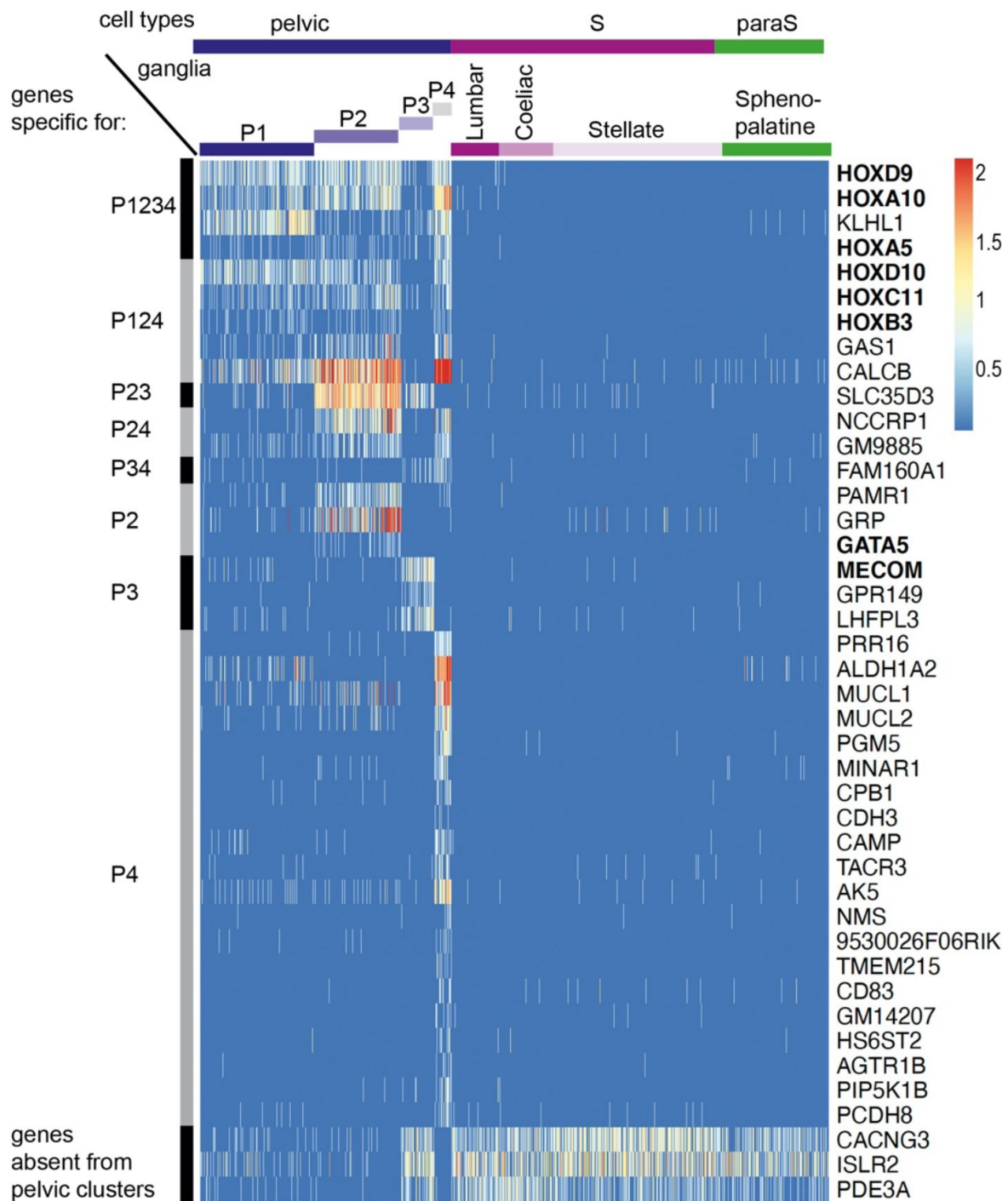


Fig. S2

Genes specific for pelvic ganglionic cells among the top 100 genes of an all-versus-all comparison.

These 42 genes correspond to pattern VII of main text. Transcription factors are indicated in bold face.

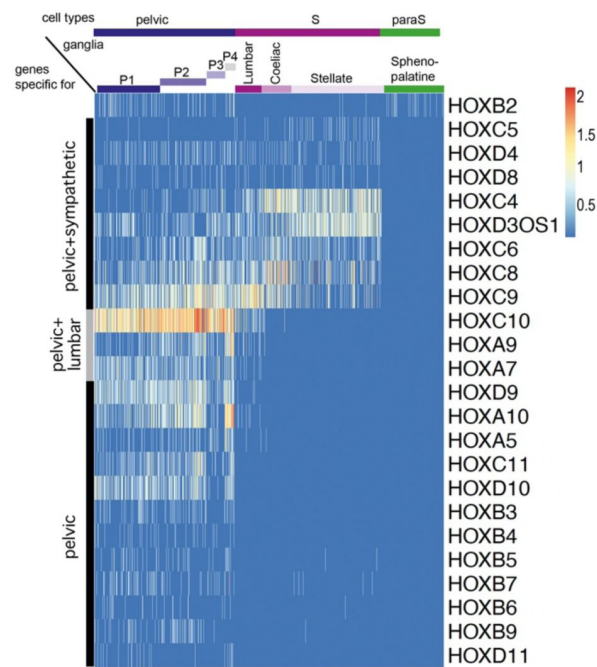


Fig. S3

Expression of all Hox genes captured by the single-cell RNA-Seq data set.

Apart from Hoxb2, all Hox genes are excluded from the sphenopalatine and are expressed either in all sympathetic and pelvic cells (8 genes), caudal sympathetic and pelvic cells (3 genes), or only in pelvic cells (12 genes). Hoxb2 appears in 177th position as {P124/Sphenopalatine} versus {P3/Lumbar/Coeliac/Stellate}, thus as a cholinergic gene in the dichotomized comparison).

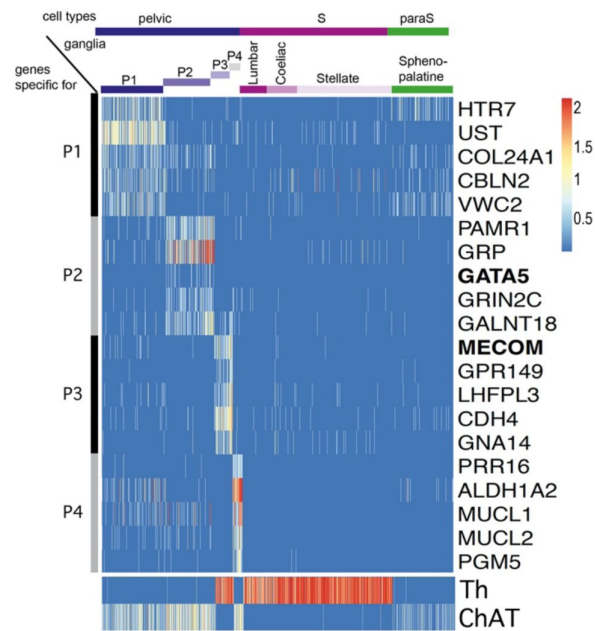


Fig. S4

Five top genes for each of the 4 individual pelvic clusters in an all-versus-all comparison.

P1 appears as the cluster the least sharply defined by specific genes. The only two transcription factors (indicated in bold face) among these top genes are Gata5, expressed in P2 and MECOM expressed in P3. Noradrenergic and cholinergic cells are indicated by Th and ChAT expression in the lower panel.

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Reviewer #1 (Public Review):

In recent years, these investigators have been engaged in a debate regarding the classification of the sacral parasympathetic system as "sympathetic" rather than "parasympathetic," based on shared developmental ontogeny of spinal preganglionic neurons. In this current study, these investigators conducted single-cell RNAseq analyses of four groups of autonomic neurons: paravertebral sympathetic neurons (stellate and lumbar train ganglia), prevertebral sympathetic neurons (coeliac-mesenteric ganglia), rostral parasympathetic ganglia (sphenopalatine ganglia), and the caudal pelvic ganglia (containing traditionally recognized sacral "parasympathetic cholinergic neurons," which the investigators sought to challenge in terms of nomenclature). The authors argued that the pelvic ganglionic neurons shared the expression of more genes with sympathetic ganglia, as opposed to parasympathetic ganglia. Additionally, the pelvic neurons did not express a set of genes observed in the rostral parasympathetic sphenopalatine ganglia. Based on these findings, they claimed that the sacral autonomic system should be considered sympathetic rather than parasympathetic.

However, noradrenergic sympathetic neurons and cholinergic neurons, by the virtue of expressing different neurotransmitters, could have distinct roles. It is true that some cholinergic neurons reside in the sympathetic train ganglia as well, such as those innervating the sweat gland and some vascular systems; in this sense, the pelvic ganglia share some features with sympathetic ganglia, except that the pelvic ganglia contain a much higher percentage of cholinergic neurons compared with sympathetic ganglia. It is much simpler and easier to divide the autonomic nervous system into sympathetic neurons that relieve noradrenaline versus parasympathetic neurons that relieve acetylcholine, and these two systems often act in antagonistic manners, even though in some cases, these two systems can work synergistically. As such, it is not justified to claim that "pelvic organs receive no parasympathetic innervation".

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Reviewer #2 (Public Review):

Summary:

Recent advances in single cell profiling of gene expression (RNA) permit the analysis of specialized cell types, an approach that has great value in the nervous system which is

characterized by prodigious neuronal diversity. The novel data in this study focus primarily on genetic profiling to compare autonomic neurons from ganglia associated with the cranial parasympathetic outflow (sphenopalatine (also known as pteropalatine), the thoraco-lumbar sympathetic outflow (stellate, coeliac) and the sacral parasympathetic outflow (pelvic). Using statistical methods to reduce the dimensionality of the data and map gene expression, the authors provide interesting evidence that cranial parasympathetic and sacral sympathetic ganglia differ from each other and from sympathetic ganglia (Figures 1, S1 - S4). The authors interpret the mapping analysis as evidence that the cranial and sacral outflows differ so that calling them both parasympathetic is unjustified. Based on anatomical localization of markers (Figure 2) (mainly transcription factors) the authors show a similarity between the sympathetic and pelvic ganglion. In Figure 3 they present evidence that some pelvic ganglionic neurons are dually innervated by sympathetic preganglionic neurons and sacral preganglionic neurons. These observations are interpreted to mean that the pelvic ganglion is not parasympathetic, but rather a modified sympathetic ganglion - hence the title of the manuscript.

Strengths:

The extensive use of single cell profiling in this work is both interesting and exciting. Although still in its early stages, it holds promise for a deepened understanding of autonomic development and function. As noted in the introduction, this study extends previous work by Professor Brunet and his associates.

Weaknesses:

This work further documents differences between the cranial and sacral parasympathetic outflows that have been known since the time of Langley - 100 years ago. The approach taken by Brunet et al. has focused on late neonatal and early postnatal development, a time when autonomic function is still maturing. In addition, the sphenopalatine and other cranial ganglia develop from placodes and the neural crest, while sympathetic and sacral ganglia develop from the neural crest alone. How then do genetic programs specifying brainstem and spinal development differ and how can this account for kinship that Brunet documents between spinal and sacral ganglia? One feature that seems to set the pelvic ganglion apart is the mixture of 'sympathetic' and 'parasympathetic' ganglion cells and the convergence of preganglionic sympathetic and parasympathetic synapses on individual ganglion cells (Figure 3). This unusual organization has been reported before using microelectrode recordings (see Crowcroft and Szurszewski, *J Physiol* (1971) and Janig and McLachlan, *Physiol Rev* (1987)). Anatomical evidence of convergence in the pelvic ganglion has been reported by Keast, *Neuroscience* (1995). It should also be noted that the anatomy of the pelvic ganglion in male rodents is unique. Unlike other species where the ganglion forms a distributed plexus of mini-ganglia, in male rodents the ganglion coalesces into one structure that is easier to find and study. Interestingly the image in Figure 3A appears to show a clustering of Chat-positive and Th-positive neurons. Does this result from the developmental fusion of mini ganglia having distinct sympathetic and parasympathetic origins. In addition, Brunet et al dismiss the cholinergic and noradrenergic phenotypes as a basis for defining parasympathetic and sympathetic neurons. However, see the bottom of Figure S4 and further counterarguments in Horn (*Clin Auton Res* (2018)). What then about neuropeptides, whose expression pattern is incompatible with the revised nomenclature proposed by Brunet et al.? Figure 1B indicates that VIP is expressed by sacral and cranial ganglion cells, but not thoracolumbar ganglion cells. The authors do not mention neuropeptide Y (NPY). The immunocytochemistry literature indicates that NPY is expressed by a large subpopulation of sympathetic neurons but never by sacral or cranial parasympathetic neurons.

The title of this paper is misleading because it implies a conclusion that is not adequately supported by the data and that is difficult for a general reader to parse. Independent assessments by two referees both agreed on title's problematic message. If one can get beyond the title, then the paper does contain data that is of interest. The authors compared

single cell gene expression in neurons from the cranial sphenopalatine ganglion, paravertebral chain ganglia (stellate and lumbar), the prevertebral coeliac ganglion and the bladder ganglion. The cranial and pelvic ganglia are parasympathetic, while the paravertebral and prevertebral ganglia are sympathetic. The gene expression data identified differences between the cranial, sympathetic, and pelvic ganglia. Based primarily on this finding the authors concluded that the sacral bladder ganglion is not parasympathetic. Since some genes suggest a kinship between the pelvic and sympathetic neurons, the authors conclude that the pelvic neurons are pelvo-sympathetic - hence the title. This nomenclature does little to improve understanding of the autonomic motor system and it ignores important anatomical and functional properties that underlie existing definitions of the sympathetic and parasympathetic systems. The idea that the cranial and sacral autonomic outflows have some differences is not new (see for example Nilsson, 1983 and Janig, 2022). Since many of the genes identified in the present study are HOX genes and other transcription factors that specify the rostro-caudal axis during development, it is also not surprising that these genes suggest a kinship between sacral parasympathetic neurons and sympathetic neurons, all of which derive from the neural crest and are supplied by the spinal cord. The different profile of cranial parasympathetic neurons is also not surprising given that they derive from a mixture of placodal and neural crest progenitors and are supplied by the brainstem. (see my previous comments for anatomical and functional criteria that further support the existing nomenclature for the sympathetic and parasympathetic motor systems.

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Author Response

Author responses to the original review:

The data we produce are not criticized as such and thus, do not require revision; the criticisms concern our interpretation of them. General themes of the reviews are that i) genetic signatures do not matter for defining neuronal types (here sympathetic versus parasympathetic); ii) that a cholinergic postganglionic autonomic neuron must be parasympathetic; and iii) that some physiology of the pelvic region would deserve the label “parasympathetic”. We answered the latter argument in (Espinosa-Medina et al., 2018) to which we refer the interested reader; and we fully disagree with the first two. Of note, part of the last sentence of the eLife assessment is misleading and does not reflect the referees’ comments. Our paper analyses genetic differences between the cranial and sacral outflow and uses them to argue that they cannot be both parasympathetic. The eLife assessment acknowledges the “genetic differences” but concludes that, somehow, they don’t detract from a common parasympathetic identity. We take issue with this paradox, of course, but it is coherent with the referee’s comments. On the other hand, the eLife assessment alone pushes the paradox one step further by stating that “functional differences” between the cranial and sacral outflows can’t either prevent them from being both parasympathetic. We would also object to this, but the only “functional differences” used by the referees to dismiss our diagnostic of a sympathetic-like character (rather than parasympathetic) for the sacral outflow are between noradrenergic and cholinergic, and between sympathetic and parasympathetic (and we also disagree with those, see above, and below) —not between cranial and sacral.

We will thus use the opportunity offered by eLife to keep the paper as it is (with a few minor stylistic changes). We respond below to the referees’ detailed remarks and hope that the publication, as per eLife new model, of the paper, the referees’ comments and our response will help move the field forward.

Public review by Referee #1

“Consistently, the P3 cluster of neurons is located close to sympathetic neuron clusters on the map, echoing the conventional understanding that the pelvic ganglia are mixed, containing both sympathetic and parasympathetic neurons”.

The greater closeness of P3 than of P1/2/4 to the sympathetic cluster can be used to judge P1/2/4 less sympathetic than P3 (and more... something else), but not more parasympathetic. There is no echo of the “conventional understanding” here.

“A closer look at the expression showed that some genes are expressed at higher levels in sympathetic neurons and in P2 cluster neurons ” [We assume that the referee means “in sympathetic neurons and in P3 cluster neurons”] but much weaker in P1, P2, and P4 neurons such as Islet1 and GATA2, and the opposite is true for SST. Another set of genes is expressed weakly across clusters, like HoxC6, HoxD4, GM30648, SHISA9, and TBX20.

These statements are inaccurate; On the one hand, the classification is not based on impression by visual inspection of the heatmap, but by calculations, using thresholds. Admittedly, the thresholds have an arbitrary aspect, but the referee can verify (by eye inspection of heatmap) that genes which we calculate as being at “higher levels in sympathetic neurons and in P3 cluster neurons, but much weaker in P1, P2, and P4 neurons” or vice versa, i.e. noradrenergic or cholinergic neurons (genes from groups V and VI, respectively), have a much bigger difference than those cited by the referee, indeed are quasi-absent from the weaker clusters or ganglia. In addition, even by subjective eye inspection:

Islet is equally expressed in P4 and sympathetics.

SST is equally expressed in P1 and sympathetics.

Tbx20 is equally expressed in P2 and sympathetics.

HoxC6, HoxD4, GM30648, SHISA9 are equally expressed in all clusters and all sympathetic ganglia.

“Since the pelvic ganglia are in a caudal body part, it is not surprising to have genes expressed in pelvic ganglia, but not in rostral sphenopalatine ganglia, and vice versa (to have genes expressed in sphenopalatine ganglia, but not in pelvic ganglia), according to well recognized rostro-caudal body patterning, such as nested expression of hox genes.”

We do not simply show “genes expressed in pelvic ganglia, but not in rostral sphenopalatine ganglia, and vice versa”, i.e. a genetic distance between pelvic and sphenopalatine, but many genes expressed in all pelvic cells and sympathetic ones, i.e. a genetic proximity between pelvic and sympathetic. This situation can be deemed “unsurprising”, but it can only be used to question the parasympathetic nature of pelvic cells (as we do), or considered irrelevant (as the referee does, because genes would not define cell types, see our response to an equivalent stance by Referee#2). Concerning Hox genes, we do take them into account, and speculate in the discussion that their nested expression is key to the structure of the autonomic nervous system, including its division into sympathetic and parasympathetic outflows.

It is much simpler and easier to divide the autonomic nervous system into sympathetic neurons that release noradrenaline versus parasympathetic neurons that release acetylcholine, and these two systems often act in antagonistic manners, though in some cases, these two systems can work synergistically. It also does not matter whether or not pelvic cholinergic neurons could receive inputs from thoracic-lumbar preganglionic neurons (PGNs), not just sacral PGNs; such occurrence only represents a minor revision

of the anatomy. In fact, it makes much more sense to call those cholinergic neurons located in the sympathetic chain ganglia parasympathetic.

This “minor revision of the anatomy” would make spinal preganglionic neurons which are universally considered sympathetic (in the thoraco-lumbar chord), synapse onto large numbers of parasympathetic neurons (in the paravertebral chains for sweat glands and periosteum, and in the pelvic ganglion), robbing these terms of any meaning.

Thus, from the functionality point of view, it is not justified to claim that "pelvic organs receive no parasympathetic innervation".

There never was any general or rigorous functional definition of the sympathetic and parasympathetic nervous systems — it is striking, almost ironic, that Langley, creator of the term parasympathetic and the ultimate physiologist, provides an exclusively anatomic definition in his *Autonomic Nervous System, Part I*. Hence, our definition cannot clash with any “functionality point of view”. In fact, as we briefly say in the discussion and explore in (Espinosa-Medina et al., 2018), it is the “sacral parasympathetic” paradigm which is unjustified from a functionality point of view, for implying a functional antagonism across the lumbo-sacral gap, which has been disproven repeatedly. It remains to be determined which neurons are antagonistic to which on the blood vessels of the external genitals; antagonism within one division of the autonomic nervous system would not be without precedent (e.g. there exist both vasoconstrictor and vasodilator sympathetic neurons, and both, inhibitor and activator enteric motoneurons). The way to this question is finally open to research, and as referee#2 says “it is early days”.

Public review by Referee #2

This work further documents differences between the cranial and sacral parasympathetic outflows that have been known since the time of Langley - 100 years ago.

We assume that the referee means that it is the “cranial and sacral parasympathetic outflows” which “have been known since the time of Langley”, not their differences (that we would “further document”): the differences were explicitly negated by Langley. As a matter of fact, the sacral and cranial outflows were first likened to each other by Gaskell, 140 years ago (Gaskell, 1886). This anatomic parallel (which is deeply flawed (Espinosa-Medina et al., 2018)) was inherited wholesale by Langley, who added one physiological argument (Langley and Anderson, 1895) (which has been contested many times (Espinosa-Medina et al., 2018) and references within).

In addition, the sphenopalatine and other cranial ganglia develop from placodes and the neural crest, while sympathetic and sacral ganglia develop from the neural crest alone.

Contrary to what the referee says, the sphenopalatine has no placodal contribution. There is no placodal contribution to any autonomic ganglion, sympathetic or parasympathetic (except an isolated claim concerning the ciliary ganglion (Lee et al., 2003)). All autonomic ganglia derive from the neural crest as determined a long time ago in chicken. For the sphenopalatine in mouse, see our own work (Espinosa-Medina et al., 2016).

One feature that seems to set the pelvic ganglion apart is [...] the convergence of preganglionic sympathetic and parasympathetic synapses on individual ganglion cells (Figure 3). This unusual organization has been reported before using microelectrode recordings (see Crowcroft and Szurszewski, J Physiol (1971) and Janig and McLachlan, Physiol Rev (1987)). Anatomical evidence of convergence in the pelvic ganglion has been reported by Keast, Neuroscience (1995).

Contrary to what the referee says, we do not provide in Figure 3 any evidence for anatomic convergence, i.e. for individual pelvic ganglion cells receiving dual lumbar and sacral inputs. We simply show that cholinergic neurons figure prominently among targets of the lumbar pathway. This said, the convergence of both pathways on the same pelvic neurons, described in the references cited by the referee, is another major problem in the theory of the “sacral parasympathetic” (as we discussed previously (Espinosa-Medina et al., 2018)).

It should also be noted that the anatomy of the pelvic ganglion in male rodents is unique. Unlike other species where the ganglion forms a distributed plexus of mini-ganglia, in male rodents the ganglion coalesces into one structure that is easier to find and study. Interestingly the image in Figure 3A appears to show a clustering of Chat-positive and Th-positive neurons. Does this result from the developmental fusion of mini-ganglia having distinct sympathetic and parasympathetic origins?

The clustering of Chat-positive and Th-positive cells could arise from a number of developmental mechanisms, that we have no idea of at the moment. This has no bearing on sympathetic and parasympathetic.

In addition, Brunet et al dismiss the cholinergic and noradrenergic phenotypes as a basis for defining parasympathetic and parasympathetic neurons. However, see the bottom of Figure S4 and further counterarguments in Horn (Clin Auton Res (2018)).

The bottom of Figure S4 simply indicates which cells are cholinergic and adrenergic. We have already expounded many times that noradrenergic and cholinergic do not coincide with sympathetic and parasympathetic. Henry Dale (Nobel Prize 1936) demonstrated this. Langley himself devoted several pages of his final treatise to this exception to his “Theory on the relation of drugs to nerve system” (Langley, 1921) (p43) (which was actually a bigger problem for him than it is for us, for reason which are too long to recount here; it is as if the theoretical difficulties experienced by Langley had been internalized to this day in the form of a dismissal of the cholinergic sympathetic neurons as a slightly scandalous but altogether forgettable oddity). (Horn, 2018) reviews the evidence that the thoracic cholinergic sympathetic phenotype is brought about by a secondary switch upon interaction with the target and argues that this would be a fundamental difference with the sacral “parasympathetic”. But in fact the secondary switch is preceded by co-expression of ChAT and VAcHT with Th in most sympathetic neurons (reviewed in (Ernsberger and Rohrer, 2018)); and we have no idea of the dynamic in the pelvic ganglion. It may also be mentioned in this context that target-dependent specification of neuronal identity has also been demonstrated of other types of sympathetic neurons ((Furlan et al., 2016)

What then about neuropeptides, whose expression pattern is incompatible with the revised nomenclature proposed by Brunet et al.?

There was never any neuropeptide-inspired criterion for a nomenclature of the autonomic nervous system.

Figure 1B indicates that VIP is expressed by sacral and cranial ganglion cells, but not thoracolumbar ganglion cells.

Contrary to what the referee says, there are VIP-positive cells in our sympathetic data set and even strongly positive ones, except they are scattered and few (red bars on the UMAP). They correspond to cholinergic sympathetics, likely sudomotor, which are known to contain VIP (e.g.(Anderson et al., 2006)(Stanke et al., 2006)). In other words, VIP is probably part of what

we call the cholinergic synexpression group (but was not placed in it by our calculations, probably because of a low expression level in sympathetic noradrenergic cells).

The authors do not mention neuropeptide Y (NPY). The immunocytochemistry literature indicates that NPY is expressed by a large subpopulation of sympathetic neurons but never by sacral or cranial parasympathetic neurons.

Contrary to what the referee says, Keast (Keast, 1995) finds 3.7% of pelvic neurons double stained for NPY and VIP in male rats, and says (Keast, 2006) that in females “co-expression of NPY and VIP is common” (thus in cholinergic neurons that the referee calls “parasympathetic”). Single cell transcriptomics is probably more sensitive than immunochemistry, and in our dichotomized data set (table S1), NPY is expressed in all pelvic clusters and all sympathetic ganglia. In other words, it is one more argument for their kinship. It does not appear in the heatmap because it ranks below the 100 top genes.

Answer to the original recommendations by Referee #2

Introduction - the use of the words 'consensual' and 'promiscuity' are not clear and rather loaded in the context of the pelvic ganglia. Pick alternative words.

There is no sexual innuendo inherent in “promiscuity”: “condition of elements of different kinds grouped or massed together without order” (Oxford English Dictionary). We replaced “never consensual” by “never generally accepted”.

Results - Page 2 - what sex were the mice? Previous works indicate significant sexual dimorphism in the pelvic ganglion.

The mice included both males and females, and male and female cells are represented in all ganglia and clusters. This is now mentioned in the Material and Methods. Thus, however unsuited to analyze sexual dimorphism, our data set ensures that all the cell types we describe are qualitatively present in both sexes.

Results line 3 - the celiac and mesenteric ganglia are prevertebral ganglia and not part of the sympathetic chain. The chain refers to the paravertebral ganglia.

We replaced “part of the prevertebral chain” by “belonging to prevertebral ganglia”. This said, there are precedents for “prevertebral chain ganglia” to designate the rostro-caudal series of prevertebral ganglia. Rita Levi-Montalcini, for example, who devoted her glorious career to sympathetic ganglia, writes in 1972 “The nerve cell population of para- and prevertebral chain ganglia is reduced to 3–5% of that of controls”. (10.1016/0006-8993(72)90405-2).

Page 3 - "as the current dogma implies". Dogma often refers to opinion or church doctrine. The current nomenclature is neither. Pick another word.

There is little in science that is proven to the point of eliminating any element of opinion. “Dogma” refers to “that which is held as a principle or tenet [...], especially a tenet authoritatively laid down by [...] a school of thought” (OED). And “dogma” is used in science to designate tenets better experimentally supported than the “sacral parasympathetic”, such as the “central dogma of molecular biology”.

Page 3 - "To give justice" implies the classical notion is unjust. How about, 'to further explore previous evidence indicating that'

The term is indeed not proper English for the meaning intended, and the right expression is “to do justice”, to mean: “to treat [a subject or thing] in a manner showing due appreciation, to deal with [it] as is right or fitting” (OED). We have corrected the paper accordingly.

Page 4 top - the convergence indicated by Figure 3 does not justify excluding cholinergic and noradrenergic genes from the analysis.

Contrary to what the referee says, Figure 3 does not show any “convergence”, see our answer to Referee#1. What Figure 3 shows is that cells that are targeted by the lumbar pathway (a pathway universally deemed “sympathetic”) are cholinergic in massive proportion. Therefore, by an uncontroversial criterion, the pelvic ganglion contains lots of sympathetic cholinergic neurons. The only other option is to declare that sympathetic preganglionic neurons synapse onto parasympathetic postganglionic ones (which is what Referee#1 proposes, and considers “much simpler”. We beg to differ).

Our justification for excluding cholinergic and noradrenergic genes from the definition of “sympathetic” and “parasympathetic” is simply that sympathetic neurons can be cholinergic (to sweat glands and periosteum; and — as we show in Figure 3 — many targets of the lumbar pathway); One can also note that anywhere else in the nervous system, classifying cell types as a function of neurotransmitter phenotype would lead to non-sensical descriptions, such as putting together pyramidal cells and cerebellar granules, or motor neurons and basal forebrain cholinergic neurons. Indeed Referee#1 proposes such a revolutionary revision, by calling all cholinergic autonomic neurons “parasympathetic” (see our answer above).

Keast (1995) did similar experiments and used presynaptic lesions to draw a different conclusion indicating preferential innervation pelvic subpopulations.

Keast found “preferential” innervation of pelvic subpopulations based on lesion experiments; Nevertheless, she concluded (at the time) that “the correct definition of these two components of the nervous system is based on neuroanatomy rather than chemistry” (Keast, 2006).

Page 4 - "In the aggregate, the pelvic ganglion is best described as a divergent sympathetic ganglion devoid of parasympathetic neurons" The notion of a divergent ganglion is completely unclear!

We take “divergent” in a developmental or evolutionary meaning: related to sympathetic ganglia, yet somewhat differing from them. Elsewhere we use the word “modified”. Importantly (and as cited in the paper), a similar situation emerges from the single cell transcriptomic analysis of the lumbar and sacral preganglionics (by other research groups).

Granted, it is devoid of neurons having the signature of cranial parasympathetics, but that is insufficient to conclude that they are not parasympathetics.

If a genetic signature which is not only un-parasympathetic, but sympathetic-like remains compatible with some version of the label “parasympathetic”, we get dangerously close to dismissing the molecular make-up of a neuron as a definition of its type. This goes against any contemporary understanding of neuron types (take (Zeisel et al., 2018) among hundreds of other examples).

Page 4 - "the entire taxonomy of autonomic ganglia could be a developmental readout of Hox genes." This reader completely agrees! We appreciate this would be difficult to test but it helps to explain possible differences along the rostral-caudal axis. Consider making this a key implication of the study!

If the reader agrees, then his/her previous points become mysterious: we speculate that the Hox code determines the structure of the autonomic nervous system, i.e. the array, along the rostrocaudal axis, of a bulbar parasympathetic, a thoracolumbar sympathetic and lumbosacral “pelvo-sympathetic”. The existence of caudal parasympathetic neurons, on the contrary, would subvert any role for Hox genes: similar neurons (similar enough to be called by the same name) would arise at completely different rostro-caudal levels, i.e. with a different Hox code.

Page 5 - "It is thus remarkable ...that we uncover in no way contradicts the physiology." Not really. The 'classical' sympathetic system innervates the limbs, and the skin and it participates in thermoregulation and in cardiovascular adjustments to exercise. The parasympathetic system does none of these things. Reclassing the pelvic outflow as pseudo-sympathetic contradicts this physiology.

We do not say that the sacral outflow is classically sympathetic; We go all the way to proposing the special name “pelvo-sympathetic”; And we insist that these special sympathetic-like neurons have special targets (detrusor muscle, helicine arteries...): there is no contradiction. Not only is there no contradiction, but we remove the mind-twister of an anatomical/genetic/cell type-based “sacral parasympathetic” combined with a lack of physiological lumbosacral antagonism (we provide a short history of this dissonance in (Espinosa-Medina et al., 2018)), which led Wilfrid Jänig to write (Jänig, 2006)(p. 357): “Thus, functions assumed to be primarily associated with sacral (parasympathetic) are well duplicated by thoracolumbar (sympathetic) pathways. This shows that the division of the spinal autonomic systems into sympathetic and parasympathetic with respect to sexual functions is questionable”. We could not agree more: this division is questionable in terms of physiology and inexistent in terms of cell types. In other words, we reconcile cell types with physiology (but “it is early days”).

Answer to the novel recommendations by Referee #2

In addition to my original comments, important anatomical and functional distinctions are not explained by the data in this paper. ANATOMY- Sympathetic ganglia are located in close proximity to major branches of the aorta. Cranial and sacral parasympathetic ganglia are located next to or within the structures they innervate (e.g. eye, lung, heart, bladder).

The pelvic ganglion, including some of its cholinergic neurons, that the referee insist are parasympathetic, is further removed from one of its major targets (the helicine arteries of the external genitals) than the sympathetic prevertebral ganglia are of some of theirs (like the gut or kidney). We discussed this issue in (Espinosa-Medina et al., 2018).

FUNCTION- The sympathetic system controls state variables (e.g. body temperature, blood pressure, serum electrolytes and fluid balance), parasympathetic neurons do not.

Even in the classical view, the sympathetic system controls the blood vessels of the external genitals or the size of the pupil, for example, which are not state variables.

[...] The data in the paper are a useful next step in defining the genetic diversity of autonomic neurons but do not justify or improve upon existing nomenclature. The future challenge is to understand distinctions between subsets of autonomic ganglion cells that innervate different targets and the principles that govern the integrative function of the autonomic motor system that controls behavior.

We thank the referee for finding our data useful; and we fully agree with the latter statement. However, neurons, like many other cell types, are hierarchically organized (Zeng and Sanes, 2017), i.e. subsets of neurons belong to sets, with defining traits. Our data argue that there is no parasympathetic neuronal set that includes any pelvic ganglionic neuron. In contrast, there is a ganglionic sympathetic set (defined by our analysis of gene expression) which includes all of them — as there is a preganglionic sympathetic set that includes sacral preganglionics (Alkaslasi et al., 2021; Blum et al., 2021)(although the direct comparison with cranial preganglionics is yet to be made).

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