Characterizing the diversity of enteric neurons using Dopamine Transporter (DAT)-Cre reporter mice

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Abstract

The enteric nervous system (ENS) comprises a complex network of neurons whereby a subset appears to be dopaminergic, although the characteristics, roles, and implications in disease are less well understood. Most investigations relating to enteric dopamine (DA) neurons rely on immunoreactivity to tyrosine hydroxylase (TH) - a rate-limiting enzyme in the production of DA. However, TH immunoreactivity is likely to provide an incomplete picture given previous work has showed that some DA neurons contain little if any TH and its levels tend to be decreased in response to cellular stress. The study herein provides a comprehensive characterization of DA neurons in the gut using a well-accepted reporter mouse line, expressing a fluorescent protein under control of the DA transporter (DAT) promoter. Our findings confirm a unique localization of DA neurons in the gut and unveil the discrete subtypes of DA neurons in this organ, which we characterized using both immunofluorescence and single-cell transcriptomics, and validated by *in situ* hybridization. We observed distinct subtypes of DAT neurons expressing co-transmitters and modulators across both plexuses; some of them likely co-releasing acetylcholine, and a smaller population likely releasing nitric oxide; while others were positive for a slew of canonical DA markers. Interestingly, we uncovered a seemingly novel population of DA neurons unique to the ENS which were ChAT/DAT-positive neurons and characterised by the expression of *Th*, *Grp*, *Calcb* and *Sst*. Given the clear heterogeneity of DAergic gut neurons, further investigation is warranted to define their functional signatures and discover any inherent vulnerabilities in disease.

Introduction

An altered gut-brain axis is increasingly recognized as central to many neurological conditions, including neuropsychiatric disorders and neurodegenerative diseases, such as autism, depression and Parkinson's disease (PD)(Björklund & Dunnett, 2007; Hamamah et al., 2022). PD is the second most prevalent neurodegenerative disease, affecting more than 10 million people globally (Ou et al., 2021). Its pathological characteristics are commonly defined by the accumulation of Lewy body inclusions and by the gradual degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) of the midbrain. The selective loss of DA is thought to underlie some of the canonical motor symptoms of PD (Greffard et al., 2006). There is growing recognition that non-motor abnormalities, including perturbed sleep, anosmia, depression and gut dysmotility precede motor deficits by up to 20 years (Gaenslen et al., 2011; Pont-Sunyer et al., 2015; Schrag et al., 2015). These are likely to result from the dysfunction or loss of multiple types of neurons, albeit quantitative evidence for the extent of neuronal loss in regions other than the SNc is fragmented (Giguère et al., 2018). Although it is likely that most PD patients suffer from some level of dysfunction of the peripheral nervous system (PNS) (Abbott et al., 2001; Borghammer, 2023; Fedorova et al., 2017; Raeder et al., 2023; Savica et al., 2009; Skjærbæk et al., 2021), whether gut dysmotility and associated constipation are driven in part by loss of enteric DA neurons remains largely unexplored (Anderson et al., 2007; Francesca et al., 2023; Liu et al., 2021; Wang et al., 2012). Various histological studies have demonstrated the presence of Lewy body inclusions in areas outside the central nervous system (CNS) predominantly in the gastrointestinal tract whereby pathological features are apparent at least 8 years prior to PD diagnosis (Braak et al., 2002; Del Tredici & Braak, 2012; Dickson et al., 2009; Hilton et al., 2014). These inclusions can be found in neurons residing in the gut and positively correlate with constipation in PD patients (Lebouvier et al., 2010). Further reports reveal neuronal death in the ganglionated plexus of patients' colons as well as in rodent models of PD (Lebouvier et al., 2010; McQuade et al., 2021; Ohlsson & Englund, 2019). Despite clear involvement of the gastrointestinal tract in PD, there are inconsistencies reported for the extent of neuronal abrogation (O'Day et al., 2022) as well as minimal studies dedicated to understanding the selectivity of neuronal types affected in the gut of PD patients.

Recent single-cell transcriptomics studies in both human and mice have started to explore the complexity of the cellular landscape within the gut including defining unique gene expression signatures within the neuronal lineage (Drokhlyansky et al., 2020; May-Zhang et al., 2021; Memic et al., 2018; Morarach et al., 2021; Wright et al., 2021). Few included the identification of catecholaminergic cell types expressing genes involved in DA synthesis, including tyrosine hydroxylase (*TH*) (Memic et al., 2018; Morarach et al., 2021; Wright et al., 2021), and of these most were described in the developing ENS of fetal mice. Experiments describing the diversity of DA neuronal populations in the gut have not yet been carried out. Historically, a neuronal subtype

is defined by the primary neurotransmitter it releases and its functional properties. With the gradual appreciation that many if not most neuronal types in the CNS and PNS utilize more than one type of neurotransmitter (El Mestikawy et al., 2011; Trudeau, 2004; Trudeau & El Mestikawy, 2018; Trudeau & Gutiérrez, 2007) and with the advent of single-cell transcriptomic approaches, assigning a molecular gene signature is progressively used to precisely define neuronal sub-classes. In the case of midbrain DA neurons, previous microarray and recent single-cell RNA sequencing (RNAseq) work has revealed extensive heterogeneity including in genes specifying their developmental origin, as well as a battery of genes involved in the synthesis and vesicular packaging of DA, but also of other neurotransmitters including glutamate and various neuropeptides (Gaertner et al., 2022; Kouwenhoven et al., 2020; Poulin et al., 2018; Poulin et al., 2020; Poulin et al., 2014).

The enteric nervous system (ENS) is a network of neurons situated in two separate regions of the gastrointestinal wall; the submucosal plexus (SMP) found underneath the mucosa and the myenteric plexus located within the muscularis propria. The two plexuses are responsible for varying functions of the gut; the SMP plays a crucial role in secretion and luminal sensing, whereas the MP regulates gastrointestinal motility through peristaltic waves. These complex intestinal functions require a diverse population of neurons including TH-positive DA neurons as well as serotonin, cholinergic and nitrergic neurons (Furness et al., 2014). Although there are several reports of the presence of DA neurons in the gut (Li et al., 2004; Walker et al., 2000), such studies typically rely solely on immunostaining for TH – the rate limiting enzyme in the synthesis of catecholamines to confirm DAergic identity. As such, distinct DA neuron subtypes that exist in the gut are minimally characterised. Indeed, neurons lacking TH, but immunoreactive for the DA transporter (DAT) are apparent in the murine ileum and vice-versa (Li et al., 2004), but no further characterisations have been performed. The use of transgenic reporter mouse lines such as those in which promoters for *DAT* drive Cre recombinase to induce the expression of fluorescent molecules, such as tdTomato (named DAT-Cre mice) has the potential to shed new light on enteric DA neuron diversity.

By taking advantage of DAT-Cre mice, we sought to deepen our understanding of DA neurons and their putative subtypes in the ENS. We conducted immunofluorescence staining in colonic tissues along with single-cell RNAseq and *in situ* hybridization of DAT-tdTomato-expressing cells to unravel the heterogeneity of DAergic neuronal subpopulations. Providing an in-depth characterization of these classes of enteric neurons will be conducive to future endeavors aimed at better understanding their functional signatures and any potential selective vulnerabilities of enteric neurons in disease.

Methods

Protocols associated with this work can be found on protocols.io: dx.doi.org/10.17504/protocols.io.3byl4q792vo5/v1

Mice and tissue processing

DAT-Ires-Cre (RRID:IMSR_JAX:006660) mice were bred with Ai9 mice (RRID: IMSR_JAX:007909) to induce the expression of tdTomato in DAT-expressing cells (namely DAT-Cre mice). Adult DAT-Cre mice of age 2-3 months were utilized in the present study. Mice (both sexes) were housed in a vivarium under pathogen-free conditions, given water and food *ad libitum.* Studies were approved by the Université de Montréal Animal Care and Use Committee (CDEA). CDEA guidelines on the ethical use and care of animals were followed. The mice were euthanized by isoflurane followed by CO_2 asphyxiation. Age-matched wild type C57Bl6/J mice (RRID:IMSR_JAX:000664) were also used as control for tdTomato expression. We collected gut tissue, and focused on colons where fewer investigations of the ENS have been made and which is affected in prodromal stages of PD (Lebouvier et al., 2010; Ohlsson & Englund, 2019). After isolation, fecal material was flushed with an 18-gauge needle and adipose tissue was removed. Colons were cut open longitudinally and coiled around themselves, resembling a *swiss-roll* (as depicted in Fig 1A), then fixed in 4% paraformaldehyde (PFA) for 24 hours and dehydrated with a 30% sucrose solution. The tissue was then embedded and cryosectioned serially to obtain coronal sections at 15 µm, then stored at -80°C.

Immunofluorescence

Following thawing, tissue sections were permeabilized and blocked (10% donkey serum with 0.5% Triton X-100) for 2h. Primary antibody stains were performed overnight at room temperature (RT) followed by secondary antibody staining for 2h. Primary and secondary antibodies used are indicated in **Table 1**. Hoechst 33258 was used for nuclear staining (1:5000, Thermo Fisher Scientific Cat# H3569, RRID:AB_2651133). Slides were mounted in Permafluor Mounting media (Thermofisher).

Table 1: Primary antibodies used in immunofluorescence staining of colonic tissue

Antigen	Host	Dilution	Company (CAT#)	RRDI
Tubb3	Mouse	1:500	Millipore (MAB5564)	RRID:AB_570921
Pgp9.5	Mouse	1:500	ThermoFisher (480012)	RRID:AB_2532243
HuD	Rabbit	1:200	Abcam (ab96474)	N/A
ChAT	Goat	1:200	Sigma Aldrich (SAB2500236)	RRID:AB_10603616
nNOS	Rabbit	1:500	ThermoFisher (61-7000)	RRID:AB_2313734
tdTomato	Goat	1:200	Rockland (200-101-379)	RRID:AB_2744552
tdTomato	Rabbit	1:200	Rockland (600-401-379)	RRID:AB_2209751
Th	Chicken	1:100	Millipore (AB9702)	RRID:AB_570923
Girk2	Rabbit	1:100	Alomone Labs (APC-006)	RRID:AB_2040115
Foxa2	Rabbit	1:100	R&D Systems (AF2400)	RRID:AB_2294104
Vmat2	Rabbit	1:200	Abcam (ab70808)	RRID:AB_1952699

Anti goat AF488	Donkey	1:200	Thermo Fisher (A11055)	RRID:AB_2534102
Anti rabbit AF488	Donkey	1:200	Thermo Fisher (A-21206)	RRID:AB_2535792
Anti goat AF555	Donkey	1:200	Thermo Fisher (A-21432)	RRID:AB_2535853
Anti mouse AF555	Donkey	1:200	Thermo Fisher (A-31570)	RRID:AB_2536180
Anti rabbit AF555	Donkey	1:200	Thermo Fisher (A-31572)	RRID:AB_162543
Anti mouse AF647	Donkey	1:200	Thermo Fisher (A-31571)	RRID:AB_162542
Anti chicken AF647	Donkey	1:200	Jackson ImmunoResearch (703-605-155)	RRID:AB_2340379

In situ hybridization

Following thawing, tissue sections were heated for 30 mins at 60°C, post-fixed in pre-chilled 4% PFA for 15 mins at 4°C, and dehydrated by subsequent immersion in 50, 70, and 100% ethanol for 5 mins at RT. *In situ* staining of fixed-frozen samples were performed according to the RNAscope Multiplex Fluorescence manufacturer's instructions (Advanced Cell Diagnostics, ACD). Briefly, tissue sections were incubated for 10 mins at 95°C for antigen retrieval and Protease III was applied for 30 mins at 40°C. After multiple washes, RNAscope multiplex assay was carried out as described in ACD's protocol followed by the aforementioned immunofluorescence protocol to co-stain with tdTomato and ChAT antibodies. All incubations were at 40°C and used a humidity control chamber. Mouse probes used were Grp-C1, Calcb-C1 and Sst-C1; and Opal dye 520 was used at 1:1500 concentration. Hoechst 33258 was used for nuclear staining (1:5000, Invitrogen H3569). Slides were mounted in Prolong Gold Antifade Mounting media (Thermofisher).

Single-cell suspension preparation

Given protocols are established for longitudinal muscle myenteric plexus (LMMP) isolations, and that this part of the ENS comprises neurons key in modulating gut motility (Furness et al., 2014), which is oftentimes disrupted in PD (Gaenslen et al., 2011; McQuade et al., 2021; Pont-Sunyer et al., 2015; Schrag et al., 2015), we prepared single cell suspensions from the MP. Isolated colons from adult DAT-Cre mice were washed, cut open longitudinally as described above, and pinned with the intestinal lumen facing down on a 4% agarose pad. The LMMP was then micro-dissected by peeling it off using fine-tip tweezers, and was then collected and cut into 1-2 cm pieces. The tissues were then enzymatically dissociated to release myenteric neurons and other cell types using 1 mg/mL collagenase IV (Sigma, C5138) and 0.05% trypsin (Sigma, T4049), adapted from (Smith et al., 2013). Dissociated cells were filtered through a 40 μm cell strainer and stained with eFluor506 fixable viability dye (ThermoFisher, 65-0866-14). About 20% live single DAT-

tdTomato-positive cells were collected by fluorescence-activated cell sorting (FACS Aria). LMMP tissue collected from wild type C57Bl6/J mice was used as control for gating.

Single-cell library preparation and analysis

Live DAT-tdTomato-positive cells (20, 000 cells total) were FACS collected (from pooled 2 male and 2 female mice) and then we prepared single-cell sequencing libraries. All cells were processed according to 10X Genomics Chromium Single Cell 3' Reagent Guidelines (https://support.10xgenomics.com/single-cell-gene-expression). Briefly, cells were partitioned into nanoliter-scale Gel Bead-In-EMulsions (GEMs) using 10X GemCode Technology. Primers containing (i) an Illumina R1 sequence, (ii) a 16 bp 10x barcode, (iii) a 10 bp Unique Molecular Identifier (UMI) and (iv) a poly-dT primer sequence were incubated with partitioned cells resulting in barcoded, full-length cDNA from poly-adenylated mRNA. Silane magnetic beads were used to remove leftover biochemical reagents/primers, then cDNA was amplified by PCR. Enzymatic fragmentation and size selection was used to optimize cDNA amplicon size prior to library construction. R1 (read 1 primer sequence) were added during GEM incubation, whereas P5, P7, a sample index (i7), and R2 (read 2 primer sequence) were added during library construction via end repair, A-tailing, adaptor ligation and PCR. Quality control and quantification was performed using an Agilent Bioanalyzer High Sensitivity chip. Sequencing was performed using NovaSeq 6000 S4 PE 100bp, which resulted in a read depth of 46 000 reads/cell. Reads were processed using the 10X Genomics Cell Ranger Single Cell 2.0.0 pipeline (RRID:SCR_017344, https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger) with default and recommended parameters, as previously described (Zheng et al., 2017). FASTQs generated from sequencing output were aligned to the mouse GRCm38.p5 (primary assembly) reference genome using the STAR algorithm 2.7.3a (RRID:SCR_004463, http://code.google.com/p/rna-star/) and to mouse tdTomato genome (Rahmani et al., 2020). Next, gene-barcode matrices were generated for each individual sample by counting unique molecular identifiers (UMIs) and filtering non-cell associated barcodes. This output was then imported into the Seurat v4.9.9.9039 R toolkit v.4.2.1 (RRID:SCR_016341, https://satijalab.org/seurat/get_started.html; RRID:SCR_001905, http://www.r-project.org/) for quality control and downstream analysis of our single-cell RNA-seq experiment (Macosko et al., 2015). All functions were run with default parameters, unless specified otherwise. Low quality cells (<200 genes/cell and >5 % of mitochondrial genes) were excluded from the overall experiment. Gene expression was log normalized to a scale factor of 10 000. A total of ~8, 000 cells and ~20, 000 genes were detected.

Tissue quantification and statistical analysis

Immunostained tissue slices were imaged using a Leica SP8 Confocal microscope and quantified with the Las X Leica software. Images were taken at 40X magnification with Z-stacks (step size of 0.9). Each ganglion was identified in either the submucosal or myenteric plexus based on morphology as shown in earlier publications (Kulkarni et al., 2018; Li et al., 2004). All quantitative analysis at the ganglia was normalized to an area of 900 μm^2 (calculated as the average size of a ganglion). Animal replicates can be found in the figure legends. All statistical analysis was performed using GraphPad Prism 5 (RRID:SCR_002798, http://www.graphpad.com/). For comparisons across multiple groups, a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison was used. For comparisons of across two groups, an unpaired

Student's t test was used. P values of < 0.05 were considered significant. Raw tabular data underlying each finding reported as a graph can be found at Zenodo.org: 10.5281/zenodo.8231677.

For quantification of single-cell RNA-seq data, unsupervised clustering of cells was performed. A selection of highly variable genes (2000 genes) was obtained and used for principal component analysis. Significant principal components were determined using JackStraw analysis. PCs 1 to 10 were used for graph-based clustering (at resolution = 1) to identify distinct groups of cells. These groups were projected onto UMAP analysis using previously computed principal components 1 to 20. Expression of selected marker genes (*Elavl4*, *Tubb3* and *Uchl1*) was used to classify neurons and these initial clusters were further divided into seven cell clusters identified by graph-based clustering.

Results

Enteric pan-neuronal markers and tdTomato expression are detectable in both colonic plexuses in DAT-tdTomato transgenic mice

DAT-Cre transgenic lines are considered more reliable in the selective identification of midbrain DA neurons compared to other reporter mouse lines such as TH-Cre (Bäckman et al., 2006; Ekstrand et al., 2007; Engblom et al., 2008; Lindeberg et al., 2004; Zhuang et al., 2005). As such, we used DAT-Cre crossed with Ai9 cre-dependent tdTomato mice and immunohistochemistry (Fig. 1A) to confirm the existence of submucosal and myenteric DA neurons, as well as characterize their heterogeneity. Using cross sectional analysis in *swiss-roll* format, we detected three subdivisions of the intestinal layers and two ganglionated plexuses, as expected. We consistently detected the submucosal plexus (SMP) between the mucosa and the muscularis propria, as well as the myenteric plexus (MP) within the muscularis propria, using a battery of pan-neuronal markers (Fig. 1B) (RNA-binding human antigen, HuD; tubulin β3, Tubb3; and ubiquitin carboxyl-terminal hydrolase-1, Uch-l1/Pgp9.5). We further explored the efficacy of these commonly used pan-neuronal markers given, in most cases, they are utilized interchangeably, with little evidence to support which markers are most robust. Tubb3 is a cytoskeletal protein involved in microtubule stability and is often used to label both neuronal soma and axonal projections in the CNS or PNS, whereas HuD and Pgp9.5 are expressed predominantly in peripheral neurons (Bolognani et al., 2010; Hibi et al., 1999; Liu et al., 2007). A direct comparison of the expression of these neuronal markers revealed that there was no statistical difference within or between plexuses across all markers (Fig. 1B). We opted to focus largely on Tubb3 as a co-label with tdTomato for consistency and due to compatibility considerations with other antibodies. We also determined that tdTomato expression was robust in colonic tissue in both the SMP and MP of DAT-Cre mice, and corroborated that the signal was not detected in wild type C57Bl/6J colonic tissue, as expected (Fig. 1C). Of note, we used a blood-myenteric-barrier marker to define MP borders, termed Agrin (which is not expressed in the SMP) (Dora et al., 2021), important for defining ganglia localization in C57Bl/6J mice (Fig. 1C).

TdTomato-immunoreactive DA neurons vary across plexuses

In keeping with previous descriptions of DA neurons in the gut demonstrating that the frequency of enteric TH-expressing neurons gradually decreases from rostral to caudal region of the gastrointestinal tract and from the SMP to MP (Chalazonitis et al., 2022; Li et al., 2004; Qu et al., 2008), we found a reduced density of tdTomato/Tubb3-positive DA neurons in the SMP compared to the MP using DAT-Cre mice (Fig. 2A). We also found that the majority of DAT-tdTomato-positive neurons were immunoreactive for TH, the rate-limiting DA synthesis enzyme, both in the MP and SMP (Fig. 2B, 2nd column). This indicated that although there are less DA neurons in the colonic MP, the majority of DA neurons that do exist in the MP appear similarly TH-positive. Interestingly, few DAT-tdTomato expressing neurons were TH-negative, while some TH-immunoreactive neurons were negative for DAT-tdTomato (Fig. 2B, 3rd column), similar to what

has been described in the ventral midbrain (Lammel et al., 2015; Poulin et al., 2018; Tiklová et al., 2019).

A large proportion of tdTomato-immunoreactive neuronal populations in the ENS exhibit dual-transmitter content

The complex neural networks in the ENS can be largely categorized as either excitatory or inhibitory motor neurons (Brehmer, 2021). The former mostly express choline acetyltransferase (ChAT), generally classified as cholinergic, whereas the latter is commonly referred to as nitrergic due to their release of nitric oxide, an inhibitory molecule synthesized by neuronal-specific nitric oxide synthase (nNOS) (Dharshika & Gulbransen, 2023). The striking abundance of DAT-tdTomato-positive neurons in the colon wall in the present study, especially in the SMP (Fig. 2A), led us to speculate that a large subset of enteric cholinergic or nitrergic neurons may also have a DAergic phenotype. This conclusion would coincide with a growing literature demonstrating that DA neurons as well as other CNS and PNS neurons use a combination of neurotransmitters (Brunet Avalos & Sprecher, 2021; Dal Bo et al., 2004; El Mestikawy et al., 2011; Trudeau & El Mestikawy, 2018). To examine this, we performed double-immunolabelling for HuD and ChAT, Tubb3 and nNOS, tdTomato and ChAT, as well as tdTomato and nNOS. Firstly, we confirmed the presence of both cholinergic and nitrergic neurons in both plexuses at similar levels (Fig. 3A). Then we assessed ChAT-and nNOS-positive neurons for DAT-tdTomato (Fig 3B). Indeed, double-phenotype DA neuron populations represented approximately 50% of either cholinergic or nitrergic neurons in both the SMP and MP (Fig 3B'). There was also a fraction of DAT-tdTomato-positive neurons that did not express ChAT or nNOS (Fig. 3B").

Single-cell transcriptomics further reveals heterogenous dopaminergic neuronal populations

Considering we identified clear heterogeneity within DA neurons in the colon, we next used single-cell transcriptomics to further examine gene expression patterns of enteric DA neurons. Dissociated DAT-tdTomato-positive cells were FACS-collected, then the 10X Genomics workflow was applied (Fig. 4A). Our flow cytometer detected that ~20% of live enteric cells were expressing tdTomato (wild type C57Bl/6J mice were used as negative control for gating, Fig. 4B, C). Following the application of computational quality control measures, we detected 247 DAT-tdTomato neurons based on the expression of *Elavl4* (HuD), *Tubb3* and *Uchl1* (Pgp9.5) (Fig. 4D), which we further classified into seven distinct cell clusters (using a resolution of 1). Consistent with our immunostaining, we identified tdTomato-positive neuronal cell clusters that expressed *Chat* (Cluster 0, 2, 3 and 6) and one cluster expressing *Nos1* (Cluster 1). Some *tdTomato*-positive neuronal populations had little to no expression of *Chat* or *Nos1* (Clusters 4, 5 and 7; Fig. 4D). We then evaluated *Th* expression and found highest expression in Clusters 2 and 5 (Fig. 5A). Notably, Cluster 5 showed high expression of canonical DA neuron markers, important in the vesicular packaging of DA (*i.e.,*vesicular monoamine transporter 2, *Vmat2*), in the regulation of SNc neurons excitability (*i.e.,* G-protein-regulated inward-rectifier potassium 2, *Girk2*), as well as previously reported genes expressed in midbrain DA neurons, such as vesicular glutamate

transporter 2, *Vglut2*, cocaine- and amphetamine-regulated transcript protein, *Cartpt* and 5-hydroxytryptamine/serotonin receptor 2B, *Htr2b* (Ásgrímsdóttir & Arenas, 2020; Carpenter et al., 2020; Doly et al., 2017; El Mestikawy et al., 2011; Trudeau, 2004; Trudeau & El Mestikawy, 2018; Trudeau & Gutiérrez, 2007) (Fig. 5B). Validating our transcriptome observations, we detected immunoreactivity for VMAT2 and GIRK2 in DAT-tdTomato-expressing neurons within the ganglionated plexuses (Fig. 5C). As for Cluster 2, the *Th*-expressing DAT-tdTomato-positive neurons had very little to no expression of the aforementioned traditional DA neuron markers (Fig. 5B); however high *Chat* expression was noted in this cluster compared to *Th*-expressing Cluster 5 (Fig. 4D). We thus further explored the transcriptomic signature of this unique *Th, Chat*-expressing, DAergic neuronal subtype (Fig. 6A); and amongst them we detected a range of genes previously reported in enteric neurons (*Grp, Calcb* and *Sst*), but not yet shown to be present in enteric DA neurons (Fig. 6B). Interestingly, these genes have also been associated with DA signaling in the brain (Charbit et al., 2009; Elina et al., 2023; Kramer et al., 2018; Salesse et al., 2020). Validation experiment using *in situ* hybridization (RNAscope technology) indicated the expression of these novel markers in a small fraction of cells immunoreactive to both tdTomato and ChAT in the colonic myenteric plexus (Fig. 6C).

Discussion

Enteric neurons are numerous and exhibit diverse neuronal subtypes, yet they are largely understudied, including for the functions and subtypes of DA neurons (Chalazonitis et al., 2020; Li et al., 2011; Li et al., 2006; Walker et al., 2000). Here we leveraged scRNAseq to investigate DA neuron heterogeneity in the colon. In particular, we identified a novel DAT/CHAT-positive enteric neuronal subtype that express many genes (*Th*, *Grp, Calcb* and *Sst*) consistent with a dual-transmitter DA neuronal phenotype. Like in the CNS, our DA enteric neuron classification has unveiled striking heterogeneity that could underlie functional heterogeneity of these cell populations in health and disease.

Our discovery of DA neurons with dual-transmitter properties in the gut suggests diversity within the enteric plexus that may contribute to functional differences. Previous work has indeed suggested that enteric DA neurons have multiple roles. For example, deletion of *Dat* in mice suggested the involvement of DAergic neurons in modulating the propagation of colonic migrating motor complexes (CMMC) to promote peristalsis – a central function of the MP (Walker et al., 2000). Meanwhile, intraperitoneal administration of neurotoxins targeting the DA system resulted not only in the ablation of enteric DA neurons, but also reduced colonic mucus content due to decreased signaling through the DA receptor D5 expressed in goblet cells (Li et al., 2019). Contrary to myenteric DA neurons, the implication of DA in mucosal secretion may be attributed to a subset of cells primarily in submucosal ganglia. Submucosal DAergic neurons were believed to further participate in gastrointestinal motility whereby mice with DA neuronal hypoplasia in the SMP manifested longer gastrointestinal transit time and slower colonic expulsion time (Chalazonitis et al., 2020). These submucosal DAergic neuronal subtypes may differ from those responsible for mucosal secretions. Optogenetic stimulation studies in transgenic mice revealed that CMMC generation is mediated in part by the activation of cholinergic neurons while nitrergic neurons are active during its tonic inhibition (Gould et al., 2019). As such, DA release from DAT-tdTomato-positive neurons co-expressing either ChAT or nNOS could be involved in governing this complex process. It has been hypothesized that repeated patterns of interconnected neural networks in the ENS are activated during peristalsis (Chalazonitis et al., 2022). To add complexity, studies have shown that DA receptor blockade conveys a suppressive action on colonic motility (Li et al., 2011; Li et al., 2006). This conclusion is contradictory to the previous work mentioned whereby inhibiting DA reuptake elicits fewer smooth muscle contraction (Walker et al., 2000). Clearly, there is a need for a robust functional classification of enteric DA neuron subtypes not only to define which cells control gut motility but also to unveil other intricate DA-related mechanisms.

Using single-cell transcriptomics, we found that traditional markers previously shown at high levels in midbrain DA neurons (eg. *Vmat2*, *Girk2*, *Vglut2*, *Cartpt* and *Htr2b*) are expressed in a DAT-tdTomato neuronal cluster that is also expressing *Th*, which may represent a subpopulation

of enteric neurons with functional similarity to certain classes of DA neurons in the brain. Interestingly, we also identified a distinct *Th*-expressing, tdTomato-positive neuronal cluster that may share similar properties with cholinergic neurons as evidenced by its high *Chat* expression. This novel neuronal subtype also upregulates genes (eg. *Grp, Calcb* and *Sst*) expressed in DA neurons found in distinct brain areas. Prior evidence for the expression of gastrin-releasing neuropeptide encoded by *Grp* in subpopulations of midbrain DA neurons substantiate its relevance as a candidate marker for DA neuron subtypes in the gut (Kramer et al., 2018; La Manno et al., 2016; Poulin et al., 2014; Viereckel et al., 2016). In contrast to previous reports of widespread expression of GRP-immunoreactive DA neurons in the midbrain (Kramer et al., 2018), our study suggests rare expression which further underscores the heterogeneity of DA subtypes across the ENS. As for *Sst* and *Calcb*; not only have their expression also been reported in DA neurons of the CNS (Charbit et al., 2009; Elina et al., 2023; Salesse et al., 2020), but these transmitters likely contribute to the multifaceted features of the DAergic system. For instance, TH-expressing neurons in the ventral tegmental area (VTA) co-release somatostatin (SST) are implicated in the response to stress (Elina et al., 2023) while calcitonin-gene related peptide (CALCB) expressed in hypothalamic DA neurons regulates pain transmission (Charbit et al., 2009). Further investigations of the differential gene expression landscape of DA neuron subtypes between the ENS and CNS would help better extrapolate shared and unique transcriptional profiles of DA neuron subpopulations and their discrete functions.

Our study sheds light on the potential heterogenous nature of the function of DA neurons in the colon. We found high, but variable expression of tdTomato across the ganglionated plexuses, with the SMP containing more DAT-tdTomato-positive DAergic neurons compared to the MP. Further analyses revealed that a small subset of these neurons does not express TH. The reason for this is unclear, albeit between embryonic days 10 to 13, a population of neuronal precursor cells termed transiently catecholaminergic (TC) cells have been reported to display high expression of TH, which progressively diminishes during terminal differentiation (Baetge & Gershon, 1989; Gershon et al., 1984). Although the majority of TC cells give rise to non-catecholaminergic cell types, the question of whether some TC cells express DAT at a specific stage and whether some of them acquire a DAergic phenotype including the expression of VMAT2 despite low levels of TH persists. Li and colleagues have in fact demonstrated only partial co-localization of DAT and TH immunoreactivities in the mouse ileum and colon, further alluding to the existence of DAT-expressing neurons that either have no or little expression of TH protein, most prominent in the SMP (Li et al., 2004). It is tempting to speculate that these divergent neuronal subtypes have corresponding functional differences whereby TH neurons may represent a more mature counterpart able to synthesize and release DA while tdTomato neurons that are TH and VMAT2-negative represent cells that are non-traditional DAergic in the sense that they should not be able to synthesize and release DA.

It remains possible that the DAergic phenotype of DAT-tdTomato neurons not expressing TH could be reactivated later in life in response to signals associated with cellular stress. Indeed, in previous work, denervation of axonal projections to the gut culminated in elevated proportions of TH-immunoreactive neurons without affecting the overall number of enteric neurons (Li et al., 2004), implying that existing pools of neurons may upregulate TH expression as a compensatory mechanism in response to injury. Hence, we postulate that a subset of molecularly defined neurons may be acting as *reserves* with submaximal DAergic function. This further begets the question whether the localization of DA neurons (being higher in the SMP) relates to the potential vulnerability of submucosal neurons (given the proximity to the intestinal lumen) and thus presumably necessitates the need to have a pool of *reserve* DA neurons in this region. In agreement with a study depicting the blood-ganglia barrier only at the myenteric plexus (Dora et al., 2021), we also noted the absence of immunoreactivity for Agrin in the SMP, thereby suggesting a lack of protection from the blood-ganglia barrier in the SMP which could render submucosal neurons more susceptible to immune cell and bacterial-related damage. A perplexing idea dominating the field of PD is the question of the origin of selective vulnerability of DA neuron subtypes and how specific gene signatures may help us infer the underlying mechanisms (Giguère et al., 2018; Poulin et al., 2020). Although enteric neurodegeneration has been examined in PD, there still exists discrepancies as to the extent and specificity of neuronal damage involved (Lebouvier et al., 2010; McQuade et al., 2021; O'Day et al., 2022; Ohlsson & Englund, 2019). In this regard, the ion channel GIRK2 implicated in modulating neuronal excitability has been hypothesized as a key factor that may influence sensitivity of specific DA neurons to degeneration. Strong GIRK2 expression is evident in most SNc DAergic cell types, but fewer (with lower levels) in less affected areas, such as the VTA, as described in post-mortem tissues and recent single-cell RNAseq dataset from a murine model of PD (Behzad Yaghmaeian et al., 2023; Reyes et al., 2012). Consistent with this correlation, we observed that tdTomato-positive neuronal *Th*-expressing Clusters 2 and 5 express *Girk2*, suggesting that a comparable selective vulnerability may be present in these gut DA subtypes.

Collectively, our study provides a steppingstone to further explore the diverse molecular and cellular properties of DAergic neurons in the gut. These data suggests that the DA neuron populations within the gut are more heterogenous than previously thought, supporting the notion that DA neurons play diverse functions in the gut. A better understanding of enteric neurons and their vulnerability in disease is critical in the face of mounting evidence suggesting roles of the gut in a myriad of brain disorders (Dinan & Cryan, 2017).

Ethics Statement

The animal study was reviewed and approved by the Université de Montréal Animal Care and Use Committee (CDEA).

Author Contributions

SJR carried out the tissue processing, single-cell preparations and bioinformatic work while SP performed immunostaining, imaging and quantification. SM bred and maintained animal colony. AM and MY supported tissue processing and bioinformatic analyses, respectively. SG and LET mentored, provided feedback on the project and contributed to writing the manuscript. SJR and SP prepared figures and drafted the manuscript. SJR and JS conceived the project, supervised the work, and prepared and approved the final manuscript. All authors contributed to the article and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data available in GSE237170 and at Zenodo.org: 10.5281/zenodo.8231677

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References

Abbott, R. D., Petrovitch, H., White, L. R., Masaki, K. H., Tanner, C. M., Curb, J. D., Grandinetti, A., Blanchette, P. L., Popper, J. S., & Ross, G. W. (2001). Frequency of bowel movements and the future risk of Parkinson's disease. *Neurology*, *57*(3), 456-462. https://doi.org/10.1212/wnl.57.3.456

Anderson, G., Noorian, A. R., Taylor, G., Anitha, M., Bernhard, D., Srinivasan, S., & Greene, J. G. (2007). Loss of enteric dopaminergic neurons and associated changes in colon motility in an MPTP mouse model of Parkinson's disease. *Exp Neurol*, *207*(1), 4-12. https://doi.org/10.1016/j.expneurol.2007.05.010

Ásgrímsdóttir, E. S., & Arenas, E. (2020). Midbrain Dopaminergic Neuron Development at the Single Cell Level: In vivo and in Stem Cells. *Front Cell Dev Biol*, *8*, 463. https://doi.org/10.3389/fcell.2020.00463

Bäckman, C. M., Malik, N., Zhang, Y., Shan, L., Grinberg, A., Hoffer, B. J., Westphal, H., & Tomac, A. C. (2006). Characterization of a mouse strain expressing Cre recombinase from the 3' untranslated region of the dopamine transporter locus. *Genesis*, *44*(8), 383-390. https://doi.org/10.1002/dvg.20228

Baetge, G., & Gershon, M. D. (1989). Transient catecholaminergic (TC) cells in the vagus nerves and bowel of fetal mice: relationship to the development of enteric neurons. *Dev Biol*, *132*(1), 189-211. https://doi.org/10.1016/0012-1606(89)90217-0

Behzad Yaghmaeian, S., Laura, L., Linda, G., Jesper Kjaer, J., Ioannis, M., Per, S., & Thomas, P. (2023). Transcriptomic atlas of midbrain dopamine neurons uncovers differential vulnerability in a Parkinsonism lesion model. *bioRxiv*, 2023.2006.2005.543445. https://doi.org/10.1101/2023.06.05.543445

Björklund, A., & Dunnett, S. B. (2007). Dopamine neuron systems in the brain: an update. *Trends Neurosci*, *30*(5), 194-202. https://doi.org/10.1016/j.tins.2007.03.006

Bolognani, F., Contente-Cuomo, T., & Perrone-Bizzozero, N. I. (2010). Novel recognition motifs and biological functions of the RNA-binding protein HuD revealed by genome-wide identification of its targets. *Nucleic Acids Res*, *38*(1), 117-130. https://doi.org/10.1093/nar/gkp863

Borghammer, P. (2023). The brain-first vs. body-first model of Parkinson's disease with comparison to alternative models. *J Neural Transm (Vienna)*, *130*(6), 737-753. https://doi.org/10.1007/s00702-023-02633-6

Braak, H., Del Tredici, K., Bratzke, H., Hamm-Clement, J., Sandmann-Keil, D., & Rüb, U. (2002). Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). *J Neurol*, *249 Suppl 3*, Iii/1-5. https://doi.org/10.1007/s00415-002-1301-4

Brehmer, A. (2021). Classification of human enteric neurons. *Histochem Cell Biol*, *156*(2), 95-108. https://doi.org/10.1007/s00418-021-02002-y

Brunet Avalos, C., & Sprecher, S. G. (2021). Single-Cell Transcriptomic Reveals Dual and Multi-Transmitter Use in Neurons Across Metazoans. *Front Mol Neurosci*, *14*, 623148. https://doi.org/10.3389/fnmol.2021.623148

Carpenter, M. D., Hu, Q., Bond, A. M., Lombroso, S. I., Czarnecki, K. S., Lim, C. J., Song, H., Wimmer, M. E., Pierce, R. C., & Heller, E. A. (2020). Nr4a1 suppresses cocaine-induced behavior via epigenetic regulation of homeostatic target genes. *Nat Commun*, *11*(1), 504. https://doi.org/10.1038/s41467-020-14331-y

Chalazonitis, A., Li, Z., Pham, T. D., Chen, J., Rao, M., Lindholm, P., Saarma, M., Lindahl, M., & Gershon, M. D. (2020). Cerebral dopamine neurotrophic factor is essential for enteric neuronal development, maintenance, and regulation of gastrointestinal transit. *J Comp Neurol*, *528*(14), 2420-2444. https://doi.org/10.1002/cne.24901

Chalazonitis, A., Rao, M., & Sulzer, D. (2022). Similarities and differences between nigral and enteric dopaminergic neurons unravel distinctive involvement in Parkinson's disease. *NPJ Parkinsons Dis*, *8*(1), 50. https://doi.org/10.1038/s41531-022-00308-9

Charbit, A. R., Akerman, S., Holland, P. R., & Goadsby, P. J. (2009). Neurons of the dopaminergic/calcitonin gene-related peptide A11 cell group modulate neuronal firing in the trigeminocervical complex: an electrophysiological and immunohistochemical study. *J Neurosci*, *29*(40), 12532-12541. https://doi.org/10.1523/jneurosci.2887-09.2009

Dal Bo, G., St-Gelais, F., Danik, M., Williams, S., Cotton, M., & Trudeau, L. E. (2004). Dopamine neurons in culture express VGLUT2 explaining their capacity to release glutamate at synapses in addition to dopamine. *J Neurochem*, *88*(6), 1398-1405. https://doi.org/10.1046/j.1471-4159.2003.02277.x

Del Tredici, K., & Braak, H. (2012). Lewy pathology and neurodegeneration in premotor Parkinson's disease. *Mov Disord*, *27*(5), 597-607. https://doi.org/10.1002/mds.24921

Dharshika, C., & Gulbransen, B. D. (2023). Enteric Neuromics: How High-Throughput "Omics" Deepens Our Understanding of Enteric Nervous System Genetic Architecture. *Cell Mol Gastroenterol Hepatol*, *15*(2), 487-504. https://doi.org/10.1016/j.jcmgh.2022.10.019

Dickson, D. W., Fujishiro, H., Orr, C., DelleDonne, A., Josephs, K. A., Frigerio, R., Burnett, M., Parisi, J. E., Klos, K. J., & Ahlskog, J. E. (2009). Neuropathology of non-motor features of Parkinson disease. *Parkinsonism Relat Disord*, *15 Suppl 3*, S1-5. https://doi.org/10.1016/s1353-8020(09)70769-2

Dinan, T. G., & Cryan, J. F. (2017). Gut Feelings on Parkinson's and Depression. *Cerebrum*, *2017*.

Doly, S., Quentin, E., Eddine, R., Tolu, S., Fernandez, S. P., Bertran-Gonzalez, J., Valjent, E., Belmer, A., Viñals, X., Callebert, J., Faure, P., Meye, F. J., Hervé, D., Robledo, P., Mameli, M., Launay, J. M., Maldonado, R., & Maroteaux, L. (2017). Serotonin 2B Receptors in Mesoaccumbens Dopamine Pathway Regulate Cocaine Responses. *J Neurosci*, *37*(43), 10372-10388. https://doi.org/10.1523/jneurosci.1354-17.2017

Dora, D., Ferenczi, S., Stavely, R., Toth, V. E., Varga, Z. V., Kovacs, T., Bodi, I., Hotta, R., Kovacs, K. J., Goldstein, A. M., & Nagy, N. (2021). Evidence of a Myenteric Plexus Barrier and Its Macrophage-Dependent Degradation During Murine Colitis: Implications in Enteric Neuroinflammation. *Cell Mol Gastroenterol Hepatol*, *12*(5), 1617-1641. https://doi.org/10.1016/j.jcmgh.2021.07.003

Drokhlyansky, E., Smillie, C. S., Van Wittenberghe, N., Ericsson, M., Griffin, G. K., Eraslan, G., Dionne, D., Cuoco, M. S., Goder-Reiser, M. N., Sharova, T., Kuksenko, O., Aguirre, A. J., Boland, G. M., Graham, D., Rozenblatt-Rosen, O., Xavier, R. J., & Regev, A. (2020). The Human and Mouse Enteric Nervous System at Single-Cell Resolution. *Cell*, *182*(6), 1606-1622.e1623. https://doi.org/10.1016/j.cell.2020.08.003

Ekstrand, M. I., Terzioglu, M., Galter, D., Zhu, S., Hofstetter, C., Lindqvist, E., Thams, S., Bergstrand, A., Hansson, F. S., Trifunovic, A., Hoffer, B., Cullheim, S., Mohammed, A. H., Olson, L., & Larsson, N. G. (2007). Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. *Proc Natl Acad Sci U S A*, *104*(4), 1325-1330. https://doi.org/10.1073/pnas.0605208103

El Mestikawy, S., Wallén-Mackenzie, A., Fortin, G. M., Descarries, L., & Trudeau, L. E. (2011). From glutamate co-release to vesicular synergy: vesicular glutamate transporters. *Nat Rev Neurosci*, *12*(4), 204-216. https://doi.org/10.1038/nrn2969

Elina, N., Annika, S., Anni-Maija, L., Lauri, V. E., Maria, R., Juzoh, U., Ksenia, E., & Esa, R. K. (2023). Somatostatin-expressing neurons in the ventral tegmental area innervate specific forebrain regions and are involved in the stress response. *bioRxiv*, 2023.2001.2006.522982. https://doi.org/10.1101/2023.01.06.522982

Engblom, D., Bilbao, A., Sanchis-Segura, C., Dahan, L., Perreau-Lenz, S., Balland, B., Parkitna, J. R., Luján, R., Halbout, B., Mameli, M., Parlato, R., Sprengel, R., Lüscher, C., Schütz, G., & Spanagel, R. (2008). Glutamate receptors on dopamine neurons control the persistence of cocaine seeking. *Neuron*, *59*(3), 497-508. https://doi.org/10.1016/j.neuron.2008.07.010

Fedorova, T. D., Seidelin, L. B., Knudsen, K., Schacht, A. C., Geday, J., Pavese, N., Brooks, D. J., & Borghammer, P. (2017). Decreased intestinal acetylcholinesterase in early Parkinson disease: An (11)C-donepezil PET study. *Neurology*, *88*(8), 775-781. https://doi.org/10.1212/wnl.0000000000003633

Francesca, G., Connor, M., Nicholas, S., Jamie, B., Sanjid, S., Seon Woo, K., Alessandro, S., Tyler, C., Ellen, K., Dritan, A., & David, S. (2023). Interaction of an α-synuclein epitope with HLA-DRB1*15:01 triggers enteric features in mice reminiscent of prodromal Parkinson's disease. *bioRxiv*, 2022.2002.2003.479014. https://doi.org/10.1101/2022.02.03.479014

Furness, J. B., Callaghan, B. P., Rivera, L. R., & Cho, H. J. (2014). The enteric nervous system and gastrointestinal innervation: integrated local and central control. *Adv Exp Med Biol*, *817*, 39-71. https://doi.org/10.1007/978-1-4939-0897-4_3

Gaenslen, A., Swid, I., Liepelt-Scarfone, I., Godau, J., & Berg, D. (2011). The patients' perception of prodromal symptoms before the initial diagnosis of Parkinson's disease. *Mov Disord*, *26*(4), 653-658. https://doi.org/10.1002/mds.23499

Gaertner, Z., Azcorra, M., Dombeck, D. A., & Awatramani, R. (2022). Molecular heterogeneity in the substantia nigra: A roadmap for understanding PD motor pathophysiology. *Neurobiol Dis*, *175*, 105925. https://doi.org/10.1016/j.nbd.2022.105925

Gershon, M. D., Rothman, T. P., Joh, T. H., & Teitelman, G. N. (1984). Transient and differential expression of aspects of the catecholaminergic phenotype during development of the fetal bowel of rats and mice. *J Neurosci*, *4*(9), 2269-2280. https://doi.org/10.1523/jneurosci.04-09-02269.1984

Giguère, N., Burke Nanni, S., & Trudeau, L. E. (2018). On Cell Loss and Selective Vulnerability of Neuronal Populations in Parkinson's Disease. *Front Neurol*, *9*, 455. https://doi.org/10.3389/fneur.2018.00455

Gould, T. W., Swope, W. A., Heredia, D. J., Corrigan, R. D., & Smith, T. K. (2019). Activity within specific enteric neurochemical subtypes is correlated with distinct patterns of gastrointestinal motility in the murine colon. *Am J Physiol Gastrointest Liver Physiol*, *317*(2), G210-g221. https://doi.org/10.1152/ajpgi.00252.2018

Greffard, S., Verny, M., Bonnet, A. M., Beinis, J. Y., Gallinari, C., Meaume, S., Piette, F., Hauw, J. J., & Duyckaerts, C. (2006). Motor score of the Unified Parkinson Disease Rating Scale as a good predictor of Lewy body-associated neuronal loss in the substantia nigra. *Arch Neurol*, *63*(4), 584-588. https://doi.org/10.1001/archneur.63.4.584

Hamamah, S., Aghazarian, A., Nazaryan, A., Hajnal, A., & Covasa, M. (2022). Role of Microbiota-Gut-Brain Axis in Regulating Dopaminergic Signaling. *Biomedicines*, *10*(2). https://doi.org/10.3390/biomedicines10020436

Hibi, K., Westra, W. H., Borges, M., Goodman, S., Sidransky, D., & Jen, J. (1999). PGP9.5 as a candidate tumor marker for non-small-cell lung cancer. *Am J Pathol*, *155*(3), 711-715. https://doi.org/10.1016/s0002-9440(10)65169-3

Hilton, D., Stephens, M., Kirk, L., Edwards, P., Potter, R., Zajicek, J., Broughton, E., Hagan, H., & Carroll, C. (2014). Accumulation of α-synuclein in the bowel of patients in the pre-clinical phase of Parkinson's disease. *Acta Neuropathol*, *127*(2), 235-241. https://doi.org/10.1007/s00401-013-1214-6

Kouwenhoven, W. M., Fortin, G., Penttinen, A. M., Florence, C., Delignat-Lavaud, B., Bourque, M. J., Trimbuch, T., Luppi, M. P., Salvail-Lacoste, A., Legault, P., Poulin, J. F., Rosenmund, C., Awatramani, R., & Trudeau, L. (2020). VGluT2 Expression in Dopamine Neurons Contributes to Postlesional Striatal Reinnervation. *J Neurosci*, *40*(43), 8262-8275. https://doi.org/10.1523/jneurosci.0823-20.2020

Kramer, D. J., Risso, D., Kosillo, P., Ngai, J., & Bateup, H. S. (2018). Combinatorial Expression of Grp and Neurod6 Defines Dopamine Neuron Populations with Distinct Projection Patterns and Disease Vulnerability. *eNeuro*, *5*(3). https://doi.org/10.1523/eneuro.0152-18.2018

Kulkarni, S., Ganz, J., Bayrer, J., Becker, L., Bogunovic, M., & Rao, M. (2018). Advances in Enteric Neurobiology: The "Brain" in the Gut in Health and Disease. *J Neurosci*, *38*(44), 9346-9354. https://doi.org/10.1523/jneurosci.1663-18.2018

La Manno, G., Gyllborg, D., Codeluppi, S., Nishimura, K., Salto, C., Zeisel, A., Borm, L. E., Stott, S. R. W., Toledo, E. M., Villaescusa, J. C., Lönnerberg, P., Ryge, J., Barker, R. A., Arenas, E., & Linnarsson, S. (2016). Molecular Diversity of Midbrain Development in Mouse, Human, and Stem Cells. *Cell*, *167*(2), 566-580.e519. https://doi.org/10.1016/j.cell.2016.09.027

Lammel, S., Steinberg, E. E., Földy, C., Wall, N. R., Beier, K., Luo, L., & Malenka, R. C. (2015). Diversity of transgenic mouse models for selective targeting of midbrain dopamine neurons. *Neuron*, *85*(2), 429-438. https://doi.org/10.1016/j.neuron.2014.12.036

Lebouvier, T., Neunlist, M., Bruley des Varannes, S., Coron, E., Drouard, A., N'Guyen, J. M., Chaumette, T., Tasselli, M., Paillusson, S., Flamand, M., Galmiche, J. P., Damier, P., & Derkinderen, P. (2010). Colonic biopsies to assess the neuropathology of Parkinson's disease and its relationship with symptoms. *PLoS One*, *5*(9), e12728. https://doi.org/10.1371/journal.pone.0012728

Li, Y., Zhang, Y., Zhang, X. L., Feng, X. Y., Liu, C. Z., Zhang, X. N., Quan, Z. S., Yan, J. T., & Zhu, J. X. (2019). Dopamine promotes colonic mucus secretion through dopamine D(5) receptor in rats. *Am J Physiol Cell Physiol*, *316*(3), C393-c403. https://doi.org/10.1152/ajpcell.00261.2017

Li, Z., Chalazonitis, A., Huang, Y. Y., Mann, J. J., Margolis, K. G., Yang, Q. M., Kim, D. O., Côté, F., Mallet, J., & Gershon, M. D. (2011). Essential roles of enteric neuronal serotonin in gastrointestinal motility and the development/survival of enteric dopaminergic neurons. *J Neurosci*, *31*(24), 8998-9009. https://doi.org/10.1523/jneurosci.6684-10.2011

Li, Z. S., Pham, T. D., Tamir, H., Chen, J. J., & Gershon, M. D. (2004). Enteric dopaminergic neurons: definition, developmental lineage, and effects of extrinsic denervation. *J Neurosci*, *24*(6), 1330-1339. https://doi.org/10.1523/jneurosci.3982-03.2004

Li, Z. S., Schmauss, C., Cuenca, A., Ratcliffe, E., & Gershon, M. D. (2006). Physiological modulation of intestinal motility by enteric dopaminergic neurons and the D2 receptor: analysis of dopamine receptor expression, location, development, and function in wild-type and knock-out mice. *J Neurosci*, *26*(10), 2798-2807. https://doi.org/10.1523/jneurosci.4720-05.2006

Lindeberg, J., Usoskin, D., Bengtsson, H., Gustafsson, A., Kylberg, A., Söderström, S., & Ebendal, T. (2004). Transgenic expression of Cre recombinase from the tyrosine hydroxylase locus. *Genesis*, *40*(2), 67-73. https://doi.org/10.1002/gene.20065

Liu, L., Geisert, E. E., Frankfurter, A., Spano, A. J., Jiang, C. X., Yue, J., Dragatsis, I., & Goldowitz, D. (2007). A transgenic mouse class-III beta tubulin reporter using yellow fluorescent protein. *Genesis*, *45*(9), 560-569. https://doi.org/10.1002/dvg.20325

Liu, X., Du, Z. R., Wang, X., Luk, K. H., Chan, C. H., Cao, X., Zhao, Q., Zhao, F., Wong, W. T., Wong, K. H., & Dong, X. L. (2021). Colonic Dopaminergic Neurons Changed Reversely With Those in the Midbrain via Gut Microbiota-Mediated Autophagy in a Chronic Parkinson's Disease Mice Model. *Front Aging Neurosci*, *13*, 649627. https://doi.org/10.3389/fnagi.2021.649627

Macosko, E. Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A. R., Kamitaki, N., Martersteck, E. M., Trombetta, J. J., Weitz, D. A., Sanes, J. R., Shalek, A. K., Regev, A., & McCarroll, S. A. (2015). Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell*, *161*(5), 1202-1214. https://doi.org/10.1016/j.cell.2015.05.002

May-Zhang, A. A., Tycksen, E., Southard-Smith, A. N., Deal, K. K., Benthal, J. T., Buehler, D. P., Adam, M., Simmons, A. J., Monaghan, J. R., Matlock, B. K., Flaherty, D. K., Potter, S. S., Lau, K. S., & Southard-Smith, E. M. (2021). Combinatorial Transcriptional Profiling of Mouse and Human Enteric Neurons Identifies Shared and Disparate Subtypes In Situ. *Gastroenterology*, *160*(3), 755-770.e726. https://doi.org/10.1053/j.gastro.2020.09.032

McQuade, R. M., Singleton, L. M., Wu, H., Lee, S., Constable, R., Di Natale, M., Ringuet, M. T., Berger, J. P., Kauhausen, J., Parish, C. L., Finkelstein, D. I., Furness, J. B., & Diwakarla, S. (2021). The association of enteric neuropathy with gut phenotypes in acute and progressive models of Parkinson's disease. *Sci Rep*, *11*(1), 7934. https://doi.org/10.1038/s41598-021-86917-5

Memic, F., Knoflach, V., Morarach, K., Sadler, R., Laranjeira, C., Hjerling-Leffler, J., Sundström, E., Pachnis, V., & Marklund, U. (2018). Transcription and Signaling Regulators in Developing Neuronal Subtypes of Mouse and Human Enteric Nervous System. *Gastroenterology*, *154*(3), 624-636. https://doi.org/10.1053/j.gastro.2017.10.005

Morarach, K., Mikhailova, A., Knoflach, V., Memic, F., Kumar, R., Li, W., Ernfors, P., & Marklund, U. (2021). Diversification of molecularly defined myenteric neuron classes revealed by single-cell RNA sequencing. *Nat Neurosci*, *24*(1), 34-46. https://doi.org/10.1038/s41593-020-00736-x

O'Day, C., Finkelstein, D. I., Diwakarla, S., & McQuade, R. M. (2022). A Critical Analysis of Intestinal Enteric Neuron Loss and Constipation in Parkinson's Disease. *J Parkinsons Dis*, *12*(6), 1841-1861. https://doi.org/10.3233/jpd-223262

Ohlsson, B., & Englund, E. (2019). Atrophic Myenteric and Submucosal Neurons Are Observed in Parkinson's Disease. *Parkinsons Dis*, *2019*, 7935820. https://doi.org/10.1155/2019/7935820

Ou, Z., Pan, J., Tang, S., Duan, D., Yu, D., Nong, H., & Wang, Z. (2021). Global Trends in the Incidence, Prevalence, and Years Lived With Disability of Parkinson's Disease in 204 Countries/Territories From 1990 to 2019. *Front Public Health*, *9*, 776847. https://doi.org/10.3389/fpubh.2021.776847

Pont-Sunyer, C., Hotter, A., Gaig, C., Seppi, K., Compta, Y., Katzenschlager, R., Mas, N., Hofeneder, D., Brücke, T., Bayés, A., Wenzel, K., Infante, J., Zach, H., Pirker, W., Posada, I. J., Álvarez, R., Ispierto, L., De Fàbregues, O., Callén, A., . . . Tolosa, E. (2015). The onset of nonmotor symptoms in Parkinson's disease (the ONSET PD study). *Mov Disord*, *30*(2), 229-237. https://doi.org/10.1002/mds.26077

Poulin, J. F., Caronia, G., Hofer, C., Cui, Q., Helm, B., Ramakrishnan, C., Chan, C. S., Dombeck, D. A., Deisseroth, K., & Awatramani, R. (2018). Mapping projections of molecularly defined dopamine neuron subtypes using intersectional genetic approaches. *Nat Neurosci*, *21*(9), 1260-1271. https://doi.org/10.1038/s41593-018-0203-4

Poulin, J. F., Gaertner, Z., Moreno-Ramos, O. A., & Awatramani, R. (2020). Classification of Midbrain Dopamine Neurons Using Single-Cell Gene Expression Profiling Approaches. *Trends Neurosci*, *43*(3), 155-169. https://doi.org/10.1016/j.tins.2020.01.004

Poulin, J. F., Zou, J., Drouin-Ouellet, J., Kim, K. Y., Cicchetti, F., & Awatramani, R. B. (2014). Defining midbrain dopaminergic neuron diversity by single-cell gene expression profiling. *Cell Rep*, *9*(3), 930-943. https://doi.org/10.1016/j.celrep.2014.10.008

Qu, Z. D., Thacker, M., Castelucci, P., Bagyánszki, M., Epstein, M. L., & Furness, J. B. (2008). Immunohistochemical analysis of neuron types in the mouse small intestine. *Cell Tissue Res*, *334*(2), 147-161. https://doi.org/10.1007/s00441-008-0684-7

Raeder, V., Batzu, L., Untucht, R., Fehre, A., Rizos, A., Leta, V., Schmelz, R., Hampe, J., Bostantjopoulou, S., Katsarou, Z., Storch, A., Reichmann, H., Falkenburger, B., Ray Chaudhuri, K., & Klingelhoefer, L. (2023). The Gut Dysmotility Questionnaire for Parkinson's disease: Insights into development and pretest studies. *Front Neurol*, *14*, 1149604. https://doi.org/10.3389/fneur.2023.1149604

Rahmani, W., Sinha, S., & Biernaskie, J. (2020). Immune modulation of hair follicle regeneration. *NPJ Regen Med*, *5*, 9. https://doi.org/10.1038/s41536-020-0095-2

Reyes, S., Fu, Y., Double, K., Thompson, L., Kirik, D., Paxinos, G., & Halliday, G. M. (2012). GIRK2 expression in dopamine neurons of the substantia nigra and ventral tegmental area. *J Comp Neurol*, *520*(12), 2591-2607. https://doi.org/10.1002/cne.23051

Salesse, C., Charest, J., Doucet-Beaupré, H., Castonguay, A. M., Labrecque, S., De Koninck, P., & Lévesque, M. (2020). Opposite Control of Excitatory and Inhibitory Synapse Formation by Slitrk2 and Slitrk5 on Dopamine Neurons Modulates Hyperactivity Behavior. *Cell Rep*, *30*(7), 2374-2386.e2375. https://doi.org/10.1016/j.celrep.2020.01.084

Savica, R., Carlin, J. M., Grossardt, B. R., Bower, J. H., Ahlskog, J. E., Maraganore, D. M., Bharucha, A. E., & Rocca, W. A. (2009). Medical records documentation of constipation preceding Parkinson disease: A case-control study. *Neurology*, *73*(21), 1752-1758. https://doi.org/10.1212/WNL.0b013e3181c34af5

Schrag, A., Horsfall, L., Walters, K., Noyce, A., & Petersen, I. (2015). Prediagnostic presentations of Parkinson's disease in primary care: a case-control study. *Lancet Neurol*, *14*(1), 57-64. https://doi.org/10.1016/s1474-4422(14)70287-x

Skjærbæk, C., Knudsen, K., Horsager, J., & Borghammer, P. (2021). Gastrointestinal Dysfunction in Parkinson's Disease. *J Clin Med*, *10*(3). https://doi.org/10.3390/jcm10030493

Smith, T. H., Ngwainmbi, J., Grider, J. R., Dewey, W. L., & Akbarali, H. I. (2013). An in-vitro preparation of isolated enteric neurons and glia from the myenteric plexus of the adult mouse. *J Vis Exp*(78). https://doi.org/10.3791/50688

Tiklová, K., Björklund Å, K., Lahti, L., Fiorenzano, A., Nolbrant, S., Gillberg, L., Volakakis, N., Yokota, C., Hilscher, M. M., Hauling, T., Holmström, F., Joodmardi, E., Nilsson, M., Parmar, M., & Perlmann, T. (2019). Single-cell RNA sequencing reveals midbrain dopamine neuron diversity emerging during mouse brain development. *Nat Commun*, *10*(1), 581. https://doi.org/10.1038/s41467-019-08453-1

Trudeau, L. E. (2004). Glutamate co-transmission as an emerging concept in monoamine neuron function. *J Psychiatry Neurosci*, *29*(4), 296-310.

Trudeau, L. E., & El Mestikawy, S. (2018). Glutamate Cotransmission in Cholinergic, GABAergic and Monoamine Systems: Contrasts and Commonalities. *Front Neural Circuits*, *12*, 113. https://doi.org/10.3389/fncir.2018.00113

Trudeau, L. E., & Gutiérrez, R. (2007). On cotransmission & neurotransmitter phenotype plasticity. *Mol Interv*, *7*(3), 138-146. https://doi.org/10.1124/mi.7.3.5

Viereckel, T., Dumas, S., Smith-Anttila, C. J., Vlcek, B., Bimpisidis, Z., Lagerström, M. C., Konradsson-Geuken, Å., & Wallén-Mackenzie, Å. (2016). Midbrain Gene Screening Identifies a New Mesoaccumbal Glutamatergic Pathway and a Marker for Dopamine Cells Neuroprotected in Parkinson's Disease. *Sci Rep*, *6*, 35203. https://doi.org/10.1038/srep35203

Walker, J. K., Gainetdinov, R. R., Mangel, A. W., Caron, M. G., & Shetzline, M. A. (2000). Mice lacking the dopamine transporter display altered regulation of distal colonic motility. *Am J Physiol Gastrointest Liver Physiol*, *279*(2), G311-318. https://doi.org/10.1152/ajpgi.2000.279.2.G311

Wang, L., Magen, I., Yuan, P. Q., Subramaniam, S. R., Richter, F., Chesselet, M. F., & Taché, Y. (2012). Mice overexpressing wild-type human alpha-synuclein display alterations in colonic myenteric ganglia and defecation. *Neurogastroenterol Motil*, *24*(9), e425-436. https://doi.org/10.1111/j.1365-2982.2012.01974.x

Wright, C. M., Schneider, S., Smith-Edwards, K. M., Mafra, F., Leembruggen, A. J. L., Gonzalez, M. V., Kothakapa, D. R., Anderson, J. B., Maguire, B. A., Gao, T., Missall, T. A., Howard, M. J., Bornstein, J. C., Davis, B. M., & Heuckeroth, R. O. (2021). scRNA-Seq Reveals New Enteric Nervous System Roles for GDNF, NRTN, and TBX3. *Cell Mol Gastroenterol Hepatol*, *11*(5), 1548-1592.e1541. https://doi.org/10.1016/j.jcmgh.2020.12.014

Zheng, G. X., Terry, J. M., Belgrader, P., Ryvkin, P., Bent, Z. W., Wilson, R., Ziraldo, S. B., Wheeler, T. D., McDermott, G. P., Zhu, J., Gregory, M. T., Shuga, J., Montesclaros, L., Underwood, J. G., Masquelier, D. A., Nishimura, S. Y., Schnall-Levin, M., Wyatt, P. W., Hindson, C. M., . . . Bielas, J. H. (2017). Massively parallel digital transcriptional profiling of single cells. *Nat Commun*, *8*, 14049. https://doi.org/10.1038/ncomms14049

Zhuang, X., Masson, J., Gingrich, J. A., Rayport, S., & Hen, R. (2005). Targeted gene expression in dopamine and serotonin neurons of the mouse brain. *J Neurosci Methods*, *143*(1), 27-32. https://doi.org/10.1016/j.jneumeth.2004.09.020

Figure 1: Detection of enteric neurons in DAT-tdTomato transgenic mice

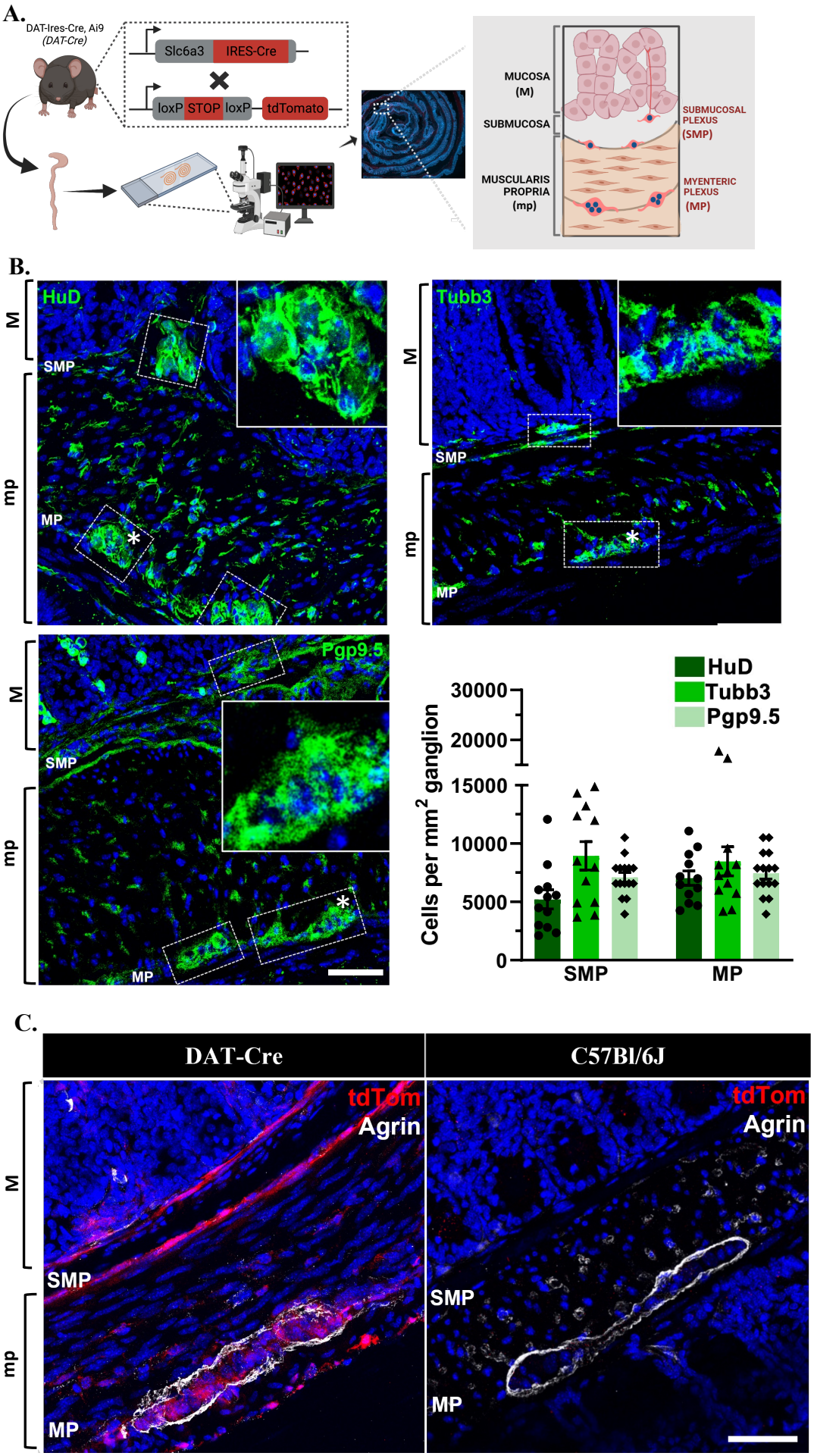
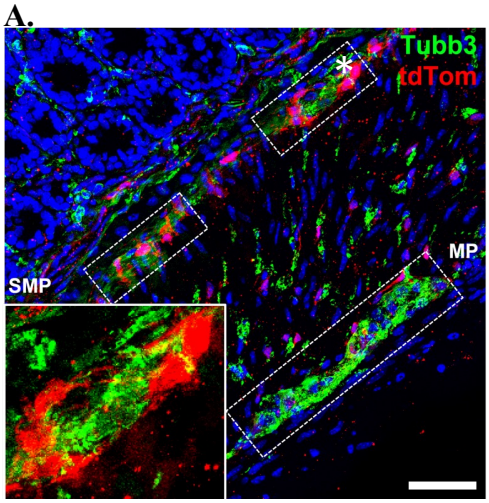
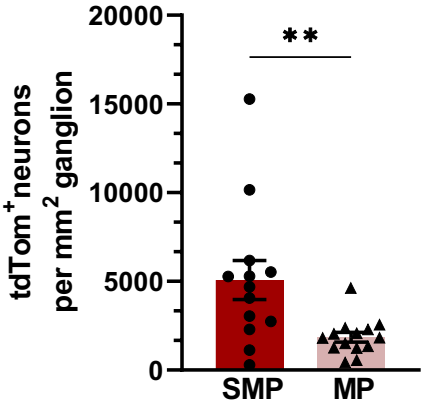


Figure 1: Detection of enteric neurons in DAT-tdTomato transgenic mice. A) Schematic diagram of immunofluorescence staining protocol for mouse colon isolated from transgenic mice, where tdTomato (Ai9) is controlled under the promoter for dopamine transporter (Slc6a3, referred to as DAT-Cre). The coronal plane of the colon mounted in a *"swiss-roll"* depicts the three subdivision of intestinal layers and the two ganglionated plexuses. The submucosal plexus (SMP) is shown between the mucosa (M) and the muscularis propria (mp), while the myenteric plexus (MP) is within the mp. **B)** Representative immunofluorescence staining with cross-sectional view of colonic tissue from DAT-Cre mice. Enteric neurons within the 2 ganglionated plexuses are detectable with several pan-neuronal markers that are commonly used to identify neurons. Neurons (green) are immunoreactive for RNA-binding human antigen (HuD), tubulin $\beta 3$ (Tubb3) and ubiquitin carboxyl-terminal hydrolase-1 (Uch-l1/Pgp9.5). Ganglia are outlined by white dotted lines and inset is a magnified view of the ganglia designated with *. The density of neurons normalized to ganglionic area (in mm^2) is quantified in both plexuses. Mean $\pm$ SEM, n=12-15. **C)** Cross-sectional view of colonic tissue from DAT-Cre mice immunoreactive for tdTomato (red). Agrin is used as a marker for blood-myenteric-barrier (white) to depict ganglia outline. Note the lack of tdTomato signal in wild type C57Bl/6J colonic tissue, as expected. Nuclei are stained with Hoechst (blue). Scale bars: 50 μm.

Figure 2: TdTomato-immunoreactivity varies across plexuses and colocalizes with tubulin $\beta 3$ and tyrosine hydroxylase-positive cells

A.





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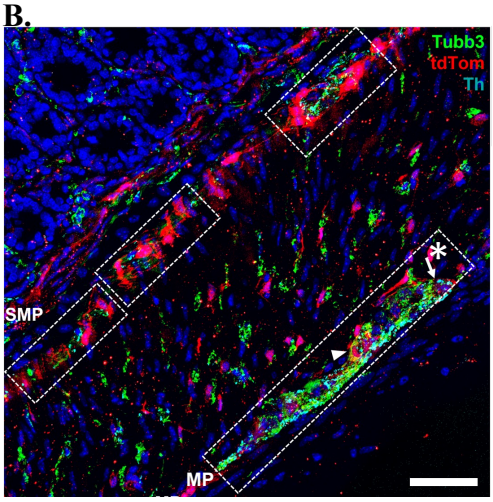
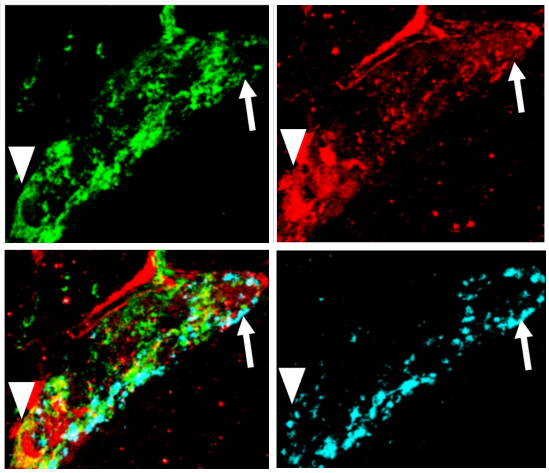




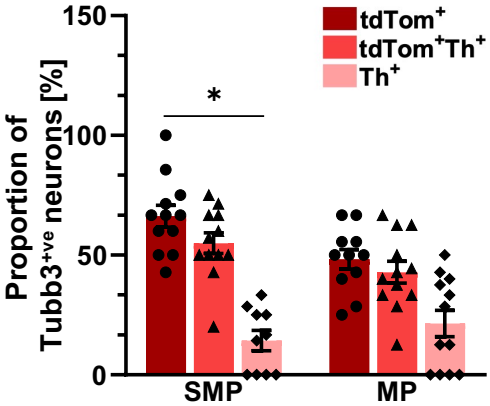


Figure 2: TdTomato-immunoreactivity varies across plexuses and colocalizes with tubulin $\beta 3$ (Tubb3) and tyrosine hydroxylase (Th)-positive cells. A) Representative coronal slice of colonic tissue illustrating that DAT-tdTomato-expressing cells (red) are immunoreactive for a pan-neuronal marker, Tubb3 (green) in the submucosal and myenteric plexuses (SMP, MP). The density of Tubb3+ tdTom$^+$ neurons normalized to ganglionic area (in mm^2) is quantified in both plexuses, which revealed significantly less DAT-tdTomato+ neurons in the MP compared to the SMP. Unpaired, two-tailed t-test, $p<0.05$. Mean $\pm$ SEM, n=13-14. **B)** Representative immunofluorescence staining for tyrosine hydroxylase (Th) (cyan), which is a rate limiting enzyme for generating L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor for dopamine. Th$^+$ cells colocalized with tdTomato (red) and Tubb3 (green) in both ganglionated plexuses. The percent abundance of Tubb3$^+$ enteric neurons expressing either DAT-tdTomato (tdTom$^+$); Th$^+$ alone (Th$^+$); or co-expressing both DAT-tdTomato and Th (tdTom$^+$Th$^+$) is presented (fold change relative to total neurons normalized to ganglionic area). This revealed that most enteric neurons (~45-60%) expressed both Th and TdTom. One-way ANOVA followed by Tukey's multiple comparison's test, $p<0.05$. Mean $\pm$ SEM, n=10-12. Ganglia are outlined by the white dotted lines and inset is a magnified view of the ganglia designated with *. Nuclei (blue). Scale bars: 50 μm.

Figure 3: A large proportion of tdTomato-immunoreactive neuronal populations in the ENS exhibit dual-transmitter content

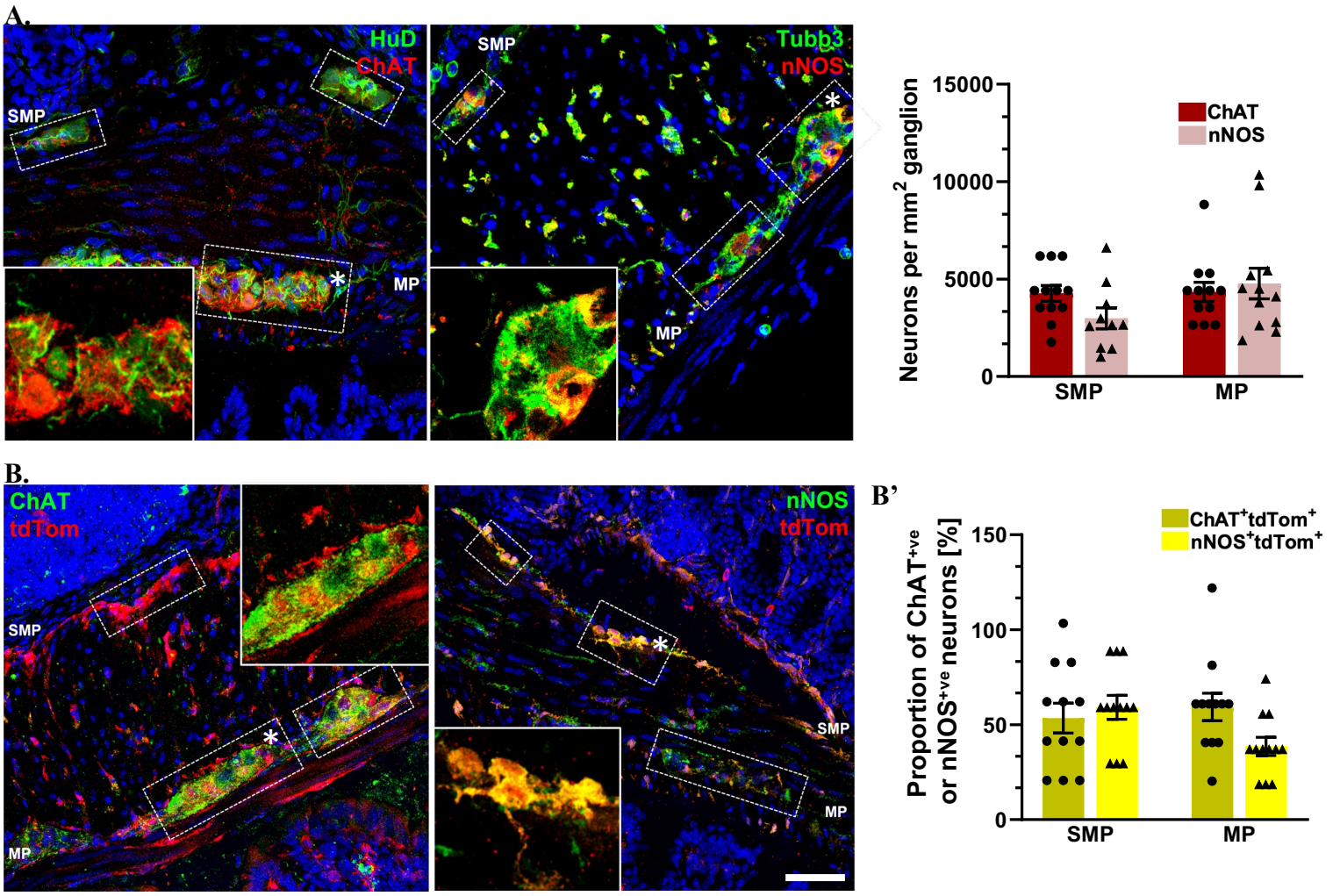


Figure 3: A large proportion of tdTomato-immunoreactive neuronal populations in the ENS exhibit dual-transmitter content. **A)** Representative cross-sectional view of colonic tissue labelling cholinergic (choline acetyl transferase, ChAT-positive) or nitrergic (neuronal nitric oxide synthase, nNOS-positive) (red) neurons (green, HuD or Tubb3) in submucosal and myenteric plexuses (SMP and MP). The density of $ChAT^+$ or $nNOS^+$ neuronal populations (colocalized with pan-neuronal markers HuD or Tubb3, respectively) are normalized to ganglionic area (in mm^2). Mean ± SEM, n=10-12. **B)** Representative immunofluorescence staining for DAT-tdTomato-expressing cells (red) co-localizing with either ChAT or nNOS (green) in both ganglionated plexuses (SMP and MP). **B')** Percent abundance of neurons immunoreactive for DAT-tdTomato co-expressing either ChAT ($ChAT^+tdTom^+$) or nNOS ($nNOS^+tdTom^+$) (fold change relative to total cholinergic or nitrergic neurons normalized to ganglionic area). **B")** Percent abundance of neurons immunoreactive for DAT-tdTomato that are not co-expressing ChAT ($ChAT^{neg}tdTom^+$) or nNOS ($nNOS^{neg}tdTom^+$) (fold change relative to total $tdTom^+$ neurons normalized to ganglionic area). Mean ± SEM, n=6-12. Nuclei (blue). Ganglia are outlined by white dotted lines, and inset is a magnified view of the ganglia designated with *. Scale bars: 50 μm.

Figure 4: Single-cell RNA sequencing of DAT-tdTomato-expressing cells confirms heterogeneity of DAT-tdTomato+ neuronal subtypes

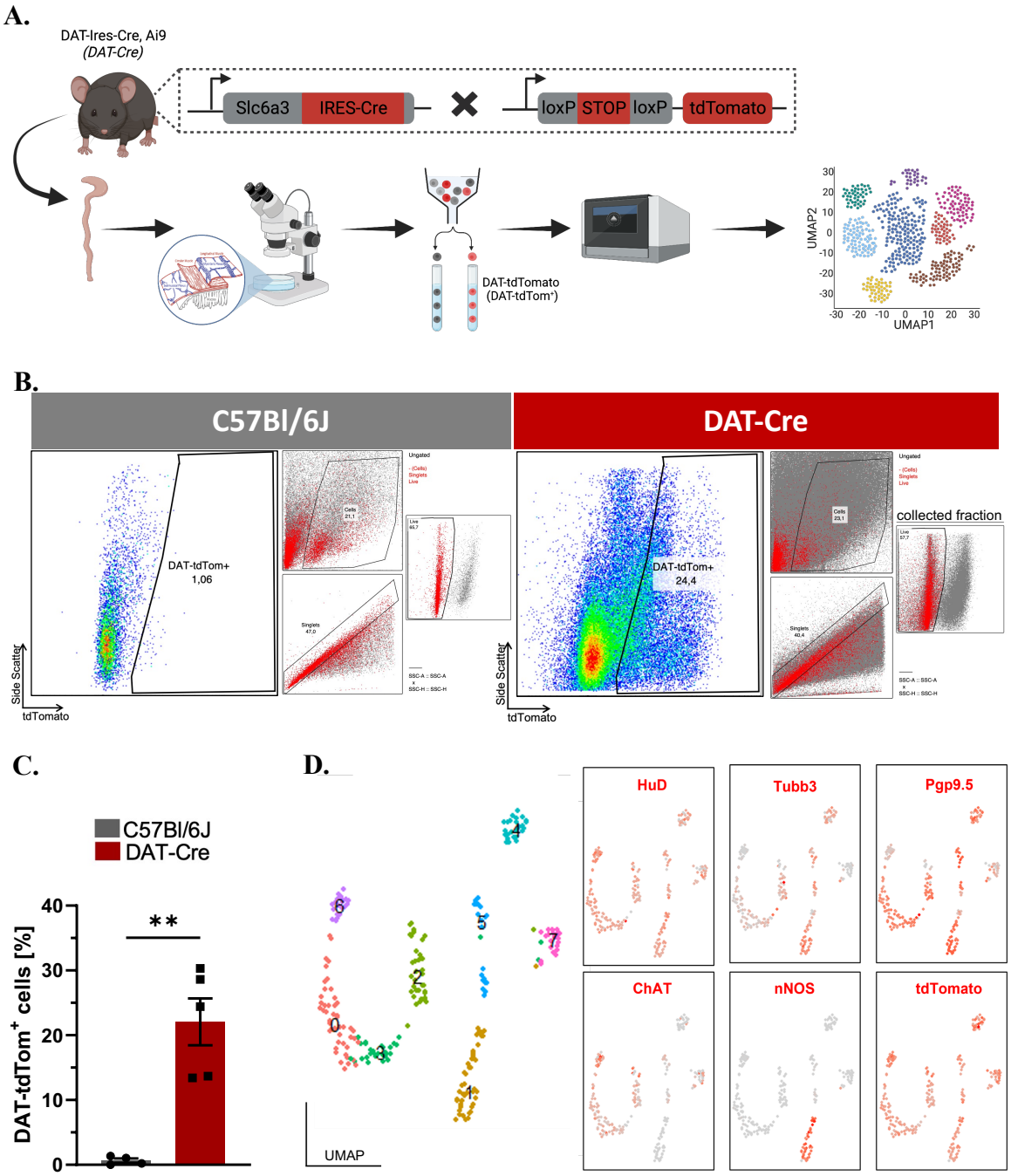
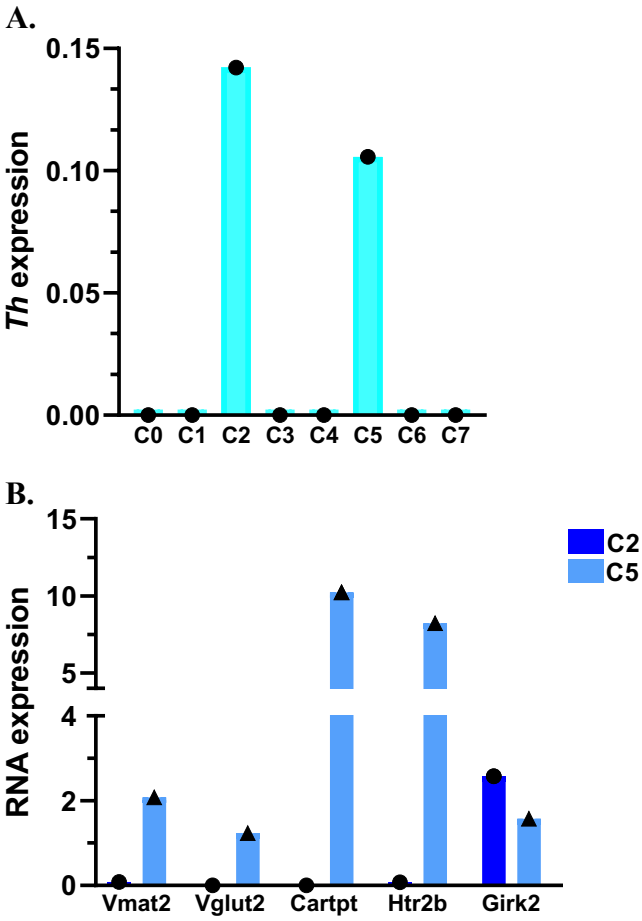


Figure 4: Single-cell RNA sequencing of DAT-tdTomato-expressing cells confirms heterogeneity of DAT-tdTomato+ neuronal subtypes. A) Schematic diagram of the colonic longitudinal muscle and myenteric plexus (LMMP) isolation and dissociation for single-cell RNA sequencing (scRNAseq). Live tdTomato-positive cells were enriched via fluorescence-activated cell sorting (FACS) then gene expression libraries were prepared using 10X Chromium Genomics. Quality control, normalization, clustering and expression analyses of data was done in R using *Seurat* with default settings. **B)** Representative flow cytometric gating of tdTomato-positive cells isolated from dissociated colons of DAT-Cre mice. Gates were set using wild type C57Bl/6J dissociated colons, and only live cells (~60% of total singlets) were used. **C)** The percent abundance of enteric DAT-tdTomato-expressing cells was determined relative to total live singlets demonstrating an enrichment in DAT-tdTom$^+$ cells from DAT-Cre mice compared to C57Bl/6J mice. Unpaired, two-tailed t-test is applied, $p<0.05$. Mean $\pm$ SEM, n=5. **D)** UMAP plot depicting seven neuronal cell subpopulations identified by confirming the expression of 3 pan-neuronal marker genes in these populations (*HuD, Tubb3, Pgp9.5*). These cells expressed *tdTomato,* and four clusters (0, 2, 3 and 6) also expressed *ChAT,* while one cluster (1) also expressed *nNOS* at high levels.

Figure 5: Two DAT-tdTomato-expressing neuronal subclusters expressed tyrosine hydroxylase along with traditional dopaminergic neuronal markers



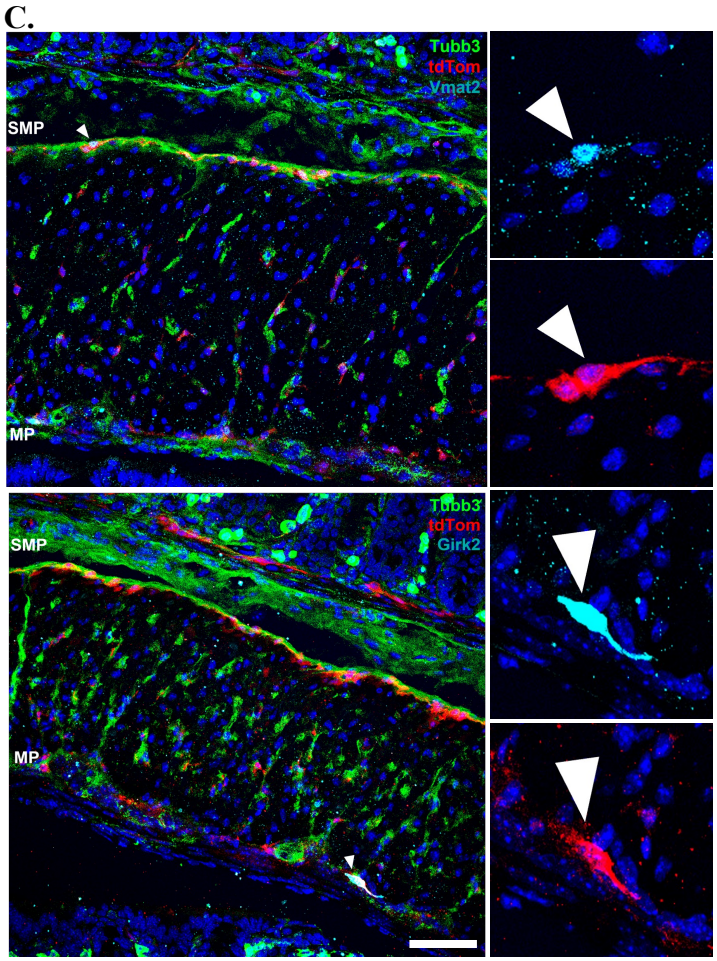


Figure 5: Two DAT-tdTomato-expressing neuronal subclusters expressed traditional dopaminergic neuronal markers. A, B) Bar graphs demonstrating the expression of known DAergic neuronal markers, *Th,* in DAT-tdTomato^{+ve} neurons, as well as other previously identified genes in DA neurons. These genes are predominantly expressed in both Clusters 2 and 5. **B)** Representative images of colonic tissue demonstrating co-labeling of tdTomato (red) with these known dopaminergic neuronal markers (cyan; Vmat2 and Girk2), indicated by arrowheads. Tubb3 is used as pan-neuronal marker (green) and nuclei are stained with Hoechst (blue). Scale bars: 50 μm.

Figure 6: Identification of novel enteric dopaminergic neuron subtype with cholinergic phenotype

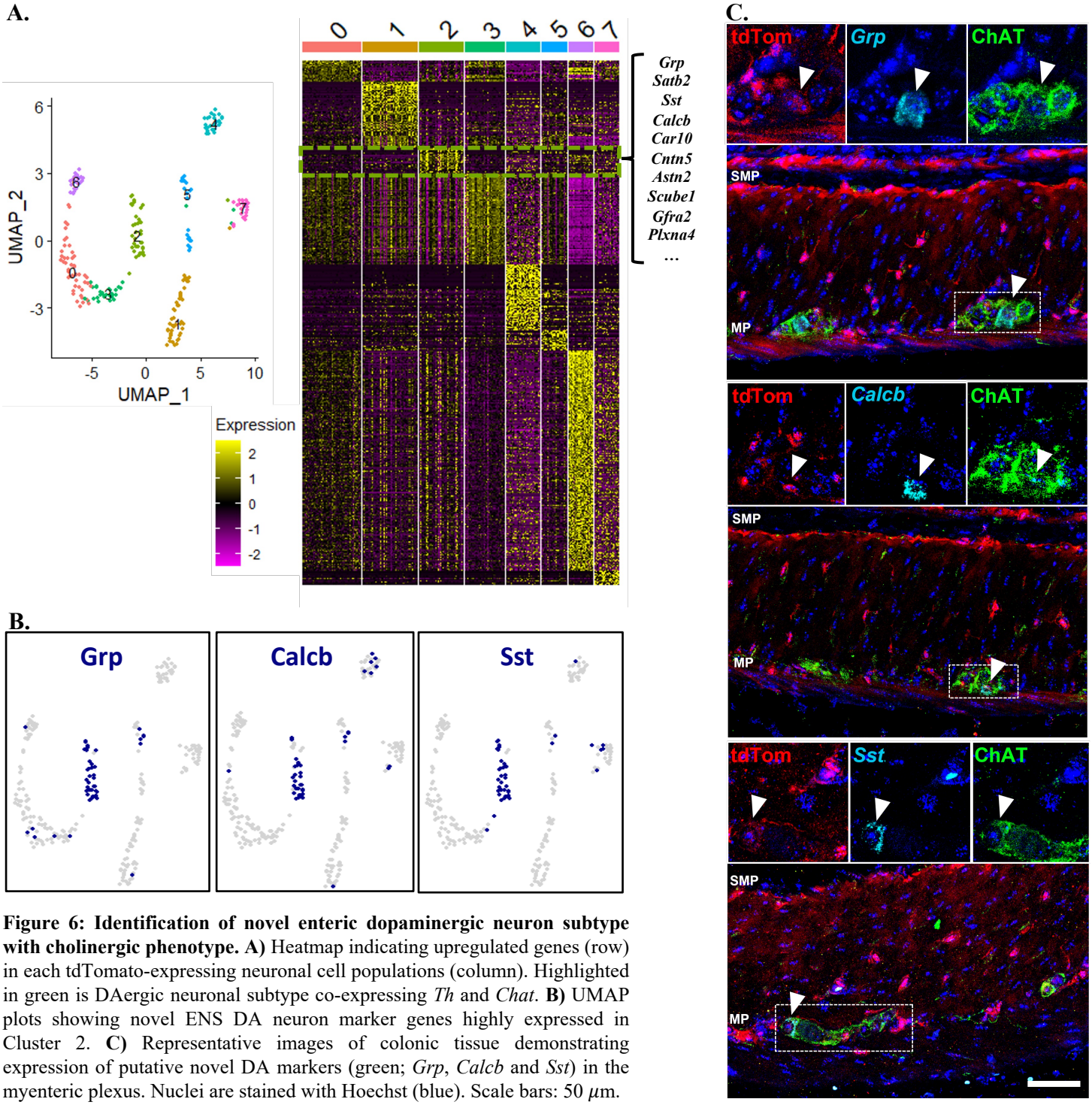


Figure 6: Identification of novel enteric dopaminergic neuron subtype with cholinergic phenotype. A) Heatmap indicating upregulated genes (row) in each tdTomato-expressing neuronal cell populations (column). Highlighted in green is DAergic neuronal subtype co-expressing *Th* and *Chat*. **B)** UMAP plots showing novel ENS DA neuron marker genes highly expressed in Cluster 2. **C)** Representative images of colonic tissue demonstrating expression of putative novel DA markers (green; *Grp*, *Calcb* and *Sst*) in the myenteric plexus. Nuclei are stained with Hoechst (blue). Scale bars: 50 μm.