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

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Abstract

Dopaminergic neurons (DA) are the predominant cell type in the midbrain that synthesize dopamine, a neurotransmitter implicated in a number of behavioural processes, including motor function, the reward pathway, and satiety. In diseases affecting these neurons, such as in Parkinson's disease (PD), there is growing evidence that the gut-brain axis and selective vulnerability of DA neurons plays a crucial role in disease. Most investigations relating to DA neurons in the gut rely on immunoreactivity to tyrosine hydroxylase (TH) - a rate-limiting enzyme in the production of dopamine. However, the reliability of TH staining as a marker of DA neurons has been questioned in recent years. Our aim is to perform a comprehensive characterization of DA neurons in the gut using a well-accepted reporter mouse line, expressing a fluorescent protein under the dopamine transporter promoter (DAT). Our findings confirm a unique localisation of DA neurons in the gut, and also unveil that there are discrete subtypes of DA neurons in the gut, which we characterised using both immunofluorescence and single cell transcriptomics. We observed distinct subtypes of DAT neurons expressing co-transmitters and modulators; 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 (Vmat2, Girk2, Foxa2). Given the clear heterogeneity of DA gut neurons, further investigation is warranted to define their functional signatures and discover their inherent biological differences that put these cells at risk for neurodegeneration.

Introduction

Parkinson's disease (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 the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) of the midbrain. The selective loss of this neuronal class in the SNc is thought to underlie the movement deficits that patients experience as disease progresses (Greffard et al., 2006). Nevertheless, it is evident that non-motor abnormalities precede motor deficits by up to 20 years (Gaenslen et al., 2011; Pont-Sunyer et al., 2015; Schrag et al., 2015), but whether this is driven in-part by the same selective loss of DA neurons, in other regions of the nervous system, remains largely unexplored. Most PD patients present with prodromal symptoms, such as gastrointestinal dysmotility or constipation, involving the peripheral nervous system (PNS) (Abbott et al., 2001; Raeder et al., 2023; Savica et al., 2009). 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 confirmed to be 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 revealed neuronal death in the ganglionated plexus of patients' colon 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 in reports 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.

The ENS is an extensive neural network embedded within the gastrointestinal wall and separated into submucosal and myenteric plexuses (SMP and MP, respectively). Located underneath the mucosal layer, the submucosal neurons are closest to the intestinal lumen and epithelium, while the myenteric ganglia are situated between the circular and longitudinal smooth muscle cells of the muscularis propria (Furness et al., 2014). The anatomical distribution of these neuronal populations concurs with their functions. Luminal sensing and modulation of secretory events are cardinal properties of the SMP and in parallel, the MP governs peristaltic waves provoking gastrointestinal motility. The ENS is regulated by bi-directional communication with the CNS, and extrinsic innervation from parasympathetic and sympathetic fibers, which are paramount for maintaining intestinal functions. The ENS also has an independent neural network allowing crosstalk between ganglionated plexuses and other non-neuronal cell types (Furness, 2008; Furness et al., 2014). Recent single cell transcriptomic studies in both human and mice have elucidated 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), which included the identification of catecholaminergic cell types, possibly including DA neurons. Confirmatory experiments validating the diversity of these neuronal populations has not been carried out.

Notably, such studies are mostly conducted on developing ENS of the fetal mice (Memic et al., 2018; Wright et al., 2021) thereby the expression patterns critical for the classification of adult DA neurons is lacking.

Historically, a neuronal subtype is defined by the neurotransmitter it releases and their functional properties. In recent years, assigning a molecular gene signature has also become a popular approach for subtyping. In the case of DA neurons whereby many canonical markers are shared across cell types, this has its challenges. For example, immune cells participate in dopamine signaling and express several dopamine-related genes (Gopinath et al., 2022; Mackie et al., 2018). Consequently, to describe a cell as a dopaminergic neuron, the cell should express neuronal signatures, genes specifying their developmental origin, as well as a battery of genes involved in dopamine synthesis and release (Poulin et al., 2020). Albeit there are several reports of DA neurons in the gut (Li et al., 2004; Walker et al., 2000), these reports often rely solely on immunostaining for tyrosine hydroxylase (TH) – the rate limiting enzyme in the synthesis of catecholamines. The heavy reliance of TH labeling for enteric DA neurons is leading to an over-generalization of DA neurons in the gut, and distinct DA subtypes that likely exist in the gut have gone unnoticed. Indeed, neurons lacking TH, but immunoreactive for the dopamine transporter (DAT) were described in the murine ileum (Li et al., 2004), while TH-positive neurons without DAT have been shown in the midbrain (Lammel et al., 2015; Poulin et al., 2018; Tiklová et al., 2019). An important approach to investigate the dopamine system in the CNS is the use of transgenic mouse lines in which promoters for *DAT* drive Cre recombinase to induce the expression of fluorescent molecules, such as tdTomato (namely *DAT-Cre*). *DAT-Cre* transgenic lines are considered more reliable in reproducing the endogenous expression of *Dat* in the midbrain compared to *TH-Cre* mice (Bäckman et al., 2006; Ekstrand et al., 2007; Engblom et al., 2008; Lindeberg et al., 2004; Zhuang et al., 2005), arguing that DAT is a more robust descriptor of *bona fide* midbrain DA neurons.

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 as well as single-cell RNA sequencing of DAT-tdTomato-expressing cells to unravel the heterogeneity of DA neuronal populations. Providing an in-depth characterization of this class of enteric neurons will be conducive to future endeavours aimed at attaining insights into the intrinsic mechanisms that may leave specific dopaminergic cell types at risk for neurodegeneration.

Methods

Mice and tissue processing

DAT-Ires-Cre (also referred to as *DAT-Cre*) mice were bred and aged for two-three months to obtain adult mice. Mice (both sexes) were housed in a vivarium under pathogen-free conditions, given water and food ad-libitum. Studies were approved by the University of Montreal Animal Care and Use Committee (UACC). UACC guidelines on the ethical use and care of animals were followed. The mice were euthanized by isoflurane followed by CO_2 asphyxiation and subsequently perfused with 4% paraformaldehyde (PFA). Age-matched wild type C57Bl6/J mice were also used as control for tdTomato expression. We collected the colons where fewer investigations of the ENS have been made and in which prodromal phenotypes in PD takes part in. After isolation, fecal material was flushed with an 18-gauge needle and adipose tissue was removed. Finally, the colon was cut longitudinally and fixed in *swiss-roll* in 4% PFA for 24 hours then dehydrated with 30% sucrose solution. The tissue was then embedded and cryosectioned serially 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 2 hours. Primary antibody stains were performed overnight at room temperature followed by secondary antibody staining (1:200) for 2 hours. Primary antibodies used are indicated in Table 1. Hoechst 33258 was used for nuclear staining (1:5000, Invitrogen H3569).

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

Antigen	Antibody	Dilution	Company (CAT#)
Tubb3	Mouse	1:500	Millipore (MAB5564)
Pgp9.5	Mouse	1:500	ThermoFisher (480012)
HuD	Rabbit	1:200	Abcam (ab96474)
ChAT	Rabbit	1:200	Sigma Aldrich (AB144P)
nNOS	Rabbit	1:500	ThermoFisher (61-7000)
tdTomato	Goat	1:200	ThermoFisher (200-101-379)
Th	Chicken	1:100	Millipore (AB9702)
Dat	Rabbit	1:100	Millipore (AB1591P)
Girk2	Rabbit	1:100	Alomone Labs (APC-006)
Foxa2	Rabbit	1:100	Abcam (AF2400)
Vmat2	Rabbit	1:200	Abcam (ab70808)

Single cell suspension preparation

Given that established protocols are more conveniently adopted for the longitudinal muscle and myenteric plexus (LMMP) isolation and that this part of the ENS comprises neurons key in modulating gut motility, which is oftentimes disrupted in PD, we prepared single cell suspensions from the MP. Isolated colons from adult *DAT-Cre* mice were washed, cut longitudinally as

described above, and pinned with intestinal lumen facing down on a 4% agarose pad. The longitudinal muscle and myenteric plexus (LMMP) were then micro dissected by peeling it off using fine tip tweezers which was then collected and cut into 1-2 cm pieces. The tissues were then enzymatically dissociated to release myenteric neurons 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 40 µm cell strainer and stained with eFluor506 fixable viability dye (ThermoFisher, 65-0866-14). Live single DAT-tdTomato^{+ve} cells were collected by fluorescence-activated cell sorting (FACS Aria). Wild type C57Bl6/J was used as control for gating.

Single cell library preparation and analysis

Live DAT-tdTomato^{+ve} cells (20, 000 cells total) were FACS collected (from n=4 mice, pooled male and female) and then 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 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 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 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

The 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 nuclear morphology and from earlier publications (Kulkarni et al., 2018; Li et al., 2004). All quantitative analysis at the ganglia was normalized to an area of 900um^2 (calculated as the average size of submucosal ganglion). Animal replicates can be found in the figure legends. All statistical analysis was performed using GraphPad Prism 5. For comparisons of groups across multiple groups, a One-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison was used. For comparisons of groups across 2 groups, an unpaired Student's t test was used. p values of < 0.05 was considered significant.

For quantification of single cell RNA-seq data, firstly unsupervised clustering of cells was performed. A selection of highly variable genes (2000 genes) was obtained and used for principle component analysis. Significant principle components were determined using JackStraw analysis. PCs 1 to 10 were used for graph-based clustering (at res = 1) to identify distinct groups of cells. These groups were projected onto UMAP analysis using previously computed principle components 1 to 20. Expression of selected marker genes (*Elavl4*, *Tubb3* and *Uchl1*) was used to classify neurons which was further divided into 7 cell clusters identified by graph-based clustering.

Results

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

DAT-Cre transgenic lines are considered more reliable at reproducing the endogenous expression of *Dat* compared to other DA neuron reporter mice used in midbrain studies, such as *TH-Cre* mice (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-tdTomato transgenic mice (DAT-Cre), to confirm the existence of submucosal and myenteric DA neurons and characterise their heterogeneity. Via standard immunohistological approaches, we first set-out to confirm that our methodological approach to detect enteric neurons in DAT-Cre mice was effective. Indeed, using cross sectional analysis in *swiss-role* format, we were able to detect three subdivisions of the intestinal layers and two ganglionated plexuses, as expected (Figure 1A, B). We could consistently detect 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 (RNA-binding human antigen, HuD; tubulin β3, Tubb3; and ubiquitin carboxyl-terminal hydrolase-1, Uch-l1/Pgp9.5). We were interested in further exploring the efficacy of these commonly used pan-neuronal markers given, in most cases, they are utilized interchangeably, with little evidence to support which one/s might be 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). Albeit high variation made this comparison challenging to interpret, qualitative assessments suggested that HuD had less complete labeling of the neuronal population in the SMP (~5000 cells/mm^2 compared to ~7000+ cells/mm^2 using Tubb3 or Pgp9.5). 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 confirmed that tdTomato expression was detectable in colonic tissue in both the SMP and MP of DAT-Cre mice, and that the signal was not detected in wild type C57Bl/6J colonic tissue, as expected (Figure 1C).

TdTomato-immunoreactive DA neurons vary across plexuses

A previous description of DA neurons in the gut demonstrated 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). Given our focus is on the colonic region, we aimed to confirm the observation of reduced DA neurons in the SMP compared to the MP using DAT-Cre mice. Indeed, we detected reduced density of DAT-tdTom^{+ve} neurons in the myenteric plexus compared to those in the submucosal plexus using co-labeling with Tubb3 and tdTomato (Fig. 2A). We then determined whether these DAT-Cre-expressing neurons also expressed other DA neuron markers to give us more confidence that these

cells are bone fide DA neurons. Tyrosine hydroxylase (Th), crucial for DA synthesis, indeed coincided with the majority of the tdTomato signal, and this was in equal proportions across both plexuses (Fig. 2B). This indicated that although there are less DA neurons in the MP, the DA neurons that do exist in the MP appear to have a similar profile as the SMP. Interestingly, some DAT-tdTom^{+ve} neurons were deficient in Th, while other Th^{+ve} neurons were deficient in DAT-tdTom^{+ve} (Fig. 2B), similar to what has been described in the 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 expresses choline acetyltransferase (ChAT), generally classified as cholinergic; whereas, the latter is commonly referred to as nitrergic due to their release of an inhibitory molecule synthesized by neuronal-specific nitric oxide synthase (nNOS) (Dharshika & Gulbransen, 2023). The striking abundance of DAT-tdTom^{+ve} neurons in the gut, especially in the SMP (Fig. 2A) led us to speculate that subpopulations of enteric DAT-tdTom^{+ve} neurons may co-release other neurotransmitters in addition to dopamine, as increasing evidence accumulates supporting the notion that a substantial proportion of neurons in the CNS possess a combination of multiple transmitters (Brunet Avalos & Sprecher, 2021). Firstly, we detected the presence of cholinergic and nitrergic neurons in both plexuses at similar levels; however there was a trend for reduced density of inhibitory neurons (nNOS^{+}) in the SMP compared to MP (Fig. 3A). Next, we stained ChAT or nNOS immunoreactive neurons with DAT-tdTomato to determine the existence of co-transmitter DA neuronal subtypes (Fig 3B). Interestingly, ChAT^{+ve} or nNOS^{+ve} neurons expressed tdTomato in the majority of cells (~50%), although in the myenteric plexus, nNOS^{+ve} DAT-tdTom^{+ve} neuronal populations appeared at lower frequencies (~30% of nNOS^{+ve} neurons) (Fig 3B'). There is also a fraction of DAT-tdTom^{+ve} neurons that do not express ChAT or nNOS (Fig. 3B"), which likely represents a more traditional DA neuron subtype.

Single cell transcriptomics reveals heterogenous dopaminergic neuronal populations

Considering that a subset of DAT-tdTom^{+ve} neurons is not immunoreactive for either ChAT or nNOS, we sought to unravel the gene expression patterns attributed to these subtypes where dopamine may be the dominant neurotransmitter. Dissociated DAT-tdTom^{+ve} cells from the colon were FACS collected, then the 10X Genomics workflow was applied (Fig. 4A). Our flow cytometer detected ~20% of live enteric cells were DAT-tdTom^{+ve} (wild type C57Bl/6J mice were used as a control for gating, Fig. 4B, C). From this DAT-tdTom^{+ve} population, we were able to capture 247 DAT-tdTom^{+ve} neurons based on the expression of *Elavl4* (HuD), *Tubb3* and *Uchl1* (Pgp9.5)(Figure 4D). Consistent with our immunostaining, we identified several *tdTom*$^{+ve}$ neuronal cell clusters that expressed *Chat* (Cluster 0, 2, 3 and 6) and a defined cell cluster highly expressing *Nos1* (Cluster 1). Some *tdTom*$^{+ve}$ neuronal populations had little to no expression of

Chat or *Nos1* (Clusters 4, 5 and 7; Fig. 4D), and so we suspected these populations might be more traditional DA neurons. We evaluated cell clusters for the expression of canonical DA markers, important in the vesicular packaging of dopamine (*i.e.,*vesicular monoamine transporter 2, *Vmat2*), in the generation of mesodiencephalic floorplate-derived neurons (*i.e.,* forkhead box A2, *Foxa2*), and in the regulation of SNc neurons excitability (*i.e.,* G-protein-regulated inward-rectifier potassium 2, *Girk2*). These genes are mostly expressed in Clusters 2 and 5 where *Th* expression also predominated (Fig. 5A). We therefore validated the expression of these canonical markers in DAT-tdTomato^{+ve} neurons using immunofluorescence staining. Indeed, we noted DAT-tdTom^{+ve} neurons immunoreactive to either Foxa2, Vmat2 or Girk2 within both ganglionated plexuses (Fig. 5B). Given the co-expression of canonical DA markers in Cluster 2 and 5*,* we further explored upregulated genes in these cell clusters; and amongst them discovered novel ENS DA neuron marker genes, as well as discovered neurotransmitter/neuromodulator genes that have been associated with dopamine signaling in the SNc (Fig. 5C, D). In summary, these data suggest that the DA neuron population within the gut is highly heterogenous, supporting the notion that DA neurons play diverse functions in the gut, and that they may also be selective vulnerability to stress or disease.

Discussion

ENS neurons exhibit diverse neuronal phenotypes analogous to the CNS, yet the detailed characterisation of these neurons is largely unexplored. Single-cell sequencing is a promising tool to explore neuronal diversification in the ENS; and we have leveraged this tool to explore dopaminergic neurons in the gut. Dopamine plays pivotal roles in both the brain and the gut whereby dysfunction in dopamine signaling could underpin a plethora of neuropsychiatric disorders and neurodegenerative diseases (Björklund & Dunnett, 2007; Hamamah et al., 2022). Through advances in DA cell classification in the CNS, striking findings have underscored the functional heterogeneity of these cell populations in healthy and diseased brains. Mounting evidence nonetheless demonstrates the role of the gut in a myriad of these brain disorders, such as in depression and PD (Dinan & Cryan, 2017), making it imperative that we deepen our understanding of the cellular and molecular properties of enteric DA neurons.

Our study aimed to shed light on the heterogenous nature of DA neurons in the colon using DAT-tdTomato reporter mice. Here, we found high, but variable expression of DAT-tdTomato across the ganglionated plexuses; submucosal ganglia containing more DAT-tdTom^{+ve} dopaminergic neurons than in the MP. Further analyses revealed that a subset of these DA neurons does not express the rate-limiting enzyme for dopamine synthesis (Th), which has been posited by several studies looking at ENS development. Between embryonic days 10 to 13, a population of neuronal precursor cells termed transiently catecholaminergic (TC) display high expression of Th which progressively diminishes during terminal differentiation (Baetge & Gershon, 1989). Although the majority of TC cells give rise to non-catecholaminergic cell types, it remains unclear whether some TC cells acquire DA phenotypes despite low levels of Th. Li and colleagues have in fact demonstrated only partial co-localisation of Dat and Th immunoreactivity in the mouse ileum and colon, further providing proof that a proportion of DAT-expressing neurons either have no or little expression of Th protein (Li et al., 2004). Interestingly, these subpopulations of DA neurons were also described prominently in the SMP (Li et al., 2004). Of note, the relative abundance of these DA markers is also not identical in all regions of the bowel (Chalazonitis et al., 2022). It is tempting to speculate that these divergent DA neuronal subtypes have corresponding functional differences whereby TH neurons may represent a more mature counterpart able to synthesize its own neurotransmitter while DAT neurons are dependent on the availability of dopamine in the synaptic cleft. Denervation of axonal projections to the gut culminated in elevated proportions of TH-immunoreactive neurons without affecting overall number of enteric neurons (Li et al., 2004), implying that existing pools of neurons may have the capacity to upregulate Th expression as a compensatory mechanism in response to injury. Hence, we postulate that a subset of molecularly defined DA neurons may be acting as *reserves* with submaximal dopaminergic function. This further begets the question whether the localization of these subtypes (being mostly in 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 DA *reserve* in this region. In

agreement with a study depicting the blood-ganglia barrier only at the myenteric plexus (Dora et al., 2021), we also detected 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 SMP neurons vulnerable to immune cell and bacterial-related damage.

The heterogeneity of DA neuronal populations across the plexuses may account for disparate roles of dopamine within the ENS. Deletion of *Dat* in mice demonstrated the involvement of dopaminergic 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 neurotoxin 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 dopamine receptor D5 expressed in goblet cells. Contrary to myenteric DA neurons, the implication of dopamine in mucosal secretion may be attributed to a subset of cells primarily in the submucosal ganglia. Furthermore, the discovery of DA neurons with dual-transmitter properties in the gut could insinuate diversity within enteric plexus that may also contribute to differences in functions. Submucosal dopaminergic neurons were in fact believed to also participate in gastrointestinal motility whereby mice with DA neuronal hypoplasia in the SMP manifest longer gastrointestinal transit time and slower colonic expulsion time (Chalazonitis et al., 2020). These submucosal DA 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 tonic inhibition (Gould et al., 2019) . As such, dopamine from DAT-tdTomato^{+ve} neurons co-expressing either ChAT or nNOS could be involved in governing this complex process. Indeed 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 dopamine receptor blockade conveys a suppressive action on colonic motility (Li et al., 2011; Li et al., 2006), whereas inhibiting dopamine reuptake elicited fewer smooth muscle contractions (Walker et al., 2000). Clearly, there is a need for the classification of DA neuron subtypes not only to define which cells control gut motility but also to discover other complex DA-related mechanisms.

Single cell transcriptomics prompted a surge of interest in molecular profiling of dopaminergic neurons in the CNS which may be instrumental in uncovering novel avenues for precise therapeutic interventions aimed at mitigating a myriad of DA-related diseases. We therefore applied this enticing approach to better characterize DAT-tdTomato^{+ve} neurons in the gut with the hopes of unraveling novel cellular markers that are able to demarcate specific DA neuronal subtypes. Consistent with our observations *in situ*, we identified DAT-tdTomato^{+ve} cell clusters upregulating either *Chat* or *Nos1*, thereby further supporting the possibility of co-transmitter release in these cell types. Traditional markers used to identify midbrain DA neurons are highly expressed in two clusters which may represent the subpopulation of enteric neurons emulating

certain roles of the DA system in the brain. Interestingly, several of the top upregulated genes in Clusters 2 and 5 (*e.g., Grp, Calcb, Satb2*) are similarly expressed in DA neurons found in the SNc (Anderegg et al., 2015). A perplexing idea dominating the field of PD is selective vulnerability of SNc DA subtypes and how specific gene signatures may help us infer the underlying mechanisms. In this regard, the ion channel Girk2 involved in modulating neuronal excitability has been debated as a key factor that leaves specific DA neurons susceptible for degeneration in the SNc. Strong Girk2 expression is evident in most SNc dopaminergic cell types, but fewer (with lower levels) DA neurons express Girk2 in the ventral tegmented area, where DA neurons are known to be less affected in PD (Reyes et al., 2012). Consistent with this, we observed that DAT-tdTomato^{+ve} neuronal Clusters 2 and 5 express Girk2 at high levels, suggesting that a comparable selective vulnerability may also exist in these gut DA subtypes. Collectively, our study provides a steppingstone to further garner interest in exploring the diverse molecular and cellular properties of dopaminergic neurons in the gut.

Ethics Statement

The animal study was reviewed and approved by University of Montreal Animal Care and Use Committee.

Author Contributions

SJR carried out the tissue processing, single cell preparations and most bioinformatic work while SP performed most of immunostaining, imaging and quantification. SM bred and maintained animal colony. AM and CG supported tissue processing. SG and LET mentored and provided feedback on the project. 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.

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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

Anderegg, A., Poulin, J. F., & Awatramani, R. (2015). Molecular heterogeneity of midbrain dopaminergic neurons--Moving toward single cell resolution. *FEBS Lett*, *589*(24 Pt A), 3714-3726. https://doi.org/10.1016/j.febslet.2015.10.022

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

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

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

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

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*.

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

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

Furness, J. B. (2008). The enteric nervous system: normal functions and enteric neuropathies. *Neurogastroenterol Motil*, *20 Suppl 1*, 32-38. https://doi.org/10.1111/j.1365-2982.2008.01094.x

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

Gopinath, A., Mackie, P., Hashimi, B., Buchanan, A. M., Smith, A. R., Bouchard, R., Shaw, G., Badov, M., Saadatpour, L., Gittis, A., Ramirez-Zamora, A., Okun, M. S., Streit, W. J., Hashemi, P., & Khoshbouei, H. (2022). DAT and TH expression marks human Parkinson's disease in peripheral immune cells. *NPJ Parkinsons Dis*, *8*(1), 72. https://doi.org/10.1038/s41531-022-00333-8

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

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

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, 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

Mackie, P., Lebowitz, J., Saadatpour, L., Nickoloff, E., Gaskill, P., & Khoshbouei, H. (2018). The dopamine transporter: An unrecognized nexus for dysfunctional peripheral immunity and signaling in Parkinson's Disease. *Brain Behav Immun*, *70*, 21-35. https://doi.org/10.1016/j.bbi.2018.03.020

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

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

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

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

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

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

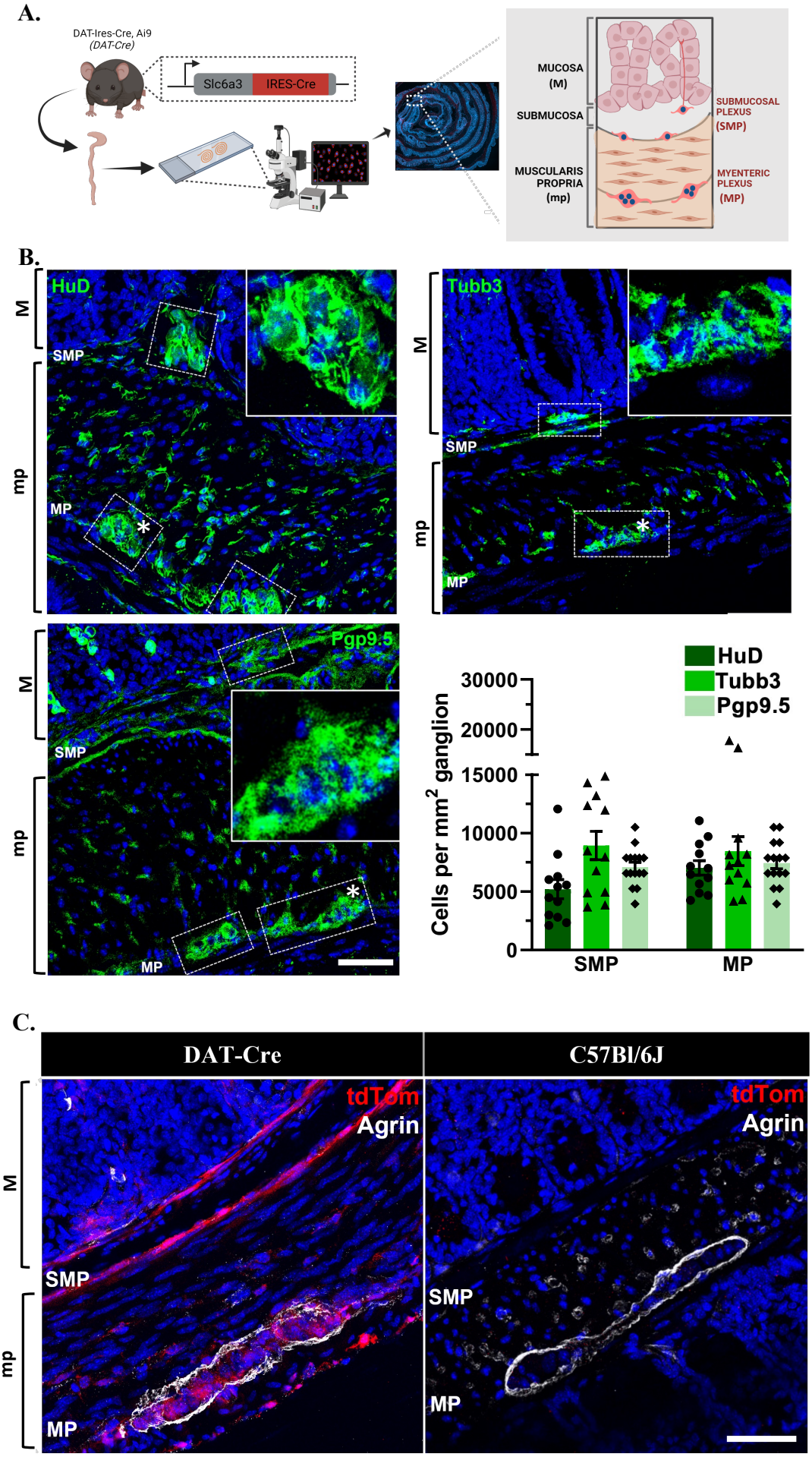
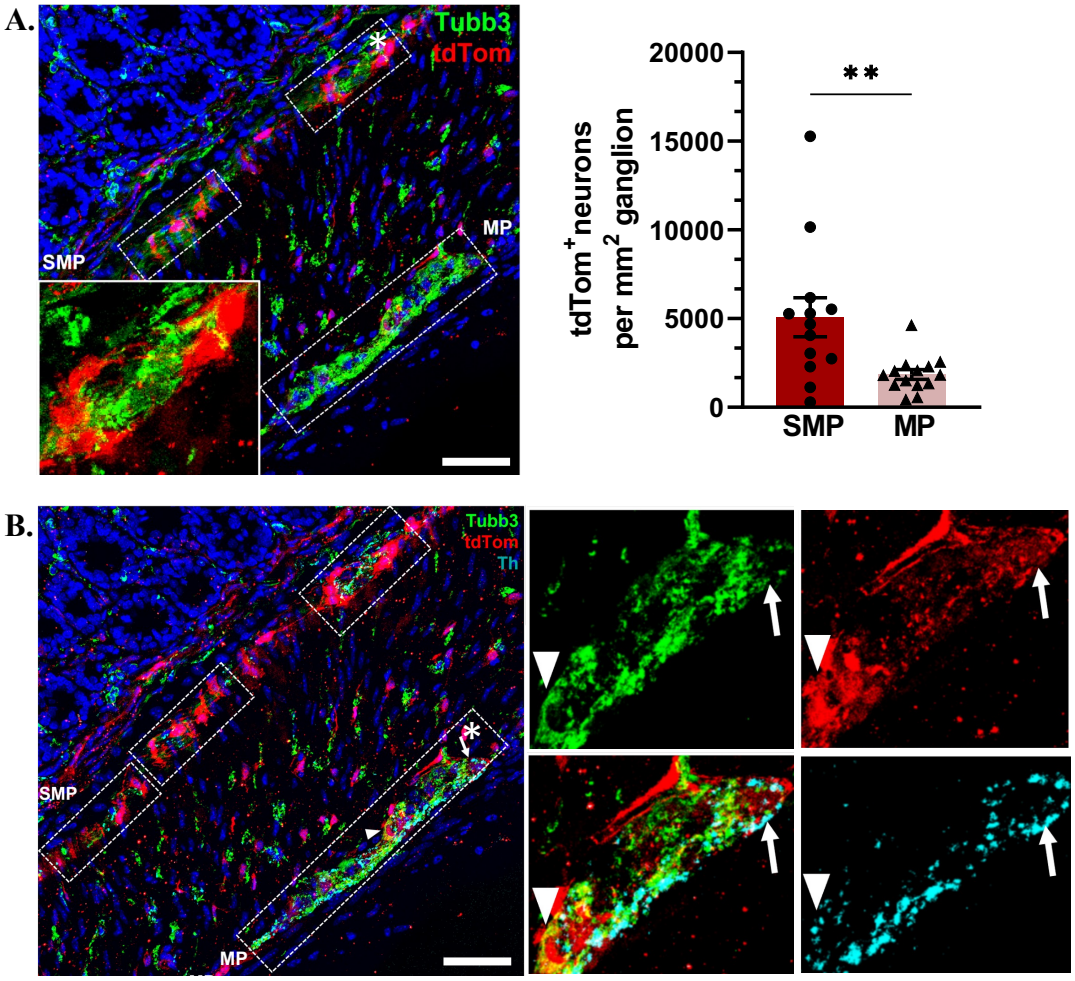


Figure 1: Detection of enteric neurons in DAT-tdTomato transgenic mice. A) Schematic diagram of immunofluorescence staining protocol for mouse colon isolated from transgenic mice, where tdTomato (Ai9) is controlled under the promoter for dopamine transporter (Slc6a3, referred to as DAT-Cre). The coronal 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 enteric 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



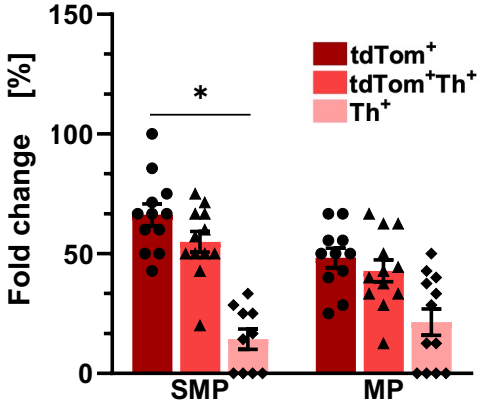
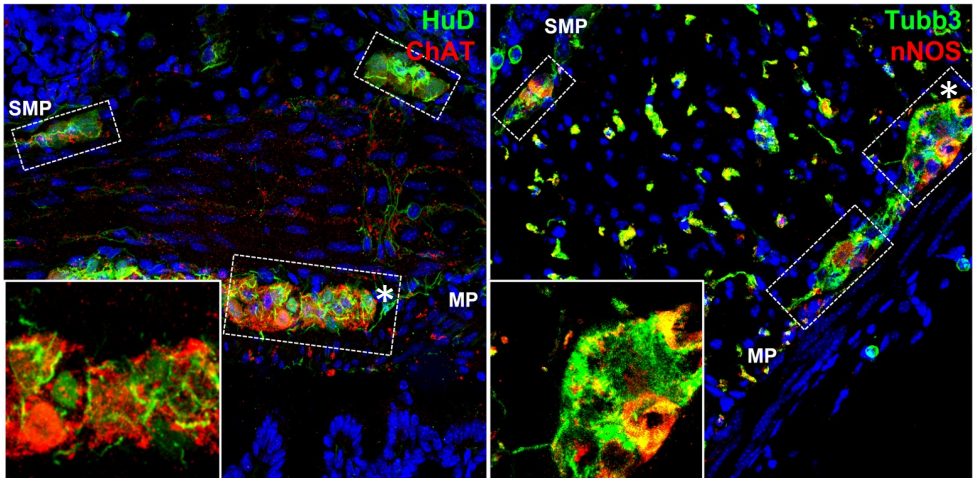
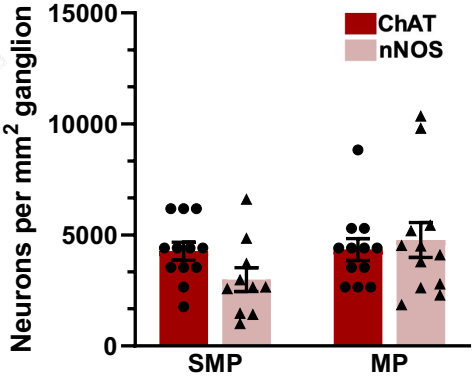


Figure 2: TdTomato-immunoreactivity varies across plexuses and colocalizes with tubulin $\beta 3$ (Tubb3) and tyrosine hydroxylase (Th) positive cells. A) Representative coronal slice of colonic tissue illustrating that DAT-tdTomato-expressing cells (red) are immunoreactive for a pan-neuronal marker, 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$^+$ tdTom^{neg}); 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 the majority of 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.

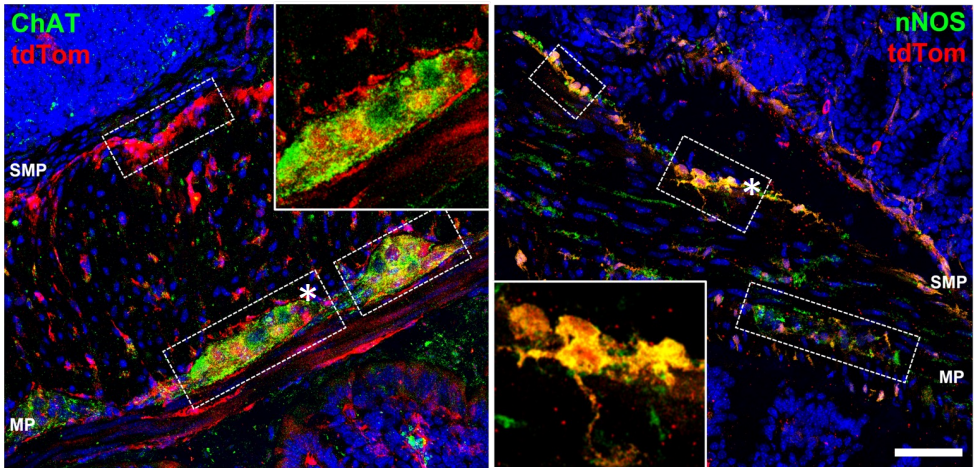
Figure 3: A large proportion of TdTomato-immunoreactive neuronal populations in the ENS exhibit dual-transmitter content

A.





B.



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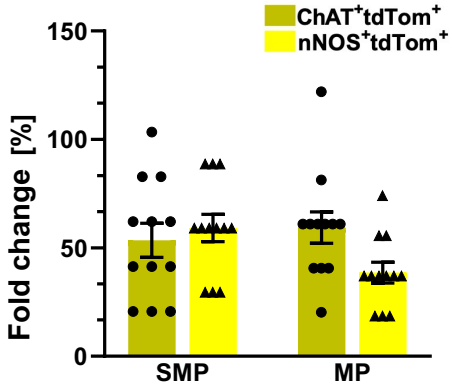

B"

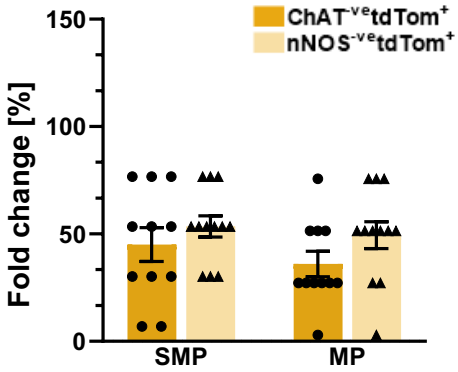

Figure 3: A large proportion of TdTomato-immunoreactive neuronal populations in the ENS exhibit dual-transmitter content. **A)** Representative cross-sectional view of colonic tissue labelling cholinergic (choline acetyl transferase, ChAT-positive) or nitrergic (neuronal nitric oxide synthase, nNOS-positive) (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=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 confirm heterogeneity of DAT-tdTomato+ neuronal subtypes

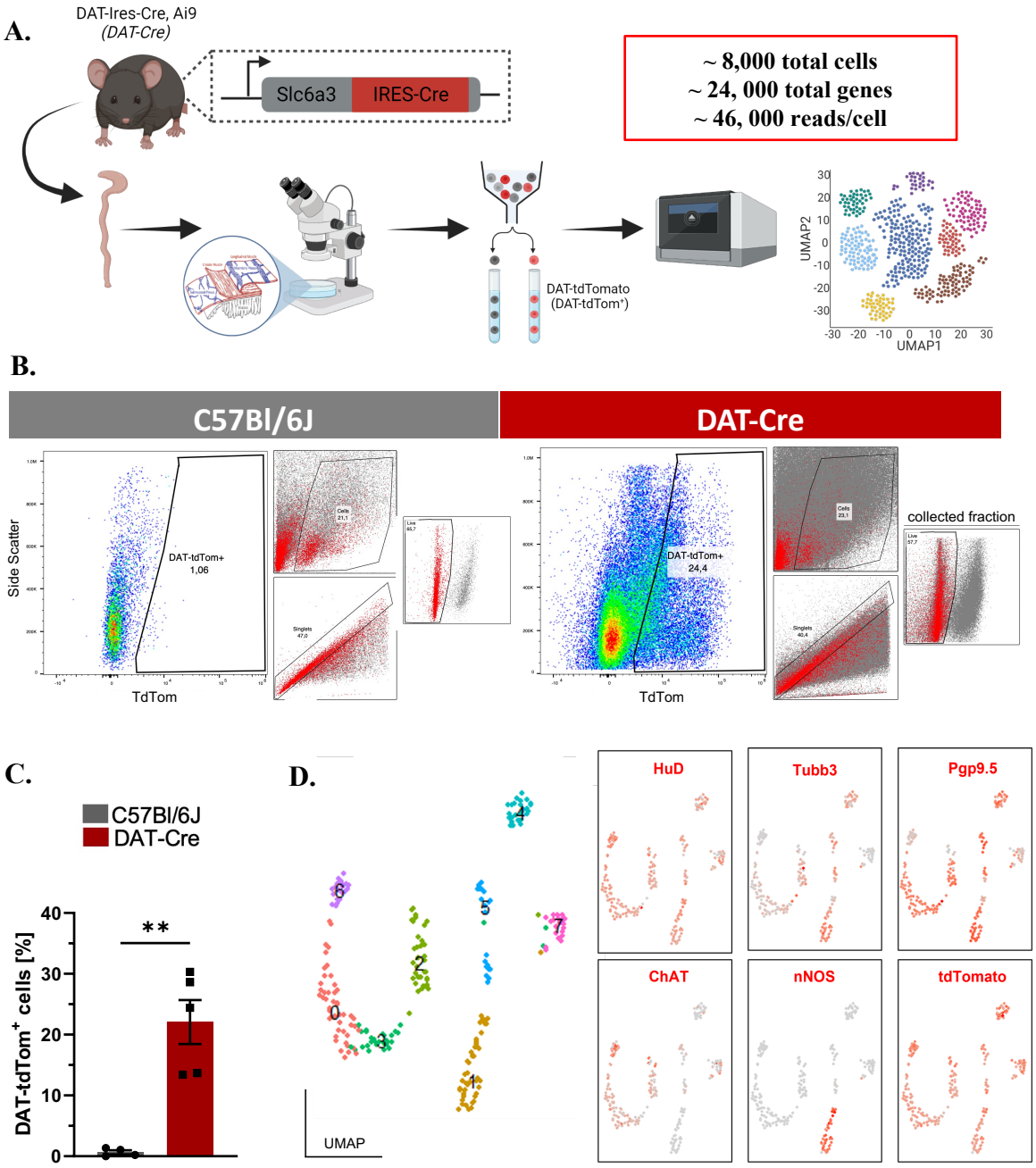

Figure 4: Single cell RNA sequencing of DAT-tdTomato-expressing cells confirm heterogeneity of DAT-tdTomato+ neuronal subtypes. A) Schematic diagram of the colonic longitudinal muscle and myenteric plexus (LMMP) isolation and dissociation for single cell RNA sequencing (scRNAseq). Live tdTomato-positive 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* using standard 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 traditional dopaminergic neuronal markers at high levels

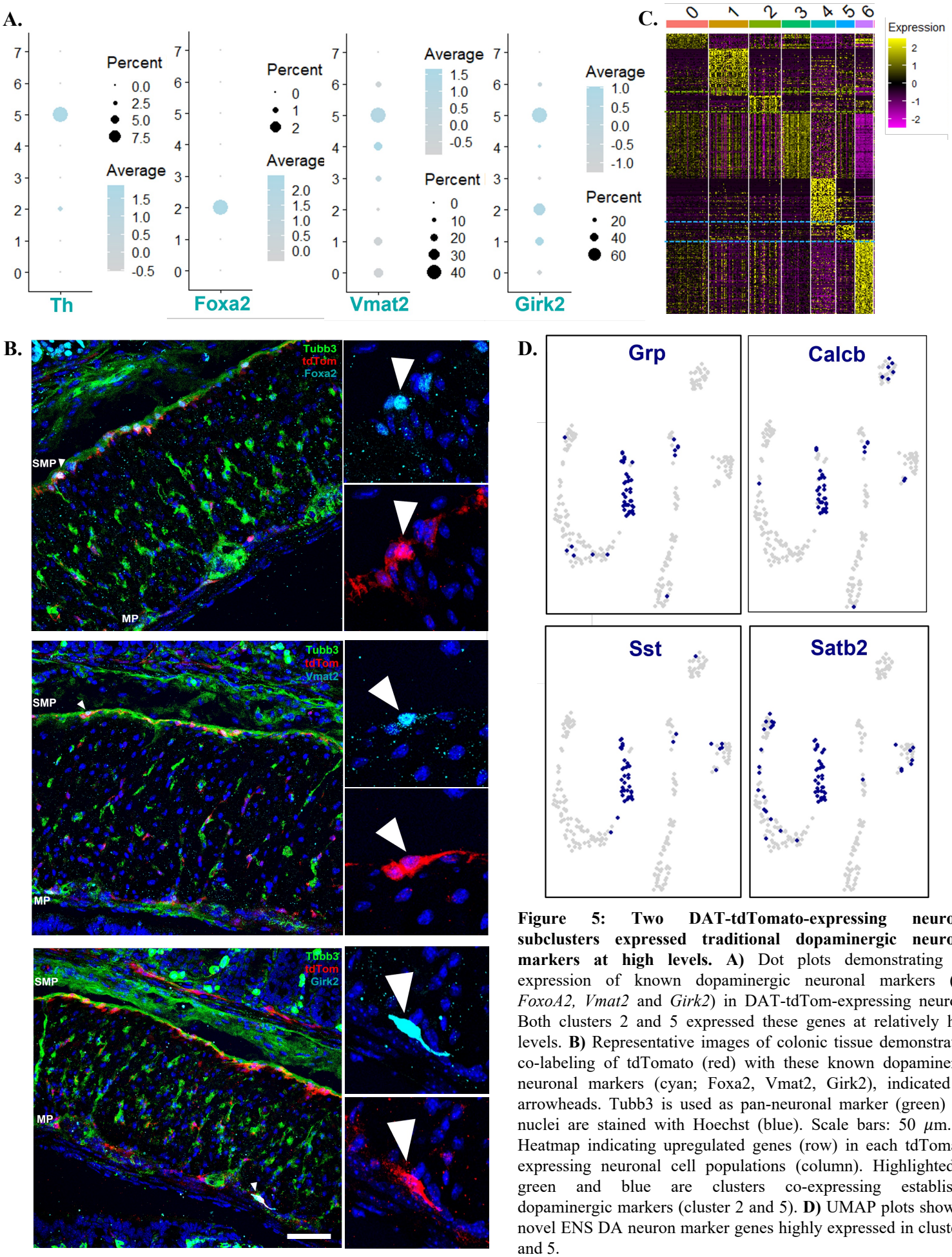

Figure 5: Two DAT-tdTomato-expressing neuronal subclusters expressed traditional dopaminergic neuronal markers at high levels. A) Dot plots demonstrating the expression of known dopaminergic neuronal markers (*Th, FoxoA2, Vmat2* and *Girk2*) in DAT-tdTom-expressing neurons. Both clusters 2 and 5 expressed these genes at relatively high levels. **B)** Representative images of colonic tissue demonstrating co-labeling of tdTomato (red) with these known dopaminergic neuronal markers (cyan; Foxa2, Vmat2, Girk2), indicated by arrowheads. Tubb3 is used as pan-neuronal marker (green) and nuclei are stained with Hoechst (blue). Scale bars: 50 μm. **C)** Heatmap indicating upregulated genes (row) in each tdTomato-expressing neuronal cell populations (column). Highlighted in green and blue are clusters co-expressing established dopaminergic markers (cluster 2 and 5). **D)** UMAP plots showing novel ENS DA neuron marker genes highly expressed in cluster 2 and 5.