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Single cell transcriptomic profiling of large intestinal enteroendocrine cells in mice — Identification of selective stimuli for insulin-like peptide-5 and glucagon-like peptide-1 co-expressing cells

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ABSTRACT

Objective: Enteroendocrine cells (EECs) of the large intestine, found scattered in the epithelial layer, are known to express different hormones, with at least partial co-expression of different hormones in the same cell. Here we aimed to categorize colonic EECs and to identify possible targets for selective recruitment of hormones.

Methods: Single cell RNA-sequencing of sorted enteroendocrine cells, using NeuroD1-Cre x Rosa26-EYFP mice, was used to cluster EECs from the colon and rectum according to their transcriptome. G-protein coupled receptors differentially expressed across clusters were identiﬁed, and, as a proof of principle, agonists of Agtr1a and Avpr1b were tested as candidate EEC secretagogues in vitro and in vivo.

Results: EECs from the large intestine separated into 7 clear clusters, 4 expressing higher levels of Tph1 (enzyme required for serotonin (5-HT) synthesis; enterochromaffin cells), 2 enriched for Gcg (encoding glucagon-like peptide-1, GLP-1, L-cells), and the 7th expressing somatostatin (D-cells). Restricted analysis of L-cells identiﬁed 4 L-cell sub-clusters, exhibiting differential expression of Gcg, Pyy (Peptide YY), Nts (neurotensin), Ins5 (insulin-like peptide 5), Cck (cholecystokinin), and Sct (secretin). Expression proﬁles of L- and enterochromaffin cells revealed the clustering to represent gradients across the crypt-surface (cell maturation) and proximal-distal gut axes. Distal colonic/rectal L-cells differentially expressed Agtr1a and the ligand angiotensin II was shown to selectively increase GLP-1 and PYY release in vitro and GLP-1 in vivo.

Conclusion: EECs in the large intestine exhibit differential expression gradients across the crypt-surface and proximal-distal axes. Distal L-cells can be differentially stimulated by targeting receptors such as Agtr1a.

Keywords: Single cell RNA-sequencing; Enteroendocrine cells; Glucagon-like peptide-1 (GLP-1); Insulin-like peptide-5 (Ins5); Serotonin (5-HT)

1. INTRODUCTION

Enteroendocrine cells (EECs) are a rare subset of gastrointestinal epithelial cells that regulate physiological processes including intestinal motility and secretion, glycemia, and appetite. They represent a diverse cellular population, collectively producing more than twenty different hormones.1,2 Gut hormone secretion after a meal is stimulated by nutrient absorption and is dominated by EECs from the small intestine, whereas the physiological role of the large number of EECs in the large intestine is less clear. However, EECs and the hormones they produce are candidate targets for drug development, as highlighted by the success of therapies based on Glucagon-like peptide-1 (GLP-1) for the treatment of type 2 diabetes and obesity. The aim of this project was to improve our understanding of the physiology of EECs in the large intestine (colon and rectum) and whether they could usefully be targeted therapeutically.

EEC populations vary along the length of the gastrointestinal (GI) tract, with some hormones produced predominantly in the proximal gut (e.g., Glucose-dependent insulinotropic polypeptide, GIP) and others predominating more distally (e.g., Peptide YY, PYY; GLP-1; Insulin-like peptide-5, INS5) [3]. Recent transcriptomic analyses have challenged the traditional notion that distinct EEC subtypes exist, which produce separate and non-overlapping sets of gut hormones [4]. Characterization of individual EECs in the small intestine by single cell RNA-
sequencing (scRNA-seq), led to the identification of distinct EEC subgroups by cluster analysis, exhibiting overlapping expression profiles for known gut hormones [5–7]. For example, Glass et al. found that subgroups of cells expressing Gcg (encoding GLP-1), classically known as L-cells, also expressed Gip (considered a product of K-cells) as well as Tph1 (tryptophan hydroxylase-1), the enzyme required for serotonin (5-HT) production, implying overlap between L, K, and enterochromaffin (Ecm) cells [5]. Immunohistological and flow cytometric studies confirmed that these overlaps identified by transcriptomics were also reflected at the level of protein synthesis [8–10]. Most previous investigations, however, have focused on the small intestine rather than the colon.

In the large intestine, enterochromaffin cells have been reported as the most prevalent subtype of EEC [11]. These cells are defined by production of 5-HT, which exerts a critical role in regulating GI motility and peristalsis and has been associated both with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [12,13]. L-cells are also highly abundant, and distinguishable by their production of GLP-1 and PYY, peptides known to suppress appetite and stimulate insulin secretion [11,14–19]. A third and rarer population known as D-cells produces somatostatin (SST) [11], which acts as a paracrine inhibitor of other EECs and excitatory cells and influences colonic motility [20–23]. Recently, we showed that approximately half of all large intestinal L-cells produce INS5, suggesting the existence of at least two subgroups of L-cells in this region [24,25]. Expression of INS5 was restricted to the large intestine and absent in other regions of the GI tract. Large intestinal EECs are likely to sense different physiological stimuli compared with those located more proximally, as ingested nutrients do not normally reach the distal gut in high quantities, and resident microbiota produce a variety of alternative candidate signaling molecules.

EECs are generated alongside other intestinal epithelial cells by the continuous division of crypt stem cells, and in the duodenum and jejunum have been reported to have a life span of 3–10 days before they are shed into the lumen from the villus tips [26,27], although a recent paper has shown longer life spans of EECs compared to surrounding enterocytes in the small intestine [28]. Small intestinal EEC development and maturation has been modeled using 3-dimensional intestinal organoid cultures, revealing that L-cells and Ecm cells mature as they migrate from crypts into villi, developing increased expression of Sct (secretin), accompanied by reductions of Gcg expression in L-cells and of Tac1 (tachykinin) in Ecm cells [7,28]. Large intestinal epithelium, by contrast, is characterized by deep crypts and no villi, and reports that EECs in this region have longer life spans of about three weeks [29] suggest some differences in EEC maturation compared with the small intestine.

In this study, we mapped large intestinal EECs using single cell RNA-sequencing. We identified different subpopulations of L-cells and Ecm-cells, and showed that these likely represent cellular gradients mapping along the proximal-distal and crypt-surface gut axes. Selective stimulation of distal L-cells using Angiotensin-II resulted in significant elevation of plasma GLP-1 levels, suggesting that these cells can contribute to circulating gut hormone concentrations despite their distal location.

2. METHODS

2.1. Animal work and ethics

All animal procedures were approved by the University of Cambridge Animal Welfare and Ethical Review Body and carried out in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039). The animal work was performed under the UK Home Office project licences 70/7824 and PESOF6065 [30,31]. Mice were housed in ventilated cages on a 12hr light/dark cycle (lights out at 07:00 GMT) with ad libitum access to water and regular chow (unless otherwise stated) and were culled by an approved Schedule 1 method.

2.2. Flow cytometry

Single cell digests of mouse colon were prepared for FACS purification by two incubations of 30 min with 1 mg/ml collagenase (dissolved in calcium-free HBSS) at 37 °C. After each incubation, single cells were harvested in the media, washed in calcium-free HBSS with 10% FBS and filtered through 50 μm filters. The two digests were spun at 300 g for 10 min at 4 °C and resuspended together in HBSS (calcium-free) with 10% FBS and stained 5 min on ice with DAPI (1 μg/mL) and washed once before being resuspended in HBSS with 10% FBS, 10 μM Y-27632 and 5 μM DraQ5 (Biolegend).

The single cell suspension was sorted using an Influx Cell Sorter (BD Biosciences) at the Cambridge Institute of Medical Research (CIMR) Flow Cytometry Core Facility. DAPI-staining, DraQ5-staining, side scatter, forward scatter and pulse width gates were applied to remove clustered debris, dead cells and cellular debris present. Neuronal-expressing cells (from a NeuroD1-Cre x Rosa26-EYFP mouse, henceforth called NeuroD1EYFP) were purified by EYFP fluorescence into LoBind tubes (Eppendorf) with 40 μL HBSS (calcium-free), 10% FBS and 10 μM Y-27632 [30,31].

2.3. Single-cell RNA-sequencing

2.3.1. Library preparation and sequencing

3500 FACS-purified NeuroD1EYFP cells (from a single mouse colon) were loaded onto the Chromium system (10x Genomics 3’ GEX V2) to produce cDNA libraries, which were paired-end sequenced (26:8:98) by a HiSeq 4000 (Illumina) at the Cancer Research UK Cambridge Institute (CRUK CI). Quality control, read alignment (with reference to the mm10 genome downloaded from the UCSC genome browser [32]) and raw count quantification for each cell was achieved using the CellRanger pipeline (10x Genomics).

2.3.2. scRNA-seq analysis

Analyses from raw counts were performed using the Seurat package (v2.3.4, Butler et al., 2018 Nat biotechnologies) in R using default parameters except when indicated. Cells were first filtered based on their total number of expressed genes (min = 800), nUMI (unique molecular identifiers; min = 1250, max = 3rd quartile + interquartile) and the percentage of mitochondrial genes (between 1 and 7.5%). A first analysis was performed using standard normalization to retrieve the list of genes that are differently expressed (DE) in at least one population. As DE genes accounted for a majority proportion of UMs, cells were further filtered on their number of UMI matching DE based on a first analysis with standard normalization (min 800). Moreover, UMI normalization was performed based on the number of UMI matching nonDE genes rather than the total number of UMIs per cell. The total number of cells of filtering was 1560. Clusters were identified by shared nearest neighbor clustering optimization using the 7 most variable dimensions of a principal component analysis performed on the most variable genes. Populations were plotted using t-SNE dimension reduction or expression of specific genes plotted on distribution plots or heatmaps. Differentially expressed genes (FDR of 5%) were assessed using a Wilcoxon rank test between individual populations against the rest of the cells or
between two specific populations or groups of populations with a log-
two-fold difference between at least one of the groups 0.2.
Subpopulations were analyzed by subsetting the cells based on cluster
annotation during first analysis and performed as the general one,
using appropriate numbers of dimensions when identifying the
clusters.

2.4. Real-time quantitative PCR (RT-qPCR)
Tissue samples were harvested from the colon divided into seven equal
parts along the proximo-distal axis (P1—P7) and lysed using TR-reagent.
RNA was extracted by adding chloroform and collecting the aqueous
phase while proteins and peptides were retained in the phenol phase.
RNA was purified by isopropanol and ethanol precipitation and resus-
pended in nuclease free water and treated with DNase-free DNA removal kit
(Invitrogen) to remove residual genomic DNA. RNA was reverse tran-
scribed using SuperScript II using a Peltier Thermal Cycler-225 (MJ
Research) according to standard protocols. The RT-qPCR reaction
mix consisted of template cDNA, TaqMan Universal Master Mix
(Applied Biosystems) and specific primers (Applied Biosystems) for
β-actin (Mm02619580_g1), Ins5 (Mm00442241_m1), Gcg
(Mm01269055_m1), Pyy (Mm00207161_g1), Nts (Mm00481140_m1),
Cck (Mm00446170_m1), Tph1 (Mm01202614_m1), Sct
(Mm00441235_g1), Sat (Mm00436671_m1), Tac1 (Mm01166996_m1),
Piezo2 (Mm01265858_m1), Ffar1 (Mm00809442_s1), Agtr1a
(Mm01957722_s1) and Avpr2b (Mm01700416_m1) and qPCR were
performed and analyzed using an ABI QuantStudio 7 (Applied Bio-
systems). Relative expression was evaluated by calculating the difference
in cycle threshold (ΔCT) between the housekeeper gene β-actin and the
gene of interest (CTgene−CTβactin).

2.5. Peptide extraction
Proteins were extracted from the phenol phase (after DNA precipitation
by ethanol) by incubation with ice-cold acetone for 10 min at 4 °C
followed by centrifugation for 10 min, 12000 g. Following a wash in
0.3 M guanidine HCl (dissolved in 95% ethanol), the resulting pellet
was air dried and resuspended in 8 M urea using a syringe. Then 80% acetonitrile
was then added to each sample to precipitate proteins and
peptides in solution recovered and dried using a centrifugal concen-
trator [3]. Peptides were resuspended in 500 μL 0.1% formic acid
and spiked with internal standards and purified by solid phase extraction
using a prime HLB elution plate (Oasis) and eluted in 60 μL 60% methanol, 30% H2O, and 10% acetic acid. Peptides were dried and
reduced-alkylated by incubation 1 h with 10 mM DTT in 50 mM ammonium bicarbonate at 60 °C followed by 30 min incubation with
20 mM iodoacetamide. Samples were further diluted with 0.1% formic acid and 10 μL were analyzed using a nano-flow rate by a Thermo-
Fisher Ultimate 3000 nano LC system coupled to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific) [3,33]. Peptide
quantification was achieved using XCalibur (ThermoFisher) to integrate
the peak area for selected sets of m/z values at specific retention times
for each individual peptide.
For the LC-MS/MS peptide quantification, treatment supernatants were
first acidified with 50 μL 1% formic acid while homogenates were
resuspended in 500 μL 0.1% formic acid. Both were subsequently
spiked with internal standards. Subsequently peptides were extracted and
analyzed following reduction/alkylation as described previously
[3,34]. Total protein content was calculated from lysate supernatants
using a BCA protein assay (Thermo Fisher Scientific) which was used
to normalize secretory responses from different wells. In several cases
surrogate peptides, chosen on the basis of their robust observable
signal, are reported and considered to be produced from the pro-
peptide in a stable molar ratio to the established hormones. For
Gcg, we chose oxyntomodulin (Figure 3) and GRPP (Figure 4) as the
GLP-1 signal was low and split between amidated and non-amidated
forms; for CCK we chose CCK21_44, as sulfated CCK8 was not
detectable in the LC-MS/MS mode needed for the other peptides; for
INSL5, we chose the C-chain.

2.6. Immunohistochemistry
Colonie wholemounts were processed using adapted methods detailed
by Winton et al. (1990) [35]. Briefly, following isolation of the epithelial
layer, the colors were fixed for 3 h at room temperature using 4% PFA
(Alfa Aesar). Next, residual mucus was removed from the fixed tissue
by incubation with 50 ml of demucifying solution (10% glycerol, 10% 0.1 M Tris titrated to pH 8.2, 20% ethanol, 92 mM NaCl, and 20 mM DTT) for 20 min at room temperature followed by PBS washes. After-
tward, the fixed colons were placed in blocking solution (PBS with
0.1% Triton-X 100 and 10% goat serum) overnight at 4 °C. The next
day, the colons were incubated for 4 h at room temperature with primary antibodies to PYY (guinea pig; Progen 16066; 1:500), INSL5
(rat; Takeda; 1:1000), and NTS (rabbit; Merck; AB4596; 1:100) diluted
in wash solution containing 1% goat serum and 0.1% Triton-X 100 in PBS. Tissues were washed overnight and then incubated with 1:300 goat secondary antibodies (conjugated to AlexFluor 488, 555 and 633) for 3 h at room temperature. Following further washing overnight at 4 °C, the colons were incubated with 1:2000 Hoechst nuclear stain (in PBS) for 30 min at room temperature followed by PBS washes. Finally, the colons were divided in half and mounted onto microscope slides
using Hydromount (National Diagnostics).

2.7. Imaging of colonic wholemounts

2.7.1. Image acquisition
Wholemounts were imaged using the Axio Scan.Z1 system (Zeiss). Tiles of extended depth of focus (EDF) images were taken for each of
the three labeled channels using a Plan-Apochromat 20x/0.8 M27
objective, a Hamamatsu Orca Flash camera, and an inbuilt autofocus
function. The depths used for the EDF images were customized for
each wholemount and depended on tissue thickness. Following
acquisition, the tiled images were stitched together with shading
correction.

2.7.2. Counting of immunofluorescently labeled cells
For analysis of cell populations from stained whole-mount tissue, 10 ROIs (0.5 mm × 0.5 mm picked based on in focus Hoescht staining)
where selected from proximal, mid and distal areas of the large in-
testine from 3 mice (total ROIs = 90) and the number of cells for each
population counted using HALO software (Indica Labs). For automated
analysis, thresholding and size criteria were kept the same for all 3
channels and the number of cells per ROI for each of the channels was
then calculated. Cell density per region was then analyzed using a
combination of Excel (Microsoft Office) and GraphPad Prism 7.0
(GraphPad Software).

2.8. Primary cultures
Excised murine large intestines were collected in Leibovitz’s L-15
medium and divided into three equal segments. Segments from
the same region from 2 different mice were pooled together to ensure
enough tissue for each secretion plate. Colonic crypts were subse-
quently isolated from each region as described previously [36]. Briefly,
isolated tissue was cleaned of contents in PBS containing CaCl2 and
MgCl2 and the epithelium was separated from adipose, the outer
muscle layers and vasculature by manual stripping. Following this, the tissue was cut into small chunks (~2 mm³) and digested using collagenase XI (Sigma; 0.25 mg/ml). Isolated colonic crypts were resuspended in DMEM enriched with glucose (4500 mg/L), 10% FBS, 1% glutamine, and 1% penicillin/streptomycin and plated onto 2% matrigel (Corning) precoated 12 well plates. Plated crypt suspensions were placed into a 37 °C humidified incubator with 5% CO₂ to settle overnight prior to experimentation.

2.9. Secretion assays
For the secretion assays, colonic crypt cultures were processed as described in Billing et al. [29]. Briefly, following washes with saline buffer containing 1 mM glucose and 0.001% fatty acid free BSA, each well of colonic crypts was incubated with 600 μl of each treatment (made up in saline buffer and 0.001% BSA) at 37 °C for 1 h. Supernatants were collected in protein LoBind 1.5 ml tubes (Eppendorf) and centrifuged at 2000 g for 5 min at 4 °C to remove cellular debris. 500 μl of each supernatant were collected into fresh 1.5 ml LoBind tubes. Meanwhile, 200 μl lysis buffer was added to each well. After 30 min on ice, the plates were snap frozen and defrosted to ensure complete cell lysis. Lysates were collected following cell scraping and spun at 2000 g for 5 min at 4 °C and the supernatants were retained. Both supernatants and lysates were stored at −80 °C prior to further processing. Protein content from lysates was analyzed by BCA assay (Pierce) and used to normalize for cell density variability between wells. 100 μL 1% formic acid and internal standards were added to supernatants and peptides were extracted by solid phase extraction and reduced alkylated as described previously. 40 μL of 120 μL were analyzed by nano-LC-MS/MS as described previously and peptide content quantified by measuring the peak area corresponding to characterized peptides. The experiment was performed five times with duplicates for each condition. Each peptide was analyzed separately and two-way ANOVA followed by a Tukey’s test were performed to test significant differences between responses.

2.10. In vivo stimulation test
Adult male and female mice, obtained from a C57Bl6 colony maintained at the University of Cambridge, were fasted overnight (16 h) with free access to water. AVP (V8979, Sigma - 100 ng per mouse), angiotensin II (A9525, Sigma - 1 mg/kg BW) or vehicle (saline) were administered via intraperitoneal injection. For measurement of GLP-1 (cross-over design) and PYY levels (independent experiment, due to the greater plasma volume required for the assay), 50 and 80 mg/kg/C0 were administered via intraperitoneal injection. For measurement of GLP-1 and PYY levels (independent experiment, due to the greater plasma volume required for the assay), 50 and 80 mg/kg/C0 were administered via intraperitoneal injection. To determine whether the acquisition of Sct expression in L-cells is accompanied by other transcriptional changes that are common between LNS cells and LNS cells (Figure 2A), restricting our analysis to genes showing an absolute log fold-change (LFC) > 0.2 in both Sct/Gcg pairs and reaching a significant difference in at least one of the Sct/Gcg pairs. Genes enriched in LNS cells (vs LNS cells) correlated positively with those enriched in LNS cells (vs LNS cells), with Sct and Gcg exhibiting the most extreme regulation between these clusters. Other genes highlighted by the analysis included the transcription factor Id (inhibitor of differentiation) -1,2,3, which were higher in Sct-enriched clusters, and Nr4a1, which was higher in Gcg-enriched clusters.

3. RESULTS

3.1. EEC heterogeneity in the colon
EECs from a Neurod1-cre mouse crossed with a Rosa26-EYFP reporter mouse colon were flow-sorted and single-cell RNA-seq was performed using the 10xGenomics 3’ GEX V2 platform (see methods) (Figure 1A). Data were analyzed using the Seurat R package with modifications as described in material and methods. Cluster analysis identified 7 EEC subgroups (Figure 1B). Gut hormones were amongst the top differentially expressed genes distinguishing clusters, including Gcg, Nts (neurotensin), Insl5, Pyy, Cck (cholecystokinin), Sst, Sct, Tac1, and Tph1 (Suppl. Figure S1A–C and Figure 1C). Four clusters expressed high levels of Tph1, identifying them as Ecm cells (790/1560 cells, ~50%), two clusters were enriched for Gcg and Pyy characteristic of L-cells (609/1560 cells, ~40%), and the remaining cluster expressed high levels of Sct (D-cells, 161/1560 cells, ~10%) (Figure 1C). Each of the clusters expressed specific set of genes with the top5 being presented in Suppl. Figure S1A.

Two of the four Ecm-cell clusters showed enrichment for the expression of the mechanosensitive ion channel Piezo2 and have been denoted EcmPz+, with the two Piezo2-negative clusters denoted EcmPz−. Both Piezo-positive and Piezo-negative cells sub-clustered into groups that were enriched for either Sct or Tac1, resulting in clusters labeled EcmPz−/Sac, EcmPz−/Tac, EcmPz+−/Sac, and EcmPz−/Tac (Figure 1C).

Comparing the two L-cell clusters, one expressed high levels of Nts and Cck (denoted LNS-cells) whereas the other had high levels of Insl5 (LNS-cells) (Figure 1C). When the L-cell clusters were further analyzed after exclusion of all other EECs, additional sub-clustering was observed (Figure 1D and Suppl. Figure S1D). LNS-cells separated into two groups, one with higher Nts, Pyy, and Sct (LNS/Sct) and the other with higher Gcg (LNS/Gcg). LNS cells similarly separated into a group with higher Ppy and Sct (LNS/Sct) and one with higher Gcg (LNS/Gcg) (Figure 1E). Analysis of the D-cell cluster without other EEC sub-types did not reveal further D-cell sub-clusters. Differential expression heatmaps of G-protein coupled receptors (GPCRs) and transcription factors across all 7 EEC clusters, as well as across the four L-cell sub-clusters analyzed separately, are shown in Suppl. Figure S1.

3.2. Common transcriptomic patterns defining different EEC subgroups
We hypothesized that our observation of Ecm and L-cell clusters with differential expression of Sct, Tac1 (in Ecm cells), and Gcg (in L-cells) reflects cellular maturation along the crypt-surface axis in the colon and rectum, mirroring the recently described maturation of small intestinal EECs [7,28]. We examined whether the acquisition of Sct expression in L-cells is accompanied by other transcriptional changes that are common between LNS and LNS cells (Figure 2A), restricting our analysis to genes showing an absolute log fold-change (LFC) of > 0.2 in both Sct/Gcg pairs and reaching a significant difference in at least one of the Sct/Gcg pairs. Genes enriched in LNS cells (vs LNS cells) correlated positively with those enriched in LNS cells (vs LNS cells), with Sct and Gcg exhibiting the most extreme regulation between these clusters. Other genes highlighted by the analysis included the transcription factor Id (inhibitor of differentiation) -1,2,3, which were higher in Sct-enriched clusters, and Nr4a1, which was higher in Gcg-enriched clusters.

We performed a similar analysis of Ecm cell clusters to examine whether the acquisition of Sct and loss of Tac1 expression is accompanied by other common transcriptional changes between Piezo2-positive and negative Ecm clusters (Figure 2B). This comparison showed reasonable correlation, with several genes being up- and down-regulated in parallel with Sct and Tac1. Genes enriched in the Sct-groups (Gdk1n1a, Smad7) have been described as markers of colonic surface epithelium, whereas genes in the Tac1-cluster (Sec61b, Atf6) have been located towards the bottom of colonic crypts [37], consistent with the idea that downregulation of Tac1 expression and upregulation of Sct occurs during Ecm cell maturation in the colon and rectum. A similar analysis comparing all L- and Ecm-cells, each grouped by their Sct-expression status, also showed a reasonable
correlation, revealing an overlapping set of genes (Figure 2C) suggesting that these genes are commonly regulated along the crypt-surface epithelial axis in colonic EECs.

We next examined, independent of these maturity markers, what separated L-cells into high Insl5 vs high Nts groups and Ecm cells into Piezo2-positive vs Piezo2-negative groups (Figure 2D). A differential expression analysis, performed as above but comparing genes enriched in Piezo2 positive (vs negative) Ecm cells with those enriched in Insl5-positive L-cells (vs LNts cells), revealed parallel transcriptional changes in EcmPz cells and LInsl5 cells. This comparison identified a number of Homeobox B (Hoxb) genes which are known to be involved in rostro-caudal differentiation thereby defining the proximal to distal axis in the large intestine, with higher Hoxb numbers assigned to more distal locations [38,39]. Hoxb6, Hoxb7, and Hoxb8 were higher in
Figure 2: Common determinants of Ecm- and L-cell sub-clustering. (A–D) Correlation plots of all genes that are differently expressed in at least one comparison and for which the L2FC is higher than 0.2 in both comparisons, plotting the log2 fold change difference between two populations plotted against the log2 fold change between two other populations. Pearson correlation coefficient is indicated. (A) compares the difference between the LInsl5/Gcg and the LInsl5/Sct sub-clusters with the difference between the LNts/Gcg and LNts/Sct sub-clusters, as defined during the L-cells sub-clustering. (B) compares the difference between the EcmPz/C0/Tac cluster and the EcmPz/C0/Sct cluster with the difference between the EcmPz+/Tac and the EcmPz+/Sct clusters. (C) compares the difference between Sct-positive (LNts/Sct+ LInsl5/Sct) and Sct-negative (LNts/Gcg+ LInsl5/Gcg) L-cells with the difference between similar Ecm sub-clusters ((EcmPz/C0/Sct+Sct) and (EcmPz/C0/Tac+Sct), respectively). (D) compares the difference between Nts-positive (LNts) and Insl5-positive (LInsl5) L-cells with the differences between Piezo-negative (EcmPz/C0/Tac+EcmPz/C0/Sct) and -positive (EcmPz+/Tac+EcmPz+/Sct) Ecm-cells. (E) Violin plots of log2 normalized unique molecular identifiers (nUMIs) counts for the original identified seven clusters for different Hoxb genes.
distal gut (Figure 3A and B). Very low and the peptide undetectable in proximal regions whereas expression and INS5 peptide levels were significantly increased in the distal gut (Figure 3A and B). Nts and Cck showed the opposite pattern, with high levels in proximal regions that dropped more distally. Gcg and Pyy (and derived peptides) only exhibited weak proximal-distal gradients along the large intestine, but, interestingly, we detected PYY3-36 as well as PYY1-36 by our LC-MS/MS analysis, and found that PYY3-36 predominated in the distal rectum, indicating region-dependent processing. We further validated the regional distribution of L-cells by co-staining for INS5, Nts, and PYY in whole mounted colons (Figure 3E). NTS positive cells were mainly localized in the proximal regions whereas INS5 positive cells were found in higher numbers in the distal colon (Figure 3F). PYY positive cells were present along the large intestine, with no evident gradient. Consistent with the proposed distal location of EcmPz cells determined from the cluster analysis, Rezo2 expression was 5 times higher in the distal tissue samples, with the exception of the distal rectum. Gradients for other examined Ecm cell markers and Sst were unremarkable (Figure 3C and D).

3.3. EEC variability along the proximo-distal axis
To confirm the proximo-distal distribution of the different clusters, gene expression and peptide levels were measured in tissue homogenates from seven regions equally distributed along the colon/rectum, from proximal (P1) to distal (P7) in three mice. INS5 gene expression was very low and the peptide undetectable in proximal regions whereas expression and INS5 peptide levels were significantly increased in the distal gut (Figure 3A and B). Nts and Cck showed the opposite pattern, with high levels in proximal regions that dropped more distally. Gcg and Pyy (and derived peptides) only exhibited weak proximal-distal gradients along the large intestine, but, interestingly, we detected PYY3-36 as well as PYY1-36 by our LC-MS/MS analysis, and found that PYY3-36 predominated in the distal rectum, indicating region-dependent processing. We further validated the regional distribution of L-cells by co-staining for INS5, Nts, and PYY in whole mounted colons (Figure 3E). NTS positive cells were mainly localized in the proximal regions whereas INS5 positive cells were found in higher numbers in the distal colon (Figure 3F). PYY positive cells were present along the large intestine, with no evident gradient. Consistent with the proposed distal location of EcmPz cells determined from the cluster analysis, Rezo2 expression was 5 times higher in the distal tissue samples, with the exception of the distal rectum. Gradients for other examined Ecm cell markers and Sst were unremarkable (Figure 3C and D).

3.4. Selective stimulation of L-cell populations by GPCR ligands
As expression of some GPCRs was found to differ between the L-cell clusters (Supp. Figure S1E), we examined whether selective GPCR agonists could be used to trigger region-specific hormone secretion from the colon or rectum. We selected the arginine-vasopressin (AVP) receptor Avpr1b and the angiotensin2 (AngII) receptor Agrp1a, which were enriched in LNS5 compared with LNS cells, and the free fatty acid receptor Ffar1, which was expressed in both L-cell clusters (Figure 4A) but at lower level. Primary crypt cultures from the proximal third, the middle third or the distal third (Figure 4C–H) of the large intestine were stimulated with AVP (10 nM), AngII (10 nm) or AM1638 (1 μM; a strong agonist for FFAl). Hormone secretion was quantified by a multiplex LC-MS/MS method [25,34], and a combination of glucose (10 mM) and IBMX (100 μM) was used as a positive control. We were unable to detect secretin reliably with this method, perhaps reflecting the enrichment of crypt over surface epithelial cells in these cultures, but, NTS, CCK, GLP-1, PYY, and INS5 secretion could be monitored reliably and simultaneously, through fragments derived from the respective pro-hormones.

As expected from the expression analysis, we could not detect secretion of NTS from distal cultures and INS5 was only detected in a few samples derived from proximal colon. ProCCK levels were also lower, and PYY3-36 levels higher, in supernatants from distal vs proximal cultures. In proximal colonic cultures, AM1638 stimulated secretion of NTS, proCCK, GRPP (a peptide from Gcg) and PYY, whereas AVP and AngII were largely without effect. By contrast, all three GPCR ligands stimulated secretion of proCCK, GRPP, PYY, and INS5 from the distal cultures, consistent with the enrichment of Avpr1b and Agrp1a in LNS5, but not LNS cells. Results from the middle section fell in between the results from the proximal and distal large intestine, both with respect to the levels of individual peptides detected and the responsiveness to the different stimuli. We next examined whether large intestinal L-cells could be stimulated specifically in vivo using agonists of AVPR1B and AGTR1A. Expression of these receptors in bulk-purified L-cells from duodenum, ileum, and colon/rectum was assessed from our published RNA-sequencing data [3], revealing that whereas Agtr1a expression was restricted to the distal gut, Avpr1b was also expressed in more proximal L-cells in the small intestine (Figure 4B). Mice were injected ip with AVP, AngII or vehicle control, and plasma GLP-1 and PYY levels measured by immuno-assay after 15 min. GLP-1 levels increased ~2-fold following stimulation with either AVP or AngII (Figure 4I and J). PYY levels were elevated significantly by AVP but not AngII (Figure 4K and L).
Figure 3: Localization of INSL5, PYY, and NTS-producing cells within the colon. (A, C) Relative expression of Lcell (A) and D or Ecm-cell (C) enriched genes along the proximal-distal axis of the murine colon divided into 7 equal segments (P1–P7) by RT-qPCR. Cycle threshold difference (ΔCT) was calculated between the gene of interest and the housekeeper β-actin (CTβ-actin-CTGene). (B, D) Peptide quantification by LC-MS/MS of proCCK (CCK21-44), Neurotensin, INSL5 (C-chain), Oxyntomodulin (OXM), PYY (1-36 and 3-36) (B) and pro-SST, pro-tachykinin (TKN)1 and SCT (D) in P1–P7. (E) Representative images of proximal, middle and distal large intestine immunofluorescently labeled for NTS (blue, left column), PYY (red, middle center column) and INSL5 (green, center right column). Merged pictures for all three regions are represented in most right column. Scale bar = 50 μm. (F) Plot showing the density (per mm²) of labeled NTS (blue), PYY (red) and INSL5 (green) cells in proximal, middle, and distal large intestine as extracted from images as shown in (D). Data from 3 mice (indicated by different symbols), with 10 ROIs per region per mouse. Analysis by non-parametric one-way ANOVA with post hoc Dunns multiple comparison (compared to proximal density) for each of the hormones. ***p < 0.001.
Figure 4: Selective stimulation of distal colonic/rectal L-cells. (A) Violin plots of log2 normalized unique molecular identifiers (nUMIs) counts in the seven colonic EEC clusters for Angiotensin-II receptor 1a (Agtr1a), Arginine-vasopressin receptor 1b (Avpr1b) and Free-fatty-acid receptor 1 (Ffar1). (B) Expression of Agtr1a and Avpr1b in Venus-labeled L-cells and non-fluorescent cells from the same sorts isolated from the duodenum, ileum, and colon from GLU-Venus mice; data from bulk RNAseq by Roberts and collaborators [3], shown as Log2 (normalized reads +1) using DESeq2 normalization, data for L-cells are shown in dark red and for negative cells in light red. (C–H) Secretion of different gut peptides as indicated in response to a FFAR1- (AM1638, 1 μM), Agtr1a- (AngII, 10 nM), and Avpr1b- (AVP, 10 nM) agonists or no stimuli or IBMX 100 μM with 10 mM glucose (IG). Colonic primary cultures from the first, middle and final third of mouse large intestine were processed separately and stimulated in parallel. Peptides were quantified by LC-MS/MS and normalized to the protein content of the crypt culture. * indicates a statistical difference between the condition and the basal (control condition) tested by a Tukey’s test if a two-way ANOVA showed different populations. (I–L) Plasma GLP-1 (I, J) and PYY (K, L) levels 15 min after i.p. vehicle, AngII (1 mg/kg) (I, K) or AVP (100 ng/mouse) (J, L) application. Data were analyzed by Student’s paired t-test for GLP-1 as mice were their own controls (cross over design) and by Student’s unpaired t-test for PYY as measurements were performed in independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001.
receptor repertoire [42], is processed by DPP4 expressed in endothelial cells [43]. Ecm-cells by contrast, exhibited a strong longitudinal gradient of Piezo2, a mechanosensitive channel previously implicated in small intestinal Ecm-cell stretch sensitivity [44]. It is currently unclear why the most distally located Ecm-cells would need a higher expression of these channels, especially as Ecm-cells seem to be redundant for the initiation of colonic peristalsis and pellet propulsion [45–47].

EC-cell and L-cell clusters exhibited differential expression of a number of GPCRs. Olfr78, Olfr558, and Flar2 were more highly expressed in Tac1-positive than Sct-positive Ecm cells, suggesting these receptors are predominantly located deeper in the crypts rather than the surface epithelium. As these receptors are believed to sense microbiota-generated short chain fatty acids [48–50], this raises an interesting question of whether microbiota residing within colonic crypts are physiologically more important for providing signals to EECs than those resident in the lumen. Confirming a previous report examining the expression profile of small intestinal and colonic Ecm cells [50], we also found that GPCRs classically involved in detecting nutrient ingestion in small intestinal L-cells, including Flar1, Gpr119, and Gpbar1, were expressed in large intestinal L-cell clusters but absent from corresponding Ecm clusters.

L-cells also exhibited differential GPCR expression along the proximo-distal axis, most notably Agrp1a and Avpr1b, which were more highly expressed in clusters localized to the distal large intestine. These receptor expression profiles were utilized to examine whether targeted activation of distal L-cells would be sufficient to elevate plasma GLP-1 and PYY levels in vivo. Further analysis of receptor expression in the small intestine, however, revealed that whereas Agrp1a was indeed restricted to large intestinal L-cells, Avpr1b was also found in small intestinal L-cells. In vivo, we showed that AVP and AngII triggered hormone release from rectal but not proximal colonic primary cultures. In vivo, we were restricted to available immunoassays, as we are currently unable to detect gut hormones at endogenous levels in mouse plasma by LC-MS. Both AVP and AngII elevated plasma GIP-1 levels approximately 2-fold, whereas plasma PYY was elevated by AVP but not AngII. We noted, however, that total PYY levels were relatively high in these plasma samples, perhaps because the immuno-assay employed polyclonal antibodies that would also detect common PYY degradation products [51,52], making it more difficult to pick up small increments in PYY secretion from plasma measurements. We speculate that AVP was the stronger PYY stimulant because it targeted some small intestinal L-cells in addition to those in the rectum. The response to AngII injection in mice indicates that targeted stimulation of L-cells in the distal colon and rectum is sufficient to elevate plasma GLP-1 levels in vivo. Interestingly, these distal L-cells characterized expressed Ins15 alongside Scg and Ppy. We previously described INS15 to have orexigenic properties [24], contrasting with the anorexigenic actions of co-released PYY and GLP-1 (Figure 4) [25]. Whilst the elevated GLP-1 levels following distal L-cell stimulation are likely sufficient to exert a stimulatory effect on insulin secretion, further studies will be required to establish the net effect on food intake of targeting this distal L-cell population.

CONTRIBUTION STATEMENT

LJB, PL, JL, DAG, and RGK performed experiments, collected and analyzed data. BL and GSHY were involved in initial scRNAseq data analysis, subsequently refined by LJB and PL. AL and JL provided NeuroD1-Cre mice. PL, FMG, and FR wrote the manuscript and all authors contributed to the final version. FMG and FR guarantee the work.

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CONFLICT OF INTEREST

FMG consults for Kailyope and the FMG/FR laboratories receive funding from AZ, Lilly and LGC for other, unrelated, research projects.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.09.001.

REFERENCES

[1] Gribble, F.M., Reimann, F., 2016. Enteroendocrine cells: chemosensors in the intestinal epithelium. Annual Review of Physiology 78(1):277–299. https://doi.org/10.1146/annurev-physiol-021115-105439.

[2] Psichas, A., Reimann, F., Gribble, F.M., 2015. Gut chemosensing mechanisms. Journal of Clinical Investigation 125(3):908–917. https://doi.org/10.1172/JCI76309.

[3] Roberts, G.P., Larraufie, P., Richards, P., Kay, R.G., Galvin, S.G., Miedzybrodzka, E.L., et al., 2019. Comparison of human and murine enteroendocrine cells by transcriptomic and peptidomic profiling. Diabetes 68(5):1062–1072. https://doi.org/10.2337/db18-0883.

[4] Fothergill, L.J., Furness, J.B., 2018. Diversity of enteroendocrine cells investigated at cellular and subcellular levels: the need for a new classification scheme. Histochemistry and Cell Biology 150(6):693–702. https://doi.org/10.1007/s00418-018-1746-x.

[5] Glass, L.L., Calero-Nieto, F.J., Jawaid, W., Larraufie, P., Kay, R.G., Gittgens, B., et al., 2017. Single-cell RNA-sequencing reveals a distinct population of proglucagon-expressing cells specific to the mouse upper small intestine. Molecular Metabolism 6(10):1296–1303. https://doi.org/10.1016/j.molmet.2017.07.014.

[6] Haber, A.L., Biton, M., Rogel, N., Herbst, R.H., Shekhtar, K., Smilie, C., et al., 2017. A single-cell survey of the small intestinal epithelium. Nature 551(7680):333–339. https://doi.org/10.1038/nature24489.

[7] Beumer, J., Artegiani, B., Post, Y., Reimann, F., Gribble, F., Nguyen, T.N., et al., 2018. Enteroendocrine cells switch hormone expression along the crypt-to-villus BMP signalling gradient. Nature Cell Biology 20(8):909–916. https://doi.org/10.1038/s41556-018-0143-y.

[8] Habib, A.M., Richards, P., Caimes, L.S., Rogers, G.J., Bannon, C.A.M., Parker, H.E., et al., 2012. Overlap of endocrine hormone expression in the mouse intestine revealed by transcriptional profiling and flow cytometry. Endocrinology 153(7):3054–3065. https://doi.org/10.1210/en.2011-2170.

[9] Egerod, K.L., Engelstoft, M.S., Gründdal, K.V., Nøhr, M.K., Secher, A., Sakata, I., et al., 2012. A major lineage of enteroendocrine cells coexpressed CCK, secretin, GI, GLP-1, PYY, and neurotensin but not somatostatin. Endocrinology 153(12):5782–5795. https://doi.org/10.1210/en.2012-1595.

[10] Gründdal, K.V., Ræther, C.F., Svendsen, B., Sommer, F., Engelstoft, M.S., Madsen, A.N., et al., 2016. Neurotensin is Co-expressed, Co-released and acts
together with GIP-1 and PYY in enteroendocrine control of metabolism. Endocrinology 157(1):176–194. https://doi.org/10.1210/en.2015-1600.
[11] Gunawardene, A.R., Corfe, B.M., Staton, C.A., 2011. Classification and functions of enteroendocrine cells of the lower gastrointestinal tract. International Journal of Experimental Pathology 92(4):219–231. https://doi.org/10.1111/j.1365-2982.2011.00767.x.
[12] Terry, N., Margulis, K.G., 2016. Serotonergic mechanisms regulating the GI tract: experimental evidence and therapeutic relevance. Handbook of experimental pharmacology, vol. 239. NIH Public Access. p. 319–42.
[13] Manocha, M., Khan, W.I., 2012. Serotonin and GI disorders: an update on clinical and experimental studies. Clinical and Translational Gastroenterology 3(4):e13. https://doi.org/10.1038/ctg.2012.8.
[14] Hare, K.J., Vilsbøll, T., Asmar, C.F., Knop, F.K., Holst, J.J., 2010. The somatostatin neuroendocrine system: physiology and mental pharmacology, vol. 239. NIH Public Access. p. 319–42.
[15] Holst, J.J., Christensen, M., Lund, A., de Heer, J., Svendsen, B., Kielgast, U., et al., 2018. Co-storage and release of insulin-like peptide-5, glucagon-like peptide-1 and peptide YY from human and murine colonic enteroendocrine cells. Molecular Metabolism 16:65–75. https://doi.org/10.1016/j.molmet.2018.07.011.
[16] López-Ferreras, L., Richard, J.E., Noble, E.E., Eerola, K., Anderberg, R.H., Koda, S., Date, Y., Murakami, N., Shimbara, T., Hanada, T., Toshinai, K., et al., 2018. Identification of enteroendocrine regulators by real-time single-cell differentiation mapping. Cell. https://doi.org/10.1016/j.cell.2018.12.029.
[17] Thompson, E.M., Price, Y.E., Wright, N.A., 1990. Kinetics of enteroendocrine cells with implications for their origin: a study of the cholecystokinin and gastrin subpopulations combining tritiated thymidine labelling with immunocytochemistry in the mouse. Gut 31(4):406–411. https://doi.org/10.1136/gut.31.4.406.
[18] Cheng, H., Leblond, C.P., 1974. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine III. Entero-endocrine cells. American Journal of Anatomy 141(4):503–519. https://doi.org/10.1002/aja.101410405.
[19] Gehart, H., van Es, J.H., Hamer, K., Beumer, J., Kettritzschm, K., Dekkers, J.F., et al., 2019. Identification of enteroendocrine regulators by real-time single-cell differentiation mapping. Cell. https://doi.org/10.1016/j.cell.2018.12.029.
[20] Psichas, A., Tolhurst, G., Brighton, C.A., Gribble, F.M., Reimann, F., 2017. Liquid chromatography/mass spectrometry based detection and semi-quantitative analysis of INSIL in human and murine tissues. Rapid Communications in Mass Spectrometry 31(23):1963–1973. https://doi.org/10.1002/rcm.7978.
[21] Casper, J., Zweig, A.S., Villarreal, C., Tyner, C., Speir, M.L., Rosenbloom, K.R., et al., 2018. The UCSC Genome Browser database: 2018 update. Nucleic Acids Research 46(01):D762–D769. https://doi.org/10.1093/nar/gkx1020.
[22] Kay, R.G., Galvin, S., Larrauife, P., Reimmann, F., Gribble, F.M., 2017. Liquid chromatography/mass spectrometry based detection and semi-quantitative analysis of INSL5 in human and murine tissues. Rapid Communications in Mass Spectrometry 31(23):1963–1973. https://doi.org/10.1002/rcm.7978.
[23] Kay, R.G., Challis, B.G., Casey, R.T., Roberts, G.P., Meek, C.L., Reimmann, F., et al., 2018. Peptidomic analysis of endogenous plasma peptides from patients with pancreatic enteroendocrine tumours. Rapid Communications in Mass Spectrometry 32(16):1414–1424. https://doi.org/10.1002/rcm.8183.
[24] Winton, D.J., Ponder, B.A.J., 1990. Stem-cell organization in mouse small intestine. Proceedings of the Royal Society of London Series B Biological Sciences 241(1300):13–18. https://doi.org/10.1098/rspb.1990.0059.
[25] Psichas, A., Tolhurst, G., Mitchell, C.A., Gribble, F.M., Reimmann, F., 2017. Mixed primary cultures of murine small intestine intended for the study of gut hormone secretion and live cell imaging of enteroendocrine cells. Journal of Visualized Experiments (122). https://doi.org/10.3791/55687.
[26] Kosinski, C., Li, V.S.W., Chan, A.S.Y., Zhang, J., Ho, C., Tsui, W.Y., et al., 2007. Gene expression patterns of human colonic top organs and basal crypts and BMP antagonists as intestinal stem cell niche factors. Proceedings of the National Academy of Sciences of the United States of America 104(39): 15418–15423. https://doi.org/10.1073/pnas.0702710104.
[27] Sakiyama, J., Yokouchi, Y., Kuroiwa, A., 2001. HoxA and HoxB cluster genes divide the digestive tract into morphological domains during chick development. Development. Mechanisms of Development 101(1–3):233–236. https://doi.org/10.1016/S0898-9686(00)00564-5.
[28] Zaccetti, G., Duboule, D., Zakany, J., 2007. Hox gene function in vertebrate gut morphogenesis: the case of the caecum. Development (Cambridge, England) 134(22):3967–3973. https://doi.org/10.1242/dev.010591.
[29] Adriaensens, A., Lamm,B.Y.H., Billing, L., Skaffington, K., Sewing, S., Reimmann, F., et al., 2015. A transcriptome-led exploration of molecular mechanisms regulating somatostatin-producing D-cells in the gastric
epithelium. Endocrinology 156(11):3924–3936. https://doi.org/10.1210/en.2015-1301.

[41] Adriaenssens, A.E., Svendsen, B., Lam, B.Y.H., Yeo, G.S.H., Holst, J.J., Reimann, F., et al., 2016. Transcriptomic profiling of pancreatic alpha, beta and delta cell populations identifies delta cells as a principal target for ghrelin in mouse islets. Diabetologia 59(10):2156–2165. https://doi.org/10.1007/s00125-016-4032-1.

[42] Cox, H.M., 2007. Neuropeptide Y receptors; antisecretory control of intestinal epithelial function. Autonomic Neuroscience 133(1):76–85. https://doi.org/10.1016/j.autneu.2006.10.005.

[43] Mulvihill, E.E., Varin, E.M., Gladanac, B., Campbell, J.E., Ussher, J.R., Baggio, L.L., et al., 2017. Cellular sites and mechanisms linking reduction of dipeptidyl peptidase-4 activity to control of incretin hormone action and glucose homeostasis. Cell Metabolism 25(1):152–165. https://doi.org/10.1016/j.cmet.2016.10.007.

[44] Wang, F., Knutson, K., Alcaino, C., Linden, D.R., Gibbons, S.J., Kashyap, P., et al., 2017. Mechanosensitive ion channel Piezo2 is important for enterochromaffin cell response to mechanical forces. The Journal of Physiology 595(1):79–91. https://doi.org/10.1113/jphysiol.2016.272216.

[45] Spencer, N.J., Nicholas, S.J., Robinson, L., Kyoh, M., Flack, N., Brookes, S.J., et al., 2011. Mechanisms underlying distension-evoked peristalsis in Guinea pig distal colon: is there a role for enterochromaffin cells? American Journal of Physiology - Gastrointestinal and Liver Physiology 301(3):G519–G527 https://doi.org/10.1152/ajpgi.00101.2011.

[46] Keating, D.J., Spencer, N.J., 2019. What is the role of endogenous gut serotonin in the control of gastrointestinal motility? Pharmacological Research 140:50–55 https://doi.org/10.1016/j.phrs.2018.06.017.

[47] Heredia, D.J., Gershon, M.D., Koh, S.D., Corrigan, R.D., Okamoto, T., Smith, T.K., 2013. Important role of mucosal serotonin in colonic propulsion and peristaltic reflexes: in vitro analyses in mice lacking tryptophan hydroxylase 1. The Journal of Physiology 591(23):5939–5957. https://doi.org/10.1113/jphysiol.2013.256230.

[48] Pluznick, J.L., Protzko, R.J., Gevorgyan, H., Peterlin, Z., Sipos, A., Han, J., et al., 2013. Olfactory receptor responding to gut microbiota-derived signals plays a role in renin secretion and blood pressure regulation. Proceedings of the National Academy of Sciences 110(11):4410–4415. https://doi.org/10.1073/pnas.1215927110.

[49] Bellono, N.W., Bayer, J.R., Leitch, D.B., Castro, J., Zhang, C., O’Donnell, T.A., et al., 2017. Enterochromaffin cells are gut chemosensors that couple to sensory neural pathways. Cell 170(1):185–198. https://doi.org/10.1016/j.cell.2017.05.016.

[50] Lund, M.L., Egerod, K.L., Engelstroft, M.S., Dmytriyeva, O., Theodorsson, E., Patel, B.A., et al., 2018. Enterochromaffin 5-HT cells – a major target for GLP-1 and gut microbial metabolites. Molecular Metabolism 11:70–83. https://doi.org/10.1016/j.molmet.2018.03.004.

[51] Töräng, S., Bojesen-Møller, K.N., Svane, M.S., Hartmann, B., Rosenkilde, M.M., Madsbad, S., et al., 2016. In vivo and in vitro degradation of peptide YY 3–36 to inactive peptide YY 3–34 in humans. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 310(9):R866–R874. https://doi.org/10.1152/ajpregu.00594.2015.

[52] Töräng, S., Veedfeld, S., Rosenkilde, M.M., Hartmann, B., Holst, J.J., 2015. The anorexic hormone Peptide YY 3-36 is rapidly metabolized to inactive Peptide YY 3-34 in vivo. Physiological Reports 3(7):e12455. https://doi.org/10.14814/phy2.12455.