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A novel ex vivo experimental setup to investigate how food components stimulate the enteroendocrine secretions of different intestinal segments

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A novel *ex vivo* experimental setup to assay the vectorial transepithelial enteroendocrine secretions of different intestinal segments

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Abstract

The enteroendocrine system coordinates gastrointestinal (GI) tract functionality and the whole organism. However, the scarcity of enteroendocrine cells and their scattered distribution make them difficult to study. Here, we glued segments of the GI wall of pigs to a silicon tube, keeping the apical and the basolateral sides separate. The fact that there was less than 1% of 70-kDa fluorescein isothiocyanate (FITC)-dextran on the basolateral side proved that the gluing was efficient. Since the lactate dehydrogenase leakage at basolateral side was lower than 0.1% (1.40 ± 0.17 nKatals)it proved that the tissue was viable. The intestinal barrier function was maintained as it is in segments mounted in Ussing chambers (the amount of Lucifer Yellow crossing it, was similar between them; respectively % LY: 0.48 ±0.13; 0.52 ± 0.09; $p > 0.05$). Finally, apical treatments with two different extract produced differential basolateral enterohormone secretions (basolateral PYY secretion vs control; animal extract: 0.35 ± 0.16; plant extract: 2.5 ± 0.74; $p<0.05$). In conclusion, we report an *ex vivo* system called “Ap-to-Bas” for assaying vectorial transepithelial processes that makes it possible to work with several samples at the same time. It is an optimal device for enterohormone studies in the intestine.

Keywords: enteroendocrine system, gut hormone secretion, *ex vivo* model, transepithelial activity, food bioactives.

Introduction

As sources of energy and the building blocks of essential constituents, food components play a key role in building and renewing the body. Also, through chemical and mechanical signalling in the gastrointestinal (GI) tract, they provide essential information for the homeostasis of the whole body. The enteroendocrine system is one of the largest endocrine organs in the body. It collects information at the entrance of the organism about what is being taken in and secretes signalling molecules in response. This information is sent to central control systems and used to coordinate homeostatic systems (for example, body energetics). The role, function and mechanisms of the enteroendocrine system are only partially understood [1], [2]. One of the reasons for this lack of understanding is its distribution: it is the sum of lots of cells scattered throughout the intestine, with a low abundance of each type of enteroendocrine cell[3].

The most physiological and integrative approach to studying the enteroendocrine system works with the whole animal. Some authors administer the substances to be tested to specific areas of the GI and then obtain samples of blood from specific draining blood vessels and/or cut nerve communication with the central nervous system [4],[5]. This approach requires a huge number of animals, which is problematical from an ethical point of view, and researchers who are highly skilled in surgical procedures. A different approach is to work in vitro with enteroendocrine cell lines. These are useful for highly controlled mechanistic studies, but the physiological response is sometimes quite different from the in vivo response. The most common enteroendocrine cell lines are STC-1 [6] and Glutag [7], which mimic L-cells. Additionally, ghrelin can be studied with attached MNG-3 (derived from mice gastric ghrelinoma [8]) and the unattached (SG-1 or PG-1[9]) cell lines. One of the reasons for the different responses from animals and cell lines may be the lack of vector flux in the treatments. Most studies were carried out in cells attached to the surface in a 2D situation, quite different from the polarised epithelial position in vivo. To overcome these culture limitations, 3D strategies such as gut-on-a-chip [10] have evolved to mimic intestinal fragments, although there are no reports on how effective they are in enteroendocrine studies.

Ex-vivo approaches, such as everted sacs, perfused intestinal loops, Ussing chambers, intestinal punches, precision-cut intestinal slices (PCIS) and organoids [11], are in between the previous options, as they use natural intact tissue structures in different controlled approaches [12]. Ussing chambers are widely used to address the need for vectorial processes [13], [14]. They locate the mucosal epithelium in an apical to basolateral position in a hermetic situation with concomitant control of barrier properties. The main drawback is that it is difficult to have enough samples to minimise variability, largely because each device only has a few chambers and is also usually very expensive [15] ,[16] [17]. To overcome these limitations some authors work with *ex vivo* tissue fragments from animal intestines [18],[19]. These crude explants from animal intestines make it possible to produce numerous replicates, depending on the animal's size. Although the treatment reaches all the exposed areas of the tissue, it does not mimic the effect of the apical stimulation that takes place in the in vivo gastrointestinal tract.

We have developed a setup called Ap-to-Bas (AtB). It is an *ex vivo* system that combines the tissue that is readily available in the intestine of pigs, an animal with a metabolism that is similar to that of a human being [20], together with the vectoriality provided by a system that mimics a Ussing chambers approach. Our setup could be a useful tool to screen agents that modulate enteroendocrine secretions throughout the apical and/or basolateral epithelial intestinal areas.

Materials & Methods

Chemicals

Most of the chemicals used – formaldehyde, ethanol, xylol, dimethyl benzene, paraffin, D-mannitol, D-glucose, HEPES, $CaCl_2$, $MgCl_2$, KCl, NaCl, $NaHCO_3$, NaH_2PO_4, 70-kDa fluorescein isothiocyanate (FITC)-dextran, IBMX (I7018) – were purchased from Sigma-Aldrich (Madrid, Spain).. The tissue adhesive used on the animals was 3M Vetbond (Cat 1469SB, St. Paul, USA). Lucifer Yellow (LY-452 Da) was from BTIU 80016, Merck, (Darmstadt, Germany). The lactate dehydrogenase (LDH) kit was obtained from QCA (Amposta, Spain).

The ELISA kits for total GLP-1 (GLP1-T) (Cat. # EZGLPT1-36k), active GLP-1 (GLP1-A) (Cat. # EGLP-35K) and acyl-ghrelin (cat. # EZRGRA-90K) were purchased from Millipore (Billerica, MA, USA). We obtained Elisa kits for CCK (Cat. No: EKE-069-04) and PYY (Cat. No: FEK-059-03) from Phoenix Pharmaceuticals (Burlingame, CA, USA).

Collection of the tissue

Intestinal tissues were obtained from female pigs (*Sus scrofa domesticus, LANDRACE X LARGEWHITE*) that were killed for meat production at a local slaughterhouse. Forty-eight pigs were used in the study, all from the same farm. For each assay the number of replicates has been indicated as “n”. Pigs were commercial breeds (18% protein; 5.7% lipid; 4.9% fibre; 6.7% ashes; 1.03% Lys; 0.3% Met; 0.78% Calcium; 0.73 Phosphorus; 0.20% sodium, Coperal, Santa Coloma de Queralt, Spain) that weighed approximately 120 kg at slaughter and had been fasted for approximately 24 h prior to slaughter. Just 5 min after slaughter, the intestines were excised, and segments of various anatomical regions were stored in ice-cold oxygenated (95% O2,5%CO2) KRB buffer (Hepes 11.5 mM, $CaCl_2$ 2.6 mM, $MgCl_2$ 1.2 mM, KCl 5.5 mM, NaCl 138 mM, $NaHCO_3$ 4.2 mM, NaH_2PO_4 1.2 mM) (Sigma-Aldarich, Madrid, Spain) with D-Manitol 10 mM (Sigma-Aldarich, Madrid, Spain). Duodenum (10 cm of intestine taken from the pylorus), distal Ileum (10 cm of intestine taken from the ileocaecal junction) and proximal colon (10 cm of intestine taken downstream of the ileocaecal junction) were collected for the experiments.

Tissues were transported in KRB buffer to the laboratory at 4 ºC and immediately used for *ex vivo* experiments. The time between excision and the beginning of the experiments was approximately 30 min.

In the laboratory, the intestine was rinsed with cold KRB buffer (with D-Manitol 10 mM) and mounted in a plastic tube to facilitate the removal of the outer muscle layers. Then, the intestinal tube was cut open longitudinally, and the mucosal tissue was placed apical side up. Circles of tissue with a diameter of 14 mm (approximately 1.54 cm^2) were punched out using a biopsy punch (Figure 1a). Twelve circles were taken from each segment from each animal. The intestinal segments were randomized, per region, in a beaker glass. The entire process took around 20 minutes, and the whole time the sample was kept at a low temperature with cold buffer and an ice-cold bath.

Building the Ap-to-Bas (AtB) system

We cut a silicon tube with an internal diameter of 8 mm and an external diameter of 12 mm into pieces 1.5 cm long with a perfectly flat surface. Tissue adhesive for animal use (3M Vetbond, Cat 1469SB, St. Paul, USA) was lightly applied to the flat side of the tube, which was then gently pressed onto the apical side of the intestinal segment [21]. After 10 seconds, the intestine was placed inside a cell culture insert with no bottom membrane (Cat MCRP12H48, 12-well hanging inserts) (Figure 1b). The entire insert containing the tissue segment and the piece of tube was placed in one of the wells of a 12-well plate prefilled with 1 ml of KRB buffer (with D-Glucose 10 mM). Apically, the tube was filled with 400 µl of KRB buffer (with D-Mannitol 10 mM). The tissues were then pre-incubated at 37 °C for 15 min in a humidified incubator (5% (v/v) CO_2) (Figure 1c).

A 70-kDa fluorescein isothiocyanate (FITC)-dextran was used (Sigma Aldrich, St. Louis, MO, USA) to assess the efficiency of the gluing process. FITC-70 kDa was added apically (0.10 mg/mL), and after 60 minutes of incubation, the apical and the basolateral media were collected, centrifuged to precipitate the debris and stored at -20 ºC for further analysis. The amount of fluorescent dye that crossed to the basolateral side was measured using a Perkin-Elmer LS- 30 fluorimeter (Beaconsfield, UK) at λexc 430 nm; λem 540 nm.

Ussing chamber methodology

Intestinal segments of 0.5 cm^2 were mounted in Ussing chambers apparatus (DIPL.-ING. K. MUSSLER-SCIENTIFIC INSTRUMENTS, Aachen, Germany). Up to 6 segments from each animal were used. Mucosal compartments were filled with 1.5 ml KRB buffer (with D-Mannitol 10 mM) and the serosal compartments were filled with KRB buffer (with D-Glucose 10 mM) [14]. The chambers were kept at 37 °C and continuously oxygenated, 95% O_2 /5% CO_2, with a circular gas flow. Before the experiments were started, the tissues were equilibrated for 15 min in the chambers to achieve steady-state conditions in transepithelial potential differences.

The transmucosal potential difference was continuously monitored under open circuit conditions and recorded using 0.8 mm Ag/AgCl Glas-Electrodes. Ohm's law was used to calculate the basal transepithelial electrical resistance (TEER) from the voltage deflections induced by bipolar constant current pulses of 50 mA (every 60 s) with a duration of 200 ms and applied through platinum wires (Mussler Scientific Instruments, Aachen, Germany).

After the 20-minute equilibration period, the mucosal side of the biopsies was subject to treatment.

Paracellular transport (Lucifer Yellow assay)

To evaluate the integrity of the intestinal barrier in AtB and Ussing chambers, a solution of Lucifer Yellow (LY-452 Da, BTIU 80016, Merck, Darmstadt, Germany) was used [22]. In this study, 0.4 ml of LY 100 μM was added to the apical side and after 30, 60 and/or 90 minutes of treatment, the apical and the basolateral media were collected, centrifuged to precipitate the debris and stored at -20 ºC for further analysis. The amount of fluorescent dye that crossed to the basolateral side was measured using a Perkin-Elmer LS-30 fluorimeter (Beaconsfield, UK) at λexc 430 nm; λem 540 nm.

Viability test

Tissue viability was checked by measuring Lactate Dehydrogenase (LDH) with an LDH kit (QCA, Amposta, Spain). Tissues were homogenized in ice-cold KRB buffer with a Tissue Lyser (Qiagen, Hilden, Germany) for 2 min at 50 oscillations/0.5 seg. After centrifugation, supernatant LDH was measured. Cell culture was centrifuged to eliminate debris, and the supernatant was used for the LDH Assay. The amount of LDH activity found in the culture media was considered to indicate the health of the tissue sample throughout the incubation period. The percentage of LDH leakage vs total LDH was used as a viability test [23][16].

Study of the enteroendocrine function

To test the enteroendocrine function, we measured the basolateral presence of enterohormes in basal (unstimulated) or apically stimulated conditions. IBMX (I7018, Sigma-Aldarich, Madrid, Spain) 20 μmol/L was used as positive control [24], and natural extracts of animal and plant origin were used to test the differential ability to stimulate enterohormone secretion in the AtB system. Animal protein homogenate was obtained from pork meat and diluted to 10 mg protein/mL in KRB with D-glucose and protease inhibitors. Vegetal Grape Seed Proanthocyanidin extract (GSPE) was diluted to 100 mg/mL in the same buffer. Treatments were initiated by replacing the apical KRB buffer solution with 400 µL of pre-warmed KRB buffer (37 °C) [24] containing the test compounds. KRB buffer with D-glucose was used as a control. After 30 minutes of the treatment an aliquot of 200 µl was picked from the basolateral side of the AP-to-Bas system. Finally, 60 minutes (for ileum and colon) or 90 minutes (for duodenum) after the beginning of the experiment, the whole of the apical and basolateral sides was frozen and stored at −80 °C for further analysis of total and active GLP-1, PYY, CCK and acyl-ghrelin.

The enterohormones were assayed using commercial ELISA kits for total GLP-1 (GLP1-T) (Cat. # EZGLPT1-36k), active GLP-1 (GLP1-A) (Cat. # EGLP-35K), acyl-ghrelin (cat. # EZRGRA-90K) (Millipore, Billerica, MA, USA), CCK (Cat. No: EKE-069-04) and PYY (Cat. No: FEK-059-03) (Phoenix Pharmaceuticals, Burlingame, CA, USA) following the manufacturer's instructions.

Histology

Intestinal segments of the duodenum, ileum and colon samples were fixed in 4% diluted formaldehyde. After 24 hours of fixation, successive dehydration (Alcohol/Ethanol 70%, 96% and 100%; plus xylol/Dimethyl benzene) and paraffin infiltration-immersion took place at 52ºC (Citadel 2000. Thermo Scientific). Then, sections 2 µm thick (Microm HM 355S. Thermo Scientific) were obtained, deposited on slides (JP Selecta Paraffin Bath) and subjected to automated haematoxylin-eosin staining (Varistain Gemini. Shandom. Thermo) [25].

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Student's T-test was used to compare the treatments with the control. The one-way ANOVA test was used for multiple comparisons. P-values < 0.05 were considered to be statistically significant. The calculations were performed using XL-Stat 2017 software.

Results

Structure, viability and barrier properties of the intestinal fragments in the AtB

Our setup, called Ap-to-Bas (AtB), enables vectorial transepithelial processes prepared from different areas of the intestinal tract *ex vivo* to be assayed. As **Figure 2** shows, the intestinal mucosa and submucosa were easily separated from the muscularis.

The viability of the mucosa and submucosa was checked by measuring the amount of lactate dehydrogenase released to the basolateral side of the AtB setup. **Table 1** shows the amount of LDH activity of two representative segments (ileum and colon). At the beginning of incubation, it was similar for both tissues. After 30 minutes there was a significant increase in the amount of LDH, after which time it increased steadily. In both tissues, the ileum showed higher values of LDH basolaterally. However, the percentage of LDH in the basolateral side versus the total LDH (tissue plus basolateral) was lower than 0.1%. We also compared the LDH leakage of ileum samples mounted in the AtB to that of *ex vivo* free cultured ileum samples (of similar size). We found no differences (nKatals: 1.40 ± 0.17 (basolateral AtB); 1.38 ± 0.29 (free)).

For our study it is essential for us to be able to work on the apical-to-basolateral effect so, for this reason, the barrier function must be preserved. We initially used FITC-70 kDa to discount inadequate adhesion between biological tissue and the tube surface in the AtB. **Figure 3a** shows that the amount of FITC-70 was approximately 0.1% in the ileum samples and 0.5% in the colon samples, which suggests optimal adhesion. Afterwards, we checked the barrier properties and compared them to those found when a chambers system was used. Transepithelial electrical resistance (TEER) measurements of various intestinal segments, with similar characteristics and assay conditions, but mounted in an Ussing chamber apparatus provides information on the integrity of the epithelia and their tightness. **Figure 3b** shows that the TEER varies between the intestinal segments and that it decreased slightly only in duodenal segments after a 60-minute incubation. Since TEER cannot be measured in the AtB device, we measured the paracellular transport of Lucifer Yellow from apical to basolateral compartments. **Figure 3c** shows that the amount of Lucifer yellow crossing the ileum mucosa and submucosa is approximately 0.5% in both devices which had equal surface areas (Ussing chamber: 0.5 cm^2 and AtB: 0.5024 cm^2). The quantity of Lucifer Yellow in the duodenum mounted in AtB was 0.21% ± 0.06, and in the ascendant colon it was 1.62% ± 0.98.

Enteroendocrine function

The relative abundance of enteroendocrine cells in the various intestinal segments depends on the species [26]. Here we show the differential basolateral secretion pattern obtained in response to different apical stimulatory signals. **Figure 4a** shows that non-stimulated secretion of PYY is higher in the duodenum than in the distal ileum. Moreover, **figure 4b** shows that the distal ileum produces more GLP1 than the proximal colon. Since several enteroendocrine cells are located in the epithelium of the intestinal barrier, with the apical side in contact with the gastrointestinal duct, and the basolateral side draining the internal body fluids, apical stimulation by some agents should produce basolateral secretion of enterohormones (see **figure 4c).** Apically applied IBMX produces a statistically significant stimulation of basolateral secretion of active GLP1 at the ileum and only a slight stimulation in colonic segments.

To determine whether our setup could be used to screen enteroendocrine secretagogues, we subjected our AtB setup to two treatments with potential bioactivity for stimulating enterohormone secretion. **Figure 5a** shows that an animal extract increased CCK and active ghrelin secretion at the duodenum and inhibited PYY secretion. The same extract did not lead to any change in the secretion of active GLP-1 in the ileum or in the colon. A plant extract, which has been proved to be a satiating agent in rats [27], produced a different profile of secretions. It increased colonic active GLP1 secretion statistically. It showed a tendency to increase CCK and PYY in the duodenum and had no effect on active GLP1 secretion in the ileum segment (**Figure 5b)**.

Discussion

The study of enteroendocrine processes requires a sufficient amount of tissue to host enough endocrine cells to enable hormone secretions to be measured. These processes also need to be studied in different intestinal segments, since the enteroendocrine cell populations are distributed differently throughout the gastrointestinal tract [28]. Another key point is to preserve apical to basolateral separation, which is usually found in vivo. What is more, many signals cannot cross the intestinal barrier [24]. Ussing chambers are the gold standard procedure for this purpose, but they are expensive and the number of chambers is limited [14].

Our Ap-to-Bas setup is an ex vivo system derived from the pig's intestinal wall, which enables vectorial transepithelial processes to be assayed. This system has three main advantages over the gold standard Ussing chambers method. It makes it possible to work with more samples at the same time while maintaining transepithelial activity affordably; it makes it possible to work with an animal model that is more similar to human beings [20], and the size of the mucosal sample guarantees that the enterohormones will be detected.

Ex vivo systems have limitations, such as the short-time viability of the tissue. We have shown that when healthy intestines are mounted in the AtB system there is enough time for the changes in enterohormone secretions to be measured. Westerhout and col showed that LDH leakage of the intracellular enzyme into the basolateral media from pig jejunal tissue segments mounted in their setup was 3.5 ± 0.8% [29]. The percentage of leakage we found was lower than this, and the amount of LDH in the basolateral media increased as it did in free cultured equivalent segments, which suggests that the tissue was not damaged any further by being mounted in the AtB setup.

Our system maintained the vectoriality required for processes that occur across a wall. As we were working with glued surfaces, we discounted any problems in the adhesion of the tissue by apically applying fluorescein (FITC)-labelled dextran (70 kDa), an agent that is unable to cross the intestinal barrier [30] and is typically used to measure gastrointestinal transit [31]. The absence of fluorescence from the basolateral side of the AtB setup showed that the apical side had been optically insulated from the basolateral side. Pierre et al. [21] also used this same approach to develop an ex vivo intestinal segment culture (EVISC) model for studying the ex vivo effects of parenteral nutrition on the susceptibility of the ileum to invasion by extra-intestinal pathogenic Escherichia coli (ExPEC).

Evidence of the quality of the intestinal barrier was provided by various complementary approaches. Lucifer Yellow unidirectional permeable paracellular marker [13] showed that ileum segments were similarly permeable regardless of whether they were mounted in AtB or the Ussing chamber. And the permeability of the ileum and colon was similar. This similarity was also shown by Rozenhal working with Ussing chambers and an area of exposure that was quite similar to our own (0.46 cm^2) [13]. The percentage of LY leakage was also in the range that corresponded to an intact intestinal barrier (0.5%) according to Westerhout, who was working with porcine jejunal tissue and paracellular marker fluorescein isothiocyanate–dextran (FD4: MW 4 kDa) [29]. In fact, our values were slightly higher than those obtained by Westerhout et al, but our compound was smaller (LY: MW 0.54 kDa) than theirs. They also worked with different intestinal segments. Lennernäs [32] showed that MW correlated closely with the permeability coefficients of hydrophilic drugs and that high permeability drugs (BCS class I−II) showed a slightly higher permeability in the colon than the jejunum and ileum when passive diffusion is the dominant transport mechanism.

To reinforce the integrity of the intestinal barrier and its standard state we compared the TEER measures of various intestinal fragments. As we were unable to measure TEER in our AtB setup, we worked with the same samples in the Ussing chamber apparatus, which we have also used to measure human colonic samples for other unpublished studies (Ω* cm^2: 39.5 ± 2.2). Although the TEER measurements were highly dependent on the assay condition and the best approach was to compare them in the same study, the range of units obtained did not significantly differ from those obtained by Westerhout et al. [29], who found a TEER of 58 ± 7 Ω cm^2, which remained stable for 120 min when they used their device to work with porcine jejunal tissue. Also working with Ussing chambers, Gleeson et al. [33] obtained a TEER of 37 ± 9 Ω cm^2 (n=40) in jejunal mucosae, which was within the acceptable range [30]. Jejunal TEER gradually decreased over 120 min to 70–80% of the initial value. The lowest TEER values we found were in colon segments, the result of different barrier properties between intestinal segments. Permeability to small molecules and electrolytes was lower in the duodenum, higher in the ileum and highly increased in the ascendant colon. Hamilton et al. [34] and Moyano et al. [35] have shown that permeability to FITC KD4 (and also various hydrophilic drugs [32]) follows a similar pattern in rat samples..

Our main reason for designing this setup was to be able to test the effects of compounds on enterohormone secretion in a situation that more closely resembles the physiological situation (i. e. several molecules in the gastrointestinal tube can only stimulate enterohormone secretion by interaction with the apical side of these cells). Very few studies have used ex-vivo approaches to determine vectorial enterohormone secretion [14],[24]. Most studies use *ex-vivo* incubation of the intestine segment with treatment in a multiwell plate [36], [37]. Pig intestine makes it possible to obtain samples from various intestinal sections in sizes that are big enough to produce a concentration of hormones secreted on the basolateral side that can be measured by standard ELISA kits. Holst et al. described the enteroendocrine hormone abundance of the various intestinal segments in different animal species [26]. The basolateral secretion of PYY in duodenum and GLP1 in ileum was higher in our study than in their description. We should point out that we are working with basolateral secretions, although most of the available data has been published on the amount of hormone in each intestinal segment, not secreted on the basolateral side [26,38,39]. Ripken and Col studied the GLP1 and PYY released by pig intestinal sections, but they did not compare them [16]. Similarly, Agersnap and col [40] assayed the relative presence of CCK throughout the small intestine, and showed that it was more abundant in the first 20 centimetres after the pyloric sphincter, and Vitari and col [41] proved the presence of ghrelin-producing cells in the duodenum of pigs. When we assayed an extract rich in protein, we found a stimulation in CCK. Similarly, Sufian et al. compared this effect between protein-derived extracts from different animals [42]. And, in fact, protein is a very well defined secretagogue for CCK [43], [44]. We found that this protein-rich extract had the specific effect of reducing PYY and stimulating acyl-ghrelin production, although analysing this effect is beyond the scope of this manuscript.

To determine the possible physiological effects of this screening tool , we checked the profile produced by an extract (GSPE [27]), which has been shown to have satiating properties. The main components of this extract are flavanols and phenolic acids. GSPE significantly increased GLP1 secretion, as previously shown *in vivo* [27] and *ex-vivo*, by intestine tissue culture [45]. PYY, which significantly increased in our previous *ex-vivo* approach [45], tended to increase too. In contrast, our different systems gave different results for CCK. There may be several reasons for these differences: for example, different responses between rat and pig, or the method for stimulating cells (apically in the AtB vs around all the cells in an *ex-vivo* system).

In conclusion, our AtB setup is a tool for screening new agents that can act apically on enteroendocrine cells in a physiological approach. This tool could be useful for identifying new agents that can have an effect on the gut-brain axis.

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Author Contributions Statement

I.G., K. G-C. and P. R. have run all the laboratory tasks. M.P., A.A., X.T. and M.B. designed the experiments and collected tissues. Ll. A. provided the tested samples. All the authors discussed the results. I.G., M.P. and A.A. wrote the manuscript, which was checked and discussed by all authors.

Additional Information.

Competing financial interests

The investigators have no conflict of interest relating to this study.

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Figure captions.

Figure 1. Representative pictures describing the building of the AtB

a) After the outer muscle layers had been removed, the intestinal tube was cut open longitudinally. Circles of tissue with a diameter of 14 mm (approximately 1.54 cm^2) were punched out using a biopsy punch; b), the intestine was placed in a cell culture insert with no bottom membrane; c) Finally, the whole insert was placed in a well of a 12-well plate prefilled with 1 ml of KRB buffer (with D-Glucose 10 mM). Apically, the tube was filled with 400 µl of KRB buffer (with D-Mannitol 10 mM).

Figure 2 Haematoxilin-eosin staining of transversal thin sections from a pig's A) duodenum, B) ileum and C) colon mucosa (original magnification, ×6). The images show mucosa and submucosa of each section of intestine. The scale bar indicates 0.2 mm.

Figure 3a. Percentage of FITC dextran 70 kDa in the basolateral side in ileum and colon AtB. FICT 70 kDa was added to the apical side of the AtB setup and, after 60 minutes of incubation at 37 ºC, the percentage of FITC on the basolateral side was measured. Values are the mean± SEM.

Figure 3b. Transepithelial electrical resistance (TEER) of different intestinal segments during the incubation period.

Barrier integrity measured as transepithelial electrical resistance (TEER) in $\Omega^* cm^2$ at the start of incubation (black columns) and after 60 minutes of incubation at 37 ºC (white columns). Tissues were mounted in Ussing chambers and were incubated at 37 ºC for 60 minutes. Values are means ± SEM. *$P < 0.05$ when the incubation start time of each tissue is compared with 60 minutes (T-Student). One-way anova $P < 0.05$ was used to compare differences between the start time of each tissue; differences, obtained by T3-Dunnett post-hoc test, were defined by different letters.

Figure 3c. Percentage of Lucifer Yellow (LY) crossing the ileum wall on the basolateral sides of Ussing chambers and AtB.

LY was added to the apical side of both approaches and, after 60 minutes of incubation at 37 ºC, the percentage of LY on the basolateral side was measured. Values are the mean ± SEM.

Figure 4a: Basolaterally secreted PYY under unstimulated conditions at different anatomical locations.

Different Ap-to-Bas setups were mounted for each intestinal porcine duodenum (black column) and ileum (grey column) (n=8 for each section). After 60 minutes in the C-buffer, basolateral media were collected and hormone levels of peptide YY (PYY) were measured. Values are percentage ± SEM. * $p < 0.05$ vs duodenum.

Figure 4b: Ileum and colon relative basal secretion into basolateral media of GLP1.

Different Ap-to-Bas setups were mounted for each intestinal porcine ileum (black columns) and colon (squared columns) (n=5 for each section). After 60 minutes in the C-buffer, basolateral media were collected and hormone levels of total and active GLP1, were measured. Values are percentage ± SEM. * $p < 0.05$ vs ileum.

Figure 4c: Sensitivity of ileum and colon segments to apical IBMX stimulation of active-GLP-1 secretion

Different Ap-to-Bas setups were mounted for each intestinal porcine Ileum and colon (n=5 for each section). IBMX (20 μM) was apically applied (white columns). Black columns refer to unstimulated controls. At the end of the treatment (60 minutes), basolateral media were collected and active GLP1 was measured. Values are percentage ± SEM. * $p < 0.05$ compared to negative (vehicle treated) control (C-)

Figure 5a. Basolateral enteroendocrine secretions after apical stimulation with homogenates of animal origin in AtB setups

Different Ap-to-Bas setups were mounted for each intestinal porcine duodenum (black columns), ileum (grey column) and colon (striped column) (n=8 for each section). Animal extracts (10 mg protein/ mL) were apically applied. At the end of the treatment (duodenum: 90 min; others: 60 min) basolateral media were collected and hormone levels of cholecystokinin (CCK), peptide YY (PYY), active ghrelin and active glucagon(-like) peptide 1 (GLP-1) were measured. Values are percentage ± SEM. * $p < 0.05$ compared to negative (vehicle treated) control (C-)

Figure 5b. Basolateral enteroendocrine secretions after apical stimulation with plant extract in AtB setups.

Different Ap-to-Bas setups were mounted on each intestinal porcine duodenum (black columns), ileum (grey column) and colon (striped column) (n=8 replicates). Plant extracts (100 mg extract/ mL) were applied apically. At the end of the treatment, basolateral media were removed and hormone levels of cholecystokinin (CCK), peptide YY (PYY) and active glucagon such as peptide 1 (GLP-1) were measured. Values are percentage ± SEM. * $p < 0.05$, # $p<0.1$ compared to the negative control (C-).

Table 1. LDH leakage on the basolateral side of the AtB throughout the incubation

	0 minutes		30 minutes		60 minutes	
	nKatal	SEM	**nKatal**	SEM	**nKatal**	SEM
Ileum	**0.46** ^{A}	0.04	**1.40** ^{B}	0.17	**1.92** ^{B}	0.18
Colon	**0.31** ^{A}	0.05	**0.74** ^{B}	0.07	**0.99** ^{B}	0.13

LDH in the basolateral media was measured at different times. Values are the mean ± SEM. Statistical diferences were calculated using one-way ANOVA followed by a T3-Dunnett post-hoc. Different superscripts mean statitstical differences between times. A p value < 0.05 was considered to be statistically significant.

Figure 1a:

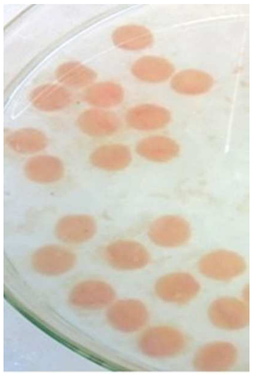
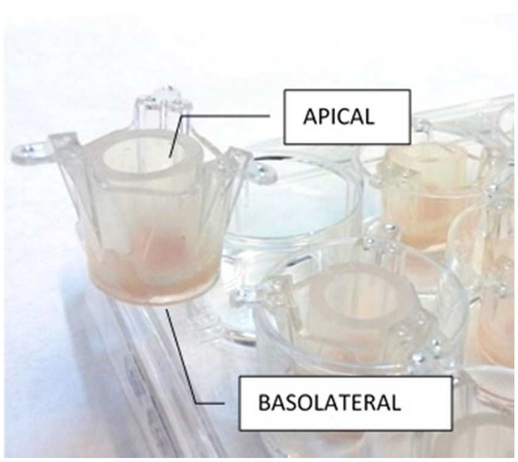


Figure 1b:

Figure 1c:



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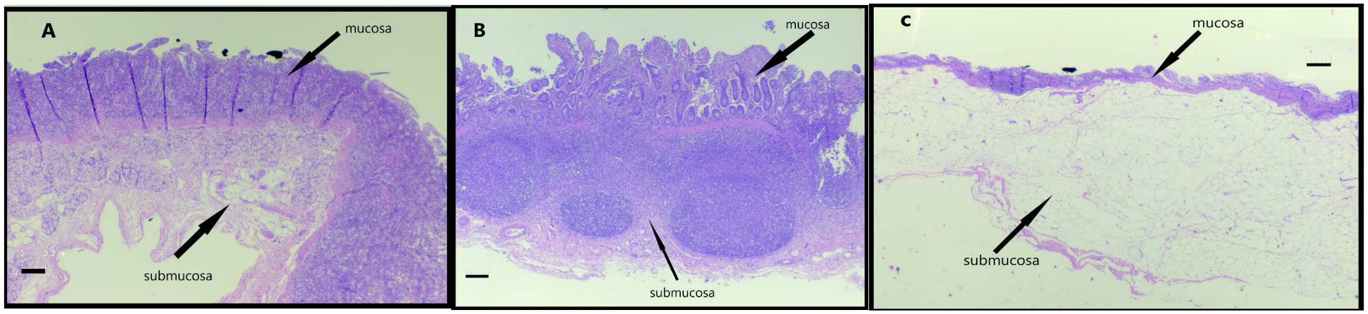
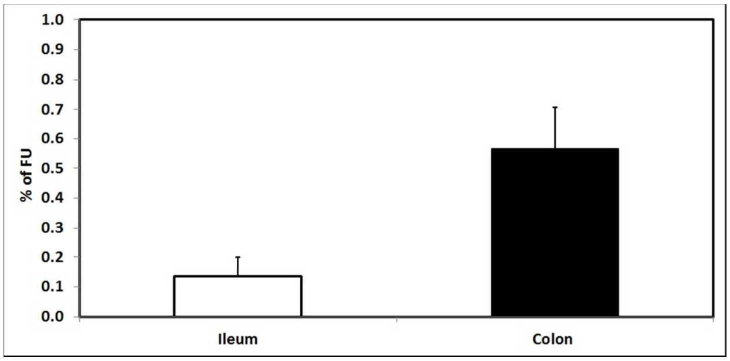


Figure 3a:



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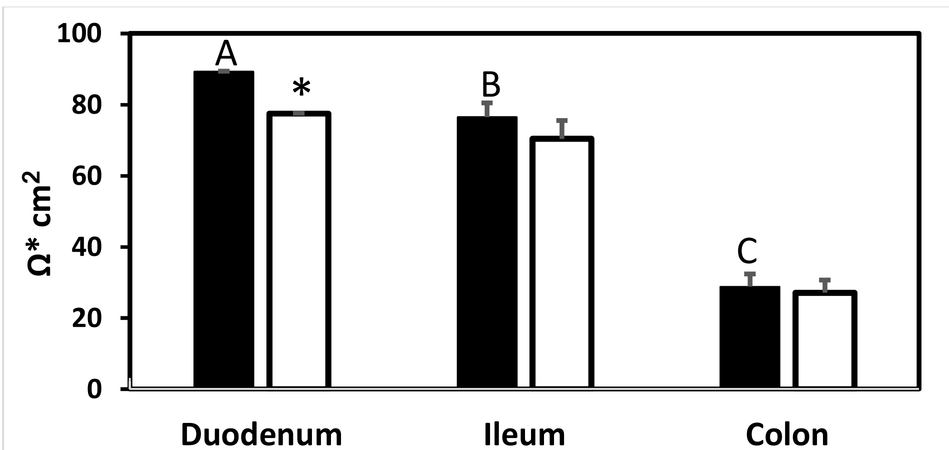

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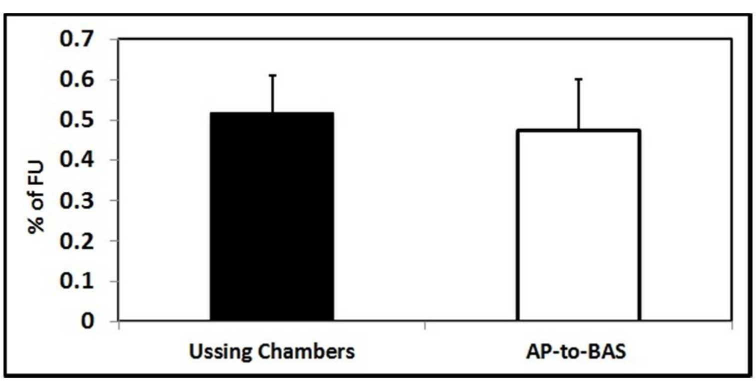
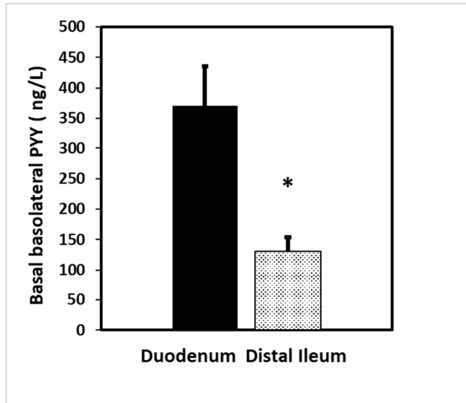


Figure 4a:



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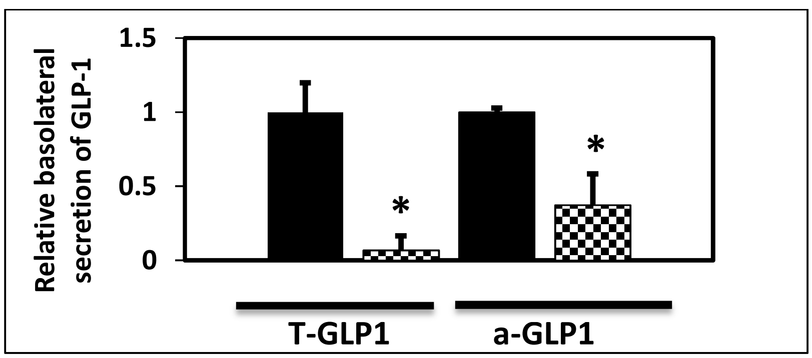

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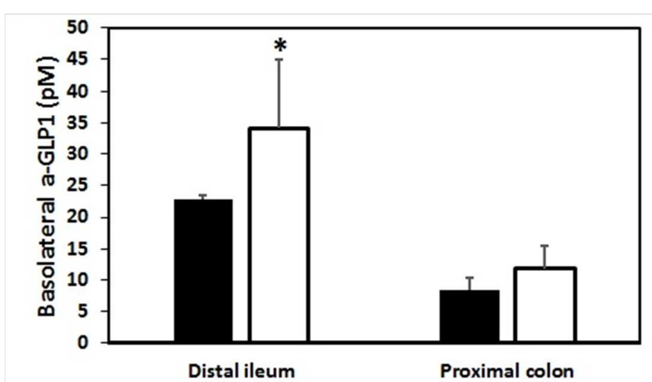

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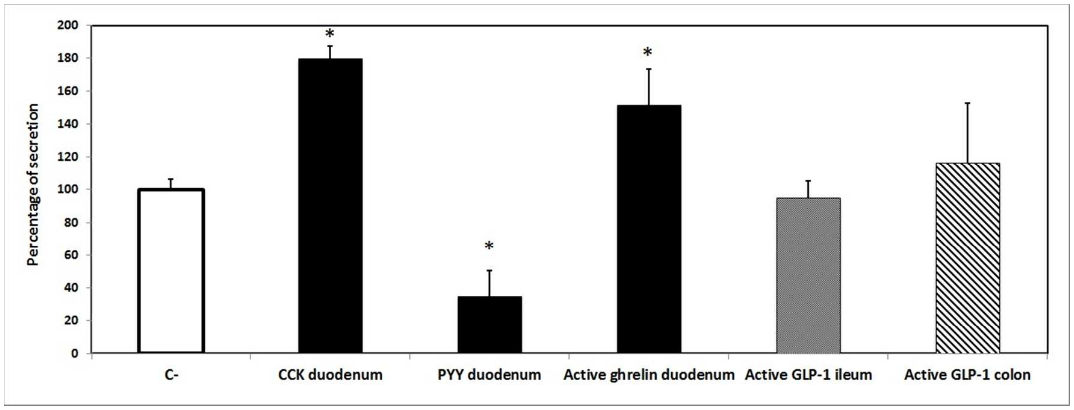

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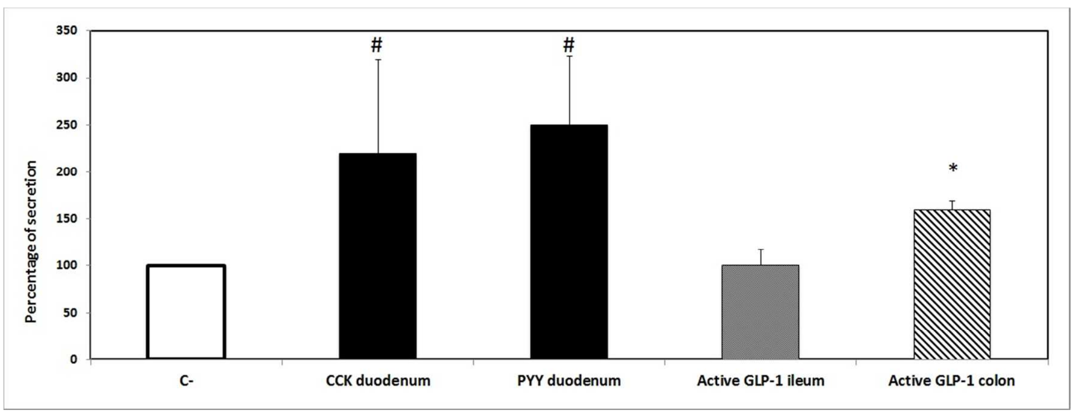
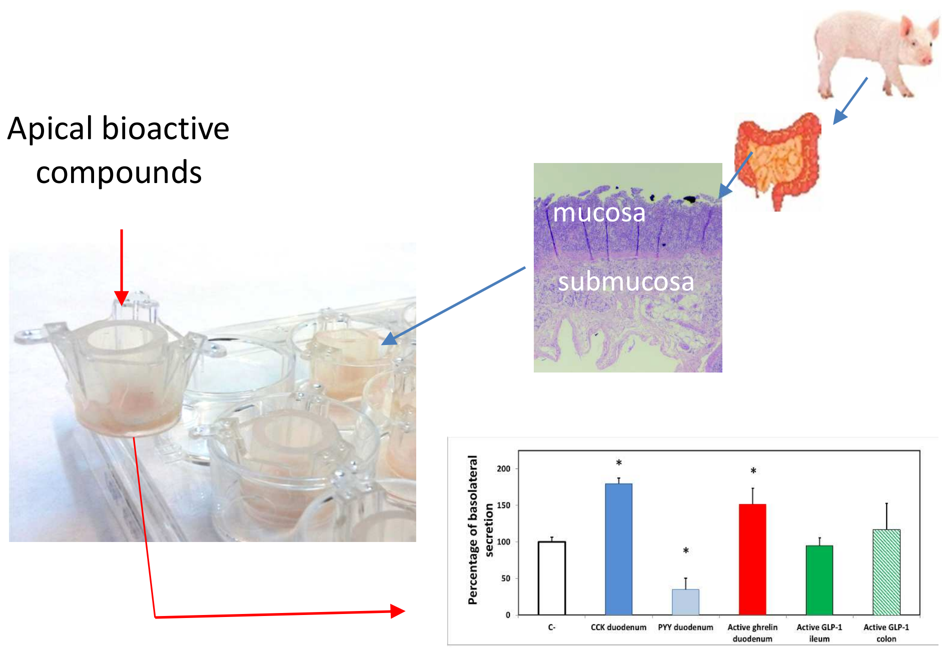
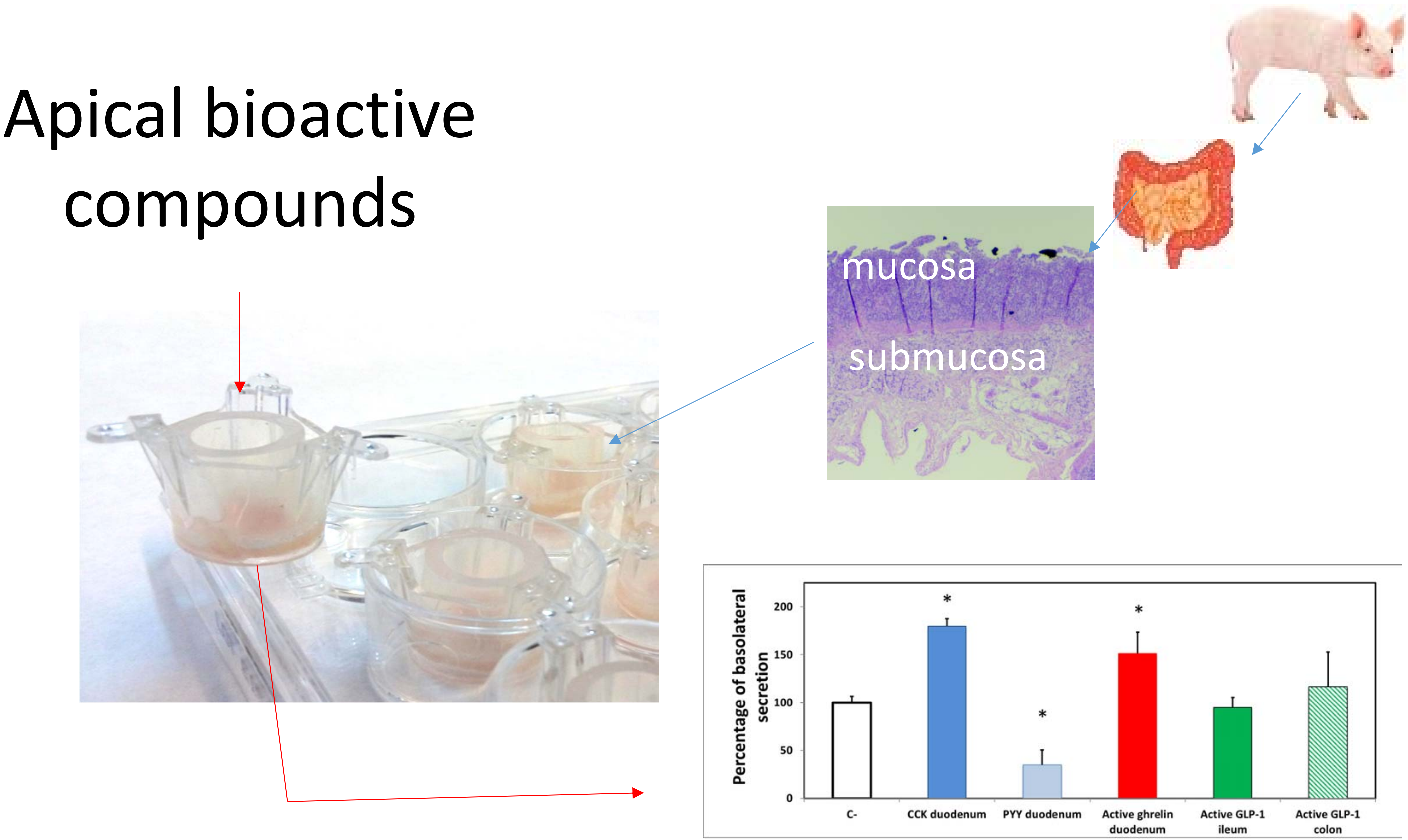


TOC


Apical bioactive compounds
mucosa
submucosa
Percentage of basolateral secretion
200
150
100
50
0
C-
CCK duodenum
PYY duodenum
Active ghrelin duodenum
Active GLP-1 ileum
Active GLP-1 colon


Apical bioactive compounds
mucosa
submucosa
Percentage of basolateral secretion
200
150
100
50
0
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C-
CCK duodenum
PYY duodenum
Active ghrelin duodenum
Active GLP-1 ileum
Active GLP-1 colon