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The endogenous preproglucagon system is not essential for gut growth homeostasis in mice

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Abstract

Objective: The prevalence of obesity and related co-morbidities is reaching pandemic proportions. Today, the most effective obesity treatments are glucagon-like peptide 1 (GLP-1) analogs and bariatric surgery. Interestingly, both intervention paradigms have been associated with adaptive growth responses in the gut; however, intestinotrophic mechanisms associated with or secondary to medical or surgical obesity therapies are poorly understood. Therefore, the objective of this study was to assess the local basal endogenous and pharmacological intestinotrophic effects of glucagon-like peptides and bariatric surgery in mice.

Methods: We used *in situ* hybridization to provide a detailed and comparative anatomical map of the local distribution of GLP-1 receptor (*Glp1r),* GLP-2 receptor (*Glp2r*), and preproglucagon (*Gcg*) mRNA expression throughout the mouse gastrointestinal tract. Gut development in GLP-1R-, GLP-2R-, or GCG-deficient mice was compared to their corresponding wild-type controls, and intestinotrophic effects of GLP-1 and GLP-2 analogs were assessed in wild-type mice. Lastly, gut volume was determined in a mouse model of vertical sleeve gastrectomy (VSG).

Results: Comparison of *Glp1r*, *Glp2r*, and *Gcg* mRNA expression indicated a widespread, but distinct, distribution of these three transcripts throughout all compartments of the mouse gastrointestinal tract. While mice null for *Glp1r* or *Gcg* showed normal intestinal morphology, *Glp2r*$^{-/-}$ mice exhibited a slight reduction in small intestinal mucosa volume. Pharmacological treatment with GLP-1 and GLP-2 analogs significantly increased gut volume. In contrast, VSG surgery had no effect on intestinal morphology.

Conclusion: The present study indicates that the endogenous preproglucagon system, exemplified by the entire Gcg gene and the receptors for GLP-1 and GLP-2, does not play a major role in normal gut development in the mouse. Furthermore, elevation in local intestinal and circulating levels of GLP-1 and GLP-2 achieved after VSG has limited impact on intestinal

morphometry. Hence, although exogenous treatment with GLP-1 and GLP-2 analogs enhances gut growth, the contributions of endogenously-secreted GLP-1 and GLP-2 to gut growth may be more modest and highly context-dependent.

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1. Introduction

Obesity and type-2 diabetes (T2D) represent increasing health and socio-economic problems worldwide [1]. The currently most effective pharmacological treatments include peptides stimulating GLP-1 receptor (GLP-1R) function [2,3], while dual agonists for the GLP-1R and other mechanisms targeting anorexigenic receptors are in development [4]. Furthermore, bariatric surgery has become increasingly attractive providing a significant, rapid, and sustainable weight loss with several positive effects on related morbidities, including resolution of T2D. Although Roux-en-Y gastric bypass (RYGB) has historically been the standard bariatric surgery method, less invasive procedures, *e.g.* vertical sleeve gastrectomy (VSG), have comparable beneficial metabolic outcomes with reduced perioperative complications, which explains why VSG is now the fastest-growing weight loss surgery option for the treatment of obesity [5]. The underlying molecular mechanisms leading to these marked metabolic effects are not fully elucidated, but several lines of evidence support an important role for nutrient-stimulated gut hormones, such as GLP-1 and GLP-2 [6–8].

High circulating levels of GLP-1 and GLP-2 have been linked to development of gut hypertrophy following RYGB [9–12]. The rise in GLP-1 and GLP-2 could provide a positive feedforward mechanism rendering the enlarged intestine more predisposed towards glucose disposal [13,14] and the release of a plethora of gut hormones with additional metabolic implications. GLP-1 and GLP-2 are co-secreted from enteroendocrine L cells in the gut and released into the circulation following enzymatic cleavage of the common propeptide proglucagon [15] see reviews [16,17]. While GLP-1 is mainly known for its metabolic effects; i.e. the increase in pancreatic glucose-dependent insulin secretion (the incretin effect), regulation of glucose flux, inhibition of gastric emptying, and reduction of appetite [18,19], GLP-2 is primarily known for its direct actions on the gut. Accordingly, peripheral administration of GLP-2 exerts potent intestinotrophic effects by increasing mesenteric blood flow and stimulating epithelial proliferation and may constitute the molecular link between nutritional status and commensurate adaptation of mucosal absorptive surface area [9,20–23]. Furthermore, GLP-2 reduces gastric emptying and gastric secretion and exhibits anti-inflammatory properties in the intestinal mucosa [24–28]. A growth-regulating role of GLP-1 has also been reported [29], demonstrating that activity of GLP-1R controls mucosal expansion in both the small and large intestine. However, even though the actions of these peptides are well described, the anatomical distribution and functional implications of the endogenous proglucagon system for normal gut growth and development remains poorly understood.

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Traditionally, the ileum and colon have been identified as the primary sites containing the majority of the proglucagon expressing L-cell populations in rat, pig, dog, primate, and man [30–34]. This general assumption focused greater attention on studies of proglucagon-related biology in the distal gut, with limited analysis of the full gastrointestinal tract. The GLP-1R is known to be expressed in several tissues, including brain, gastrointestinal tract, pancreatic islets, kidney, heart, and lung [35–38]. However, cellular localization of GLP-1R expression is confounded by the lack of validated, specific antibodies [39–41]. Similarly, GLP-2 receptor (GLP-2R) expression has been reported previously in gastrointestinal tract, mesenteric lymph nodes, fat, spleen, bladder, and hepatocytes, as well as in the central nervous system [24,42–45]. However, the exact local distribution of intestinal GLP-2Rs is disputed and thus remains unresolved [44–48].

Given the current limitations in our understanding of the localization of GLP-1R and GLP-2R expression in the gut, we aimed to provide a detailed map of *Glp1r*, *Glp2r*, and *Gcg* mRNA expression throughout the complete rostral-caudal axis of the mouse gastrointestinal tract. To gain further insight into the functional relevance of the endogenous GLP-1 and GLP-2 system on intestinal growth, we characterized intestinal volumes in *Glp1r*$^{-/-}$, *Glp2r*$^{-/-}$, and *Gcg*$^{-/-}$ mice in comparison to corresponding wild-type littermate controls. Furthermore, since bariatric surgery represents a valuable tool for studying the role of these peptide hormones in intestinal adaptation, we performed a detailed study of intestinal volume in a mouse model of VSG surgery.

2. Materials and methods

2.1 Animals

All animal experiments were approved by the Danish Committee for Animal Research under the personal license of Jacob Jelsing (2015-15-0201-00518) using internationally accepted principles for the use of laboratory animals. All animals were housed in a light-, temperature-, and humidity-controlled room (12-hour light:12-hour dark cycle, lights on/off at 4AM/4PM hour; 22 ± 1℃; 50 ± 10% relative humidity) and offered domestic quality tap water. Mice bred in Toronto were cared for in accordance with animal protocols approved by the Animal Care Committee, Toronto Centre for Phenogenomics, Mt. Sinai Hospital.

2.2 Compounds

The GLP-1 analog liraglutide was acquired commercially (Hørsholm Pharmacy). Native GLP-1, native GLP-2, and the GLP-2 analog teduglutide were prepared by automated solid-phase peptide synthesis (SPPS) using the Fmoc/tBu strategy on pre-loaded PHB TentaGel resin (Rapp polymere GmbH, Tuebingen, Germany). The couplings were performed using Fmoc-Nα-protected amino acids, N,N-diisopropylcarbodiimide and ethyl cyanoglyoxylate-2-oxime (oxyma) in N,N-dimethylformamide (Iris Biotech GmbH, Marktredwitz, Germany) for 2 x 2 h. The N-deprotections were performed using 40% piperidine in N-methyl-2-pyrrolidione (Iris Biotech GmbH, Marktredwitz, Germany) for 3 min followed by 20% piperidine in N-methyl-2-pyrrolidione for 17 min. Finally, the peptide was simultaneously side-chain deprotected and released from the solid support by a TFA cocktail containing trifluoro acetic acid (TFA) (Iris Biotech GmbH, Marktredwitz, Germany), triethylsilane (Sigma–Aldrich, Brøndby, Denmark), and H_2O (95/2.5/2.5) as scavengers for 2 h. The peptide was precipitated by the addition of diethylether (Sigma–Aldrich, Brøndby, Denmark). The peptide was purified by RP-HPLC and identified by LC-MS. The final products were obtained with >95% purity.

2.3 Sub-chronic treatment in C57/Bl6J mice

C57BL/6J mice (Janvier Labs, Saint Berthevin, Cedex, France), 8 weeks of age, were fed a regular chow diet (Altromin 1324, Brogaarden A/S, Denmark). Mice were randomized according to body weight into four individual study groups (n=10 per group): Group 1: Vehicle (SC, BID), Group 2: liraglutide (0.2 mg/kg, SC, BID), Group 3: teduglutide (1 mg/kg, SC, BID), Group 4: liraglutide (0.2 mg/kg, SC, BID) + teduglutide (1 mg/kg, SC, BID). Compounds were dissolved in PBS buffer

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containing 3% mannitol and 0.6% L-His (pH 9.0), and dosing volume was 5 ml/kg. On day 8, animals were fasted for 4 hours before being sacrificed during the light phase. The intestines were collected, and the length of the small and large intestine was measured. Intestines were cleaned by flushing with saline and finally the weight was measured. Intestines were placed in 10% natural buffered formalin until further processing. For description of mice treated with native GLP-1 and GLP-2, see supplementary information.

2.4 Histology and stereology

The gut was dissected into small and large intestine, and the lengths were measured. The intestine was sampled using systematic uniform random sampling (SURS) principles, providing a minimum of 8 systematically placed biopsies from both small and large intestine. All biopsies were embedded in blocks of paraffin enabling later identification of individual biopsies. Paraffin blocks were sectioned into 5 μm thick sections and stained with hematoxylin-eosin for subsequent stereology-based volume estimations. Stereological volume estimations were performed by point-counting on digitally scanned slides using the newCAST system (Visiopharm, Denmark) [49–51]. For studies involving double KO ($Glp1r^{-/-}$:$Glp2r^{-/-}$) mice or mice treated with native GLP-1 and GLP-2 peptides, weights of saline-flushed intestines were used (see supplementary information).

2.5 In situ hybridization (ISH)

Single-cell ISH was performed on paraffin-embedded intestinal tissue biopsies from two C57/Bl6J mice using the RNAscope 2.5 HD - RED Assay (Advanced Cell Diagnostics) to visualize cellular mRNA using specific probes directed against selected genes. Slides with tissue biopsies were treated according to RNAscope 2.5 HD – RED Assay user manual. In brief, tissue sections were pretreated, including target retrieval, hydrogen peroxide treatment, and protease treatment. Then, the specific probe was hybridized to the mRNA target, and the signal was amplified and visualized using Fast Red substrate. A probe against bacterial *dapB* mRNA was used as negative control, whereas a mouse probe against *Ppib* was used as positive control. Custom-made specific probes against *Glp1r* (REF418851), *Glp2r* (REF447061), and *Gcg* (REF400601) mRNA were employed on sections covering the entire gastro-intestinal tract (glandular and non-glandular stomach, duodenum with Brunner's glands, caudal duodenum without Brunner's glands, jejunum, ileum, cecum, and colon, for overview see supplementary information). Following ISH, the slides were counterstained in Gill's hematoxylin and coverslipped. Finally, slides were scanned under a 20X objective in a ScanScope AT slide scanner (Aperio).

2.6 VSG surgery

C57BL/6J mice were made obese by provision of a high-fat diet at least 6 weeks before surgery. In the peri-surgery period (day -3 to 4), the mice in all groups were offered a liquid diet (Osmolite). Mice were randomized based on body weight on day -1 into the following experimental groups: VSG or sham. The VSG procedure was performed as previously described [52]. In brief, mice had ~80% of the stomach resected along the major curvature and the incision was closed with staples. In sham-operated mice the stomach was taken out of the abdominal cavity and then repositioned without being cut. Pain relief was provided by subcutaneous injections of Metacam (0.25mg/100g body weight) from day 0 until day 4 post-surgery. On day 10 post-surgery animals were terminated, and intestines were placed in formalin. For a description of RYGB procedures, see supplementary information.

2.7 Knockout (KO) mice

Glp1r^{-/-} and *Glp2r*^{-/-} mice and age-matched wild-type (WT) littermates were from the Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital (Toronto, Ontario, Canada). *Gcg*^{-/-} mice and age-matched WT littermates were from the University of Michigan (Ann Arbor, Michigan, US). Single KO mice were generated as described previously [53–55]. In addition, double KO (*Glp1r*^{-/-}:*Glp2r*^{-/-}) mice were generated in Toronto as described in supplementary information.

2.8 Statistics

All data were analyzed using Graph Pad Prism 5.0 software, applying either student's t-test (gut volumes in KO mice vs. WT littermates, and VSG vs. sham controls, respectively) or one-way analysis of variance (ANOVA) with Tukey's post-hoc test (treatment with GLP-1/GLP-2 analogs). Results are presented as mean ± SEM (standard error of the mean). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Glp1r mRNA expression in the mouse GI tract

Glp1r expression was virtually absent in the non-glandular stomach (Figure 1A) but highly expressed on gastric parietal cells in the stomach glands (Figure 1B). In the proximal duodenum, *Glp1r* mRNA transcripts were abundant in the Brunner's glands (Figure 1C) in addition to a low level of expression detected in few scattered cells of the mucosa. In the caudal duodenum, *Glp1r* expression became more apparent in the mucosa (Figure 1D) with *Glp1r*-positive cells increasing in density throughout the jejunum (Figure 1E) and in the ileum (Figure 1F). *Glp1r* expression was also observed in the nerve plexus throughout the glandular stomach, small intestine, and colon (Supplementary Figure 1A-D). In the cecum, *Glp1r* expression (Figure 1G) was restricted to the muscular nerve plexus in addition to sporadic expression in the mucosa, whereas in the colon (Figure 1H), *Glp1r* expression was found predominantly in the nerve plexus with only some expression in the mucosa.

3.2 Glp2r mRNA expression in the mouse GI tract

Glp2r mRNA transcripts were abundant in both the circular and longitudinal muscle layer of the non-glandular (Figure 2A) and less abundantly but still detectable in the glandular part of the stomach (Figure 2B). In contrast to *Glp1r*, *Glp2r* was not expressed in the nerve plexuses of the glandular stomach (Supplementary Figure 1E), nor in the Brunner's glands of duodenum (Figure 2C). Within duodenum, *Glp2r* was abundantly expressed in the lamina propria of the mucosa layer (Figure 2C), as well as in the circular and longitudinal muscle layers (Supplementary Figure 1F). A similar cellular expression pattern was observed in the caudal duodenum (Figure 2D) where *Glp2r* mRNA transcripts were localized to the nerve plexuses (Supplementary Figure 1G). In the jejunum (Figure 2E) and ileum (Figure 2F), *Glp2r* was found to be highly expressed in scattered cells within the mucosa and the nerve plexus, with sporadic expression in the muscle cells of the muscularis layer. In the cecum (Figure 2G) and colon (Figure 2H), *Glp2r* mRNA was expressed in both the mucosa and nerve plexus. In addition to the expression observed in the myenteric plexuses, *Glp2r* was also detected in submucosal plexuses of the colon (Supplementary Figure 1H).

3.3 Gcg mRNA expression in the mouse GI tract

Gcg expression was virtually absent in both the non-glandular stomach (Figure 3A) and the glandular stomach (Figure 3B) as well as in the most proximal part of the small intestine (Figure

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3C). A few scattered *Gcg*-positive cells were detected in the mucosal epithelium in the caudal duodenum (Figure 3D). In the jejunum (Figure 3E), *Gcg* expression was confined to single cells of the epithelium with an increased density caudally towards the ileum (Figure 3F) and throughout the colon (Figure 3H). Likewise, *Gcg* expression was found in the cecal mucosa (Figure 3G).

3.4 Analysis of intestinal volume in Glp1r$^{-/-}$, Glp2r$^{-/-}$ and Gcg$^{-/-}$ mice

Stereological estimates of compartmental gut volumes in *Glp1r$^{-/-}$*, *Glp2r$^{-/-}$*, and *Gcg$^{-/-}$* mice, as compared to WT littermate control mice, are indicated Figure 4. *Glp1r$^{-/-}$* mice had similar small intestine, colon, and total intestine volumes, compared to wild-type controls (small intestine, 555±41 mm^3 vs. 564±28 mm^3, p=0.86; colon, 133±7 mm^3 vs 136±7 mm^3, p=0.95; total intestine, 688±37 mm^3 vs 700±31 mm^3, p=0.81), see Figure 4A-C.

In contrast, *Glp2r$^{-/-}$* mice exhibited a slight, statistically significant, reduced volume of the mucosal layer of the small intestine (13±3.7%, 547±10 mm^3 vs 475±20 mm^3, p=0.01) and total intestine (12±3.6%, 640±12 mm^3 vs 564±22 mm^3, p=0.01), (Figure 4D). Total volumes of the small intestine (668±14 mm^3 vs 603±26 mm^3, p=0.06) and whole intestine (814±15 mm^3 vs. 746±30 mm^3, p= 0.09) in *Glp2r$^{-/-}$* mice trended lower, while the colon volume was unaltered (139±15 mm^3 vs. 143±6 mm^3, p=0.79, see Figure 4E). *Glp2r$^{-/-}$* mice exhibited a non-significant increase in volume of immune cells of the small intestine (4.3±0.8mm^3 vs. 9.2±2.1mm^3, p=0.06), see Supplementary Figure 2A-C).

Similar to *Glp1r$^{-/-}$* mice, *Gcg*-deficient mice also showed normal gut development, as compared to wild-type controls (Figure 4G-I) (small intestine, 662±23mm^3 vs. 671±26mm^3, p=0.79; colon, 282±32mm^3 vs. 267±24mm^3, p=0.71; total intestine, 944±43mm^3 vs. 938±44mm^3, p=0.92).

As single disruption of either the *Glp1r* or *Glp2r* alone might result in compensatory gut growth arising from enhanced activation of the remaining functional receptor, we generated double knock out (DKO) *Glp1r$^{-/-}$:Glp2r$^{-/-}$* mice. A very modest reduction of small intestine weight (14±2.1%, 1215±44mg vs. 1047±25mg, p=0.002), thus also being reflected in total intestine weight (1466±44mg vs. 1301±28mg, p=0.003; see Supplementary Figure 3A), was observed in *Glp1r$^{-/-}$:Glp2r$^{-/-}$* mice. Colon weight (251±10mg vs 254±9mg, p=0.80) and body weight (41±1.5g vs 38±1.2g, p=0.18) were not different in *Glp1r$^{-/-}$:Glp2r$^{-/-}$* mice (Supplementary Figure 3A-B).

3.5 GLP-1 and GLP-2 analogs increase gut volumes in C57Bl/6J mice

The lack of major changes in gut volumes in mice with disruption of the endogenous proglucagon system prompted us to reassess the pharmacological consequences of GLP-1R and GLP-2R agonism in the intestine. The long-acting GLP-1 analog (liraglutide) and GLP-2 analog (teduglutide) were administered individually or in combination to male C57Bl/6J mice for 7 days (Figure 5A-C). Liraglutide monotherapy induced a non-significant 20±4.3% increase in total small intestinal volume (p>0.05, one-way ANOVA). When analyzed by individual t-test, however, small intestine volume was increased significantly by liraglutide compared to vehicle treatment (p=0.003, students t-test). In contrast, teduglutide treatment led to a significant and robust 57±7.1% increase in small intestine volume (p=0.001, one-way ANOVA) while combined treatment with liraglutide and teduglutide led to a further increase (70±6.5%, p=0.001, one-way ANOVA), indicating a nearly additive effect of the drug combination treatment. Similar changes in intestinal volumes were detected in the colon (Figure 5B) but were most apparent in the small intestine, specifically in the mucosa layer (Figure 5A). The increase in small intestine, colon, and total intestine volume was also observed following 7-days treatment with the native GLP-1 and GLP-2 peptides (Supplementary Figure 4); however, the effect was less pronounced compared to treatment with stable analogs.

3.6 Intestinal growth after vertical sleeve gastrectomy

VSG led to a significant reduction (14±2.8%, p=0.002) in body weight as compared to sham-operated control mice (Supplementary Figure 5A). Similarly, a significant weight loss (7.3±0.9%, p=0.002) was observed after RYGB surgery in the rat (Supplementary Figure 5B). At the time of termination (10 days post-surgery), stereological assessment of the duodenum (first 5 cm of the small intestine), the total small intestine, colon, and total intestine did not reveal any volume alterations in any segments or layers investigated (duodenum: 290±31 mm^3 vs. 308±27 mm^3, p=0.67, total small intestine: 885±82 mm^3 vs. 879±44 mm^3, p=0.95, colon: 213±28 mm^3 vs. 235±8 mm^3, p=0.46; Figure 6A-D). In contrast, RYGB surgery led to a marked increase in alimentary limb area in the rat (Supplementary Figure 6A-D).

4. Discussion

By use of highly sensitive *in situ* hybridization techniques, we here report a widespread and distinct expression pattern of *Glp1r*, *Glp2r*, and *Gcg* mRNAs throughout the mouse gastrointestinal tract. As previously demonstrated in rodents, monkey, and man [39,56], we confirm that *Glp1r* mRNA is expressed in gastric parietal cells in mice, with a weak staining in smooth muscle cells and stomach mucosal structures. This distinct receptor expression corresponds well with GLP-1's inhibitory effects on gastric acid production and gastric emptying [18,19]. In addition, we confirm *Glp1r* to be highly expressed in Brunner's glands of the proximal duodenum [36,57], consistent with a role for GLP-1 in the regulation of intestinal mucin production [38]. *Glp1r* was also localized to nerve plexuses of the submucosa and muscularis along the full rostro-caudal extension of the intestinal tract [17,39,58]. As a novel finding, we demonstrate with a high cellular resolution *Glp1r* expression in scattered cells throughout the small intestinal mucosa. The specific phenotype of these cells is currently unknown but consistent with intraepithelial lymphocytes [59]; however, further studies are needed to confirm this assertion. Similar to *Glp1r* mRNA, *Glp2r* expression was observed in smooth muscle cells of the gastric muscular layer, supporting a role for the GLP-2R in inhibition of gastric emptying [24,60]. In contrast, *Glp2r* mRNA expression was not detected in Brunner's glands within the duodenum. *Glp2r* mRNA was, however, markedly expressed in the mucosal lamina propria throughout the intestinal tract, consistent with the distribution of subepithelial myofibroblasts [45,48]. No *Glp2r* expression was detected in the intestinal epithelium or in proliferating crypt cells. This is in line with several other reports [45,61] but contrasts with reports of GLP-2R immunoreactivity in the epithelium from rodents, pigs, and humans [44,46]. Similar to the distribution of *Glp1r*, *Glp2r* mRNA expression was abundantly expressed in nerve plexuses of the submucosa and muscularis, suggesting a potential role of these receptors in the enteric nervous system. In agreement with numerous reports [31,62,63], we also demonstrated conspicuous *Gcg* expression in the epithelium from the caudal duodenum and throughout the gut, with an increasing caudal density gradient reaching highest density in the colon [31,62]. The *Gcg* mRNA-positive cells were organized in a clear enteroendocrine pattern with minimal luminal contact intercalated between the epithelial cells of both villi and crypts. In contrast, no *Gcg* expression was observed in the muscularis or enteric nervous system.

To investigate the relevance of basal GLP-1R and GLP-2R signaling in intestinal growth, we used unbiased stereological techniques to estimate intestinal volumes in *Glp1r*$^{-/-}$, *Glp2r*$^{-/-}$, and *Gcg*$^{-/-}$ mice. Mice with GLP-1R or GCG deletion had similar intestinal volumes as compared to corresponding wild-type controls. This is in line with previous findings of normal weight and length of the small intestine in *Glp1r*$^{-/-}$ mice [29]. It should be noted, however, that loss of GLP-1R

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expression is associated with reduced intestinal polyp growth in genetic models of small bowel hyperplasia [29], possibly suggesting that intrinsic trophic effects of GLP-1R receptors may be more marked under conditions of pathological gut growth. In contrast, GLP-2R deletion resulted in a slight reduction in small intestinal mucosa volume, in agreement with previous reports of gut weights in *Glp2r*^{-/-} mice [54]. The DKO *Glp1r*^{-/-}*:Glp2r*^{-/-} mice also displayed only a slight reduction in small intestine weight, indicating no functionally overlapping compensation between the two receptors.

In comparison to the very limited effects of GLP-2R deletion on gut growth in mice, we demonstrate that exogenous administration of a long-acting GLP-2 analog (teduglutide) alone or in combination with a long-acting GLP-1 analog (liraglutide) leads to markedly increased gut mucosal volumes in mice. Although liraglutide monotherapy did not lead to a significant increase in gut volume in the present experiment (when analyzed by one-way ANOVA), others have reported GLP-1R dependent intestinotrophic effects of the GLP-1R agonist exendin-4 [29]. Similarly, the authors demonstrated that exogenous native GLP-1 and GLP-2 promoted additive hypertrophic effects in the gastrointestinal tract of WT mice. In line with this report, we also observed a significant increase in colon weight following 7 days of treatment with high doses of native GLP-1 (3mg/kg) (Supplementary Figure 4). The discrepancies in reported efficacies between experiments may be related to specific compound doses and quantification methods. Hence, while Koehler and coworkers [29] reported changes in gut mass as a function of body weight, we used stereology-based histological methods for quantification of total volumes.

In contrast to the less well described intestinal growth-promoting effects of GLP-1, the intestinotrophic effects of GLP-2 are well-established and thought to be mediated via different signaling mechanisms, which involve induction of growth factor signaling pathways, including those associated with IGF-1, FGF7, and ErbB [48,64,65], mediating crypt proliferation and decreased apoptosis [20,66]. Collectively, our data support a model for GLP-2 action via paracrine, or endocrine mechanisms to stimulate mucosal expansion, as we found no *Glp2r* expression on epithelial cells or crypt stem cells, putative targets for GLP-2 action. In contrast, strong *Glp2r* expression was detected in other cells types within mucosa and the enteric nervous system. This localization is consistent with the hypothesis that local release of growth factors together with enteric neuronal stimulation, is a more probable explanation for the intestinotrophic action of GLP-2 [67]. As our data indicate a very limited effect of *Gcg*, *Glp1r*, or *Glp2r* deletion in normal gut development, but pharmacological doses of GLP-1/GLP-2 induced intestinal mucosal expansion, these findings suggest that supraphysiological stimulation of GLP-1R/GLP-2R activity is required for evoking intestinal growth.

It has been demonstrated previously that RYGB surgery causes massive hypertrophy of the alimentary limb in both mice, rats, and pigs [68,69], and also leads to markedly increased circulating levels of GLP-1 and GLP-2 [9–12,70]. Similarly, VSG also increases plasma levels of proglucagon-derived peptides, although to levels not as high as those observed after RYGB [71–73]. Here, we demonstrate that RYGB induces intestinal hypertrophy already at 10-days post-surgery, *i.e.* before the animals have returned to consuming a normal diet (Supplementary Figure 5 and 6). Whether gut hypertrophy, associated with concomitant improved glucose homeostasis and increased gut hormone secretion, contributes to one or more metabolic effects of RYGB surgery remains unclear [10,14,70]. VSG also increases postprandial proglucagon-derived peptide levels [10], prompting us to examine the sub-acute effects of VSG surgery in mice with the use of stereological methods. Our histological data indicate similar gut tissue volumes in VSG and sham-operated mice, which is in agreement with similar observations in a rat model of VSG [74]. Although RYGB and VSG have common beneficial metabolic effects, the absence of gut hypertrophy in VSG-treated mice suggests that gut growth and adaptation is not a prerequisite for improved metabolism after bariatric surgery. In addition, our data in single receptor and *Gcg*$^{-/-}$ mice suggest that basal signaling through the GLP-1R/GLP-2R receptors is dispensable for normal gut growth.

Although we predominantly studied VSG-treated mice, post-prandial plasma GLP-1 (and by inference GLP-2) levels are higher after RYGB as compared to VSG [75]. This effect may potentially be explained by gut hypertrophy-dependent increases in GCG-expressing cells after RYGB [68], which could contribute to increased GLP-1 secretion. There is evidence from human studies that GLP-1 secretion is strongly associated with the rate of glucose appearance in the intestine [76,77]. Although it remains to be established, VSG-mediated increased gastric emptying or RYGB-induced redirection of intestinal nutrient flow could therefore possibly lead to enhanced L-cell responsiveness to glucose, as well as amino acids and bile acids. Indeed, VSG surgery procedures promote increased gastric emptying rates in rodents [78,79] as well as in humans [80–82]. Nevertheless, the VSG procedure maintains delivery of macronutrients within the proximal duodenum which exhibits very few, if any, GCG-expressing cells. In contrast, RYGB surgery will promote delivery of dietary nutrients to more distal parts of the gut which have a relatively higher density of GCG expressing cells. Thus, it cannot be ruled out that the delivery of macronutrients to the more distal parts of the gut following RYGB may be induce higher local release of GLP-1/GLP-2, which could further enhance GLP-1R/GLP-2R trophic signaling to promote hypertrophy of the alimentary and common limb. This notion is also supported by the finding of marked gut hypertrophy following ileal interposition surgery, *i.e.* in a condition where the ileal segment is transposed to more proximal parts of the gut [83].

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6. Conclusion

Collectively, our data suggest that endogenous GLP-1R signaling does not play an essential role in intestinal growth homeostasis, whereas loss of the GLP-2R produces modest reductions in gut mucosal volume. In addition, RYGB, but not VSG, surgery induced marked gut hypertrophy, suggesting that surgically-induced modification of macronutrient entry to more distal intestinal regions exhibiting relatively high L-cell density may underlie enhanced release and intestinotrophic effects of GLP-1/GLP-2. Our findings clearly dissociate pharmacological from physiological actions of GLP-1R/GLP-2R signaling on gut tissue expansion, with potential implications for different therapeutic strategies augmenting L cell activity for the treatment of metabolic disorders.

Author contribution

P.W., P.B., T.S., L.L.B., and J.A.K. performed the experiments and evaluated the data. P.W., H.B.B., D.J.D., D.A.S, P.B.J, N.V., and J.J. made substantial contributions to the study design, evaluation of data and drafted the manuscript.

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Figure captions

Figure 1: Expression of Glp1r mRNA in the mouse gastrointestinal tract.

Localization of *Glp1r* mRNA in mouse non-glandular (A), glandular stomach (B), Brunner's glands (C), duodenum (D), jejunum (E), ileum (F), cecum (G) and colon (H) using RNA scope 2.5 in situ hybridization. Inserts magnified below overview.

Figure 2: Expression of Glp2r mRNA in the mouse gastrointestinal tract.

Localization of *Glp2r* mRNA in mouse non-glandular (A), glandular stomach (B), Brunner's glands (C), duodenum (D), jejunum (E), ileum (F), cecum (G) and colon (H) using RNA scope 2.5 in situ hybridization. Inserts magnified below overview.

Figure 3: Expression of Gcg mRNA in the mouse gastrointestinal tract.

Localization of *Gcg* mRNA in mouse non-glandular (A), glandular stomach (B), Brunner's glands (C), duodenum (D), jejunum (E), ileum (F), cecum (G) and colon (H) using RNA scope 2.5 in situ hybridization. Inserts magnified below overview.

Figure 4: Glp1r$^{-/-}$ and Gcg$^{-/-}$ mice show normal gut development while Glp2r$^{-/-}$ mice display a slightly reduced mucosal volume.
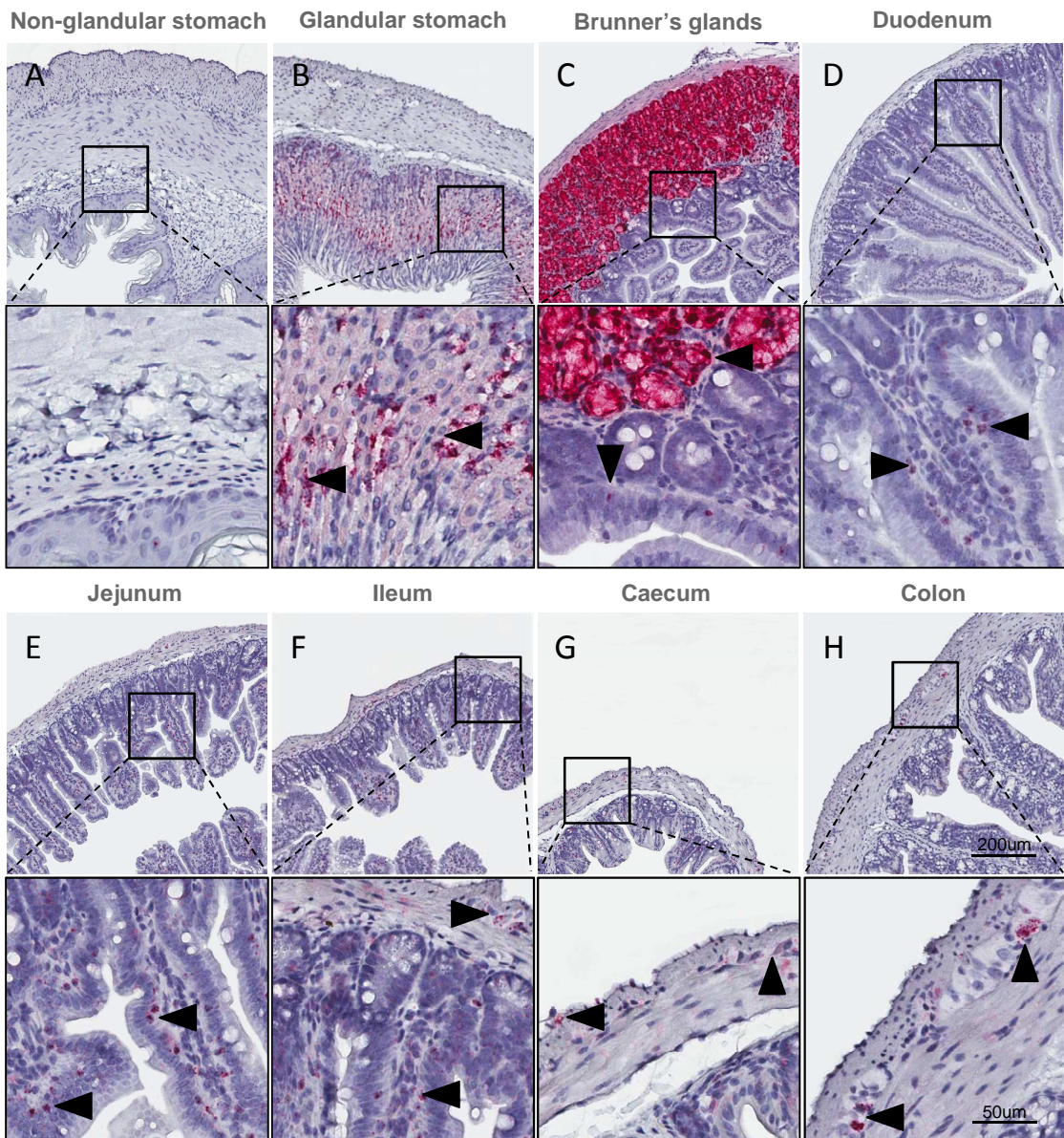
Intestine volume in *Glp1r$^{-/-}$* (A, B and C), *Glp2r$^{-/-}$* (D, E and F) and *Gcg$^{-/-}$* (G, H and I) mice as measured by stereology in WT C57/BL6J or KO mice. Small intestine volume in panel A, D and G, colon volume in panel B, E and H and total intestine volume in panel C, F and I. Volume of muscularis + submucosa (Musc.+ Subm.) and mucosa layers was measured separately.
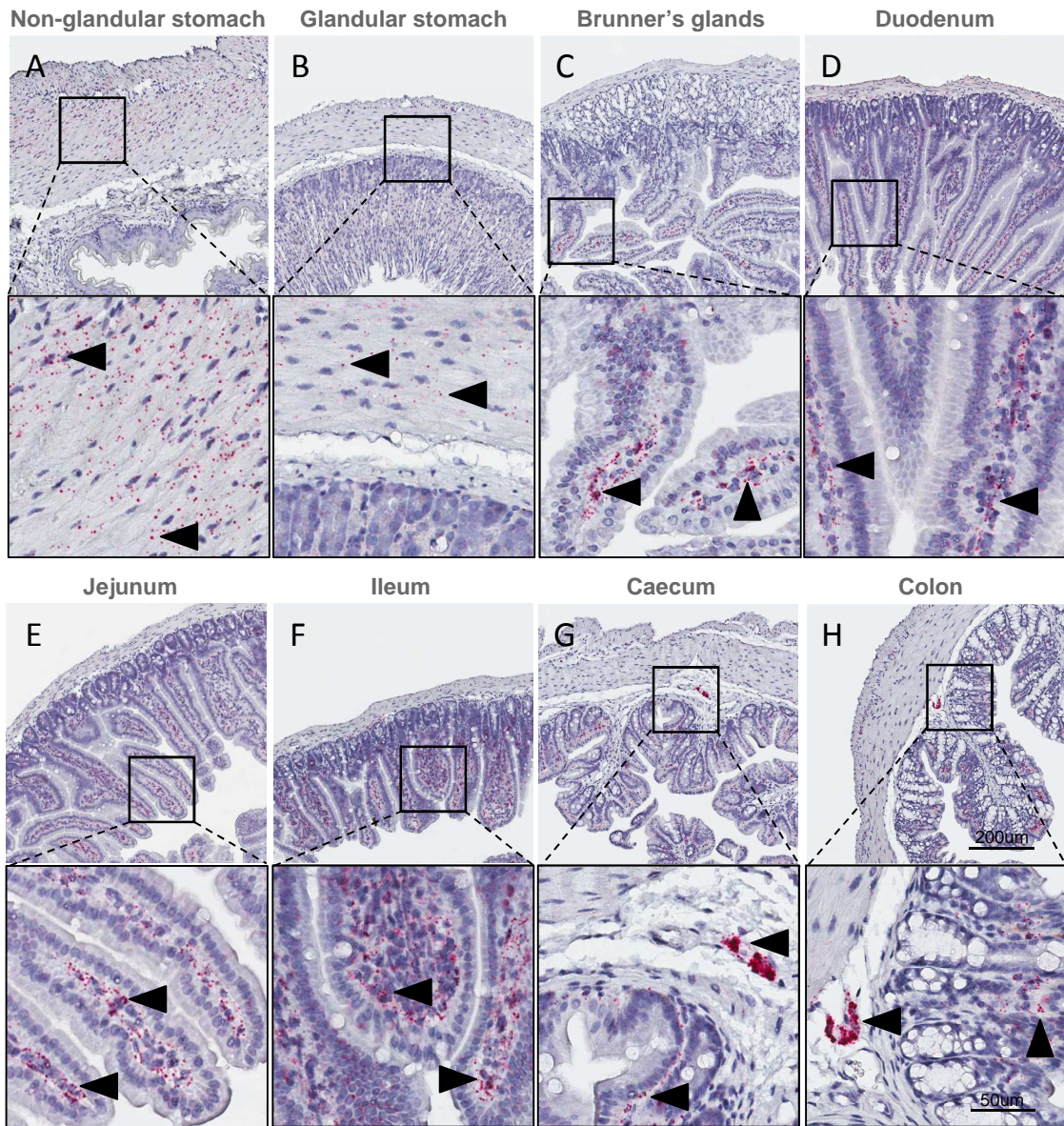
Figure 5: GLP-1 and GLP-2 analogues significantly increase gut volumes in C57BL/6J mice
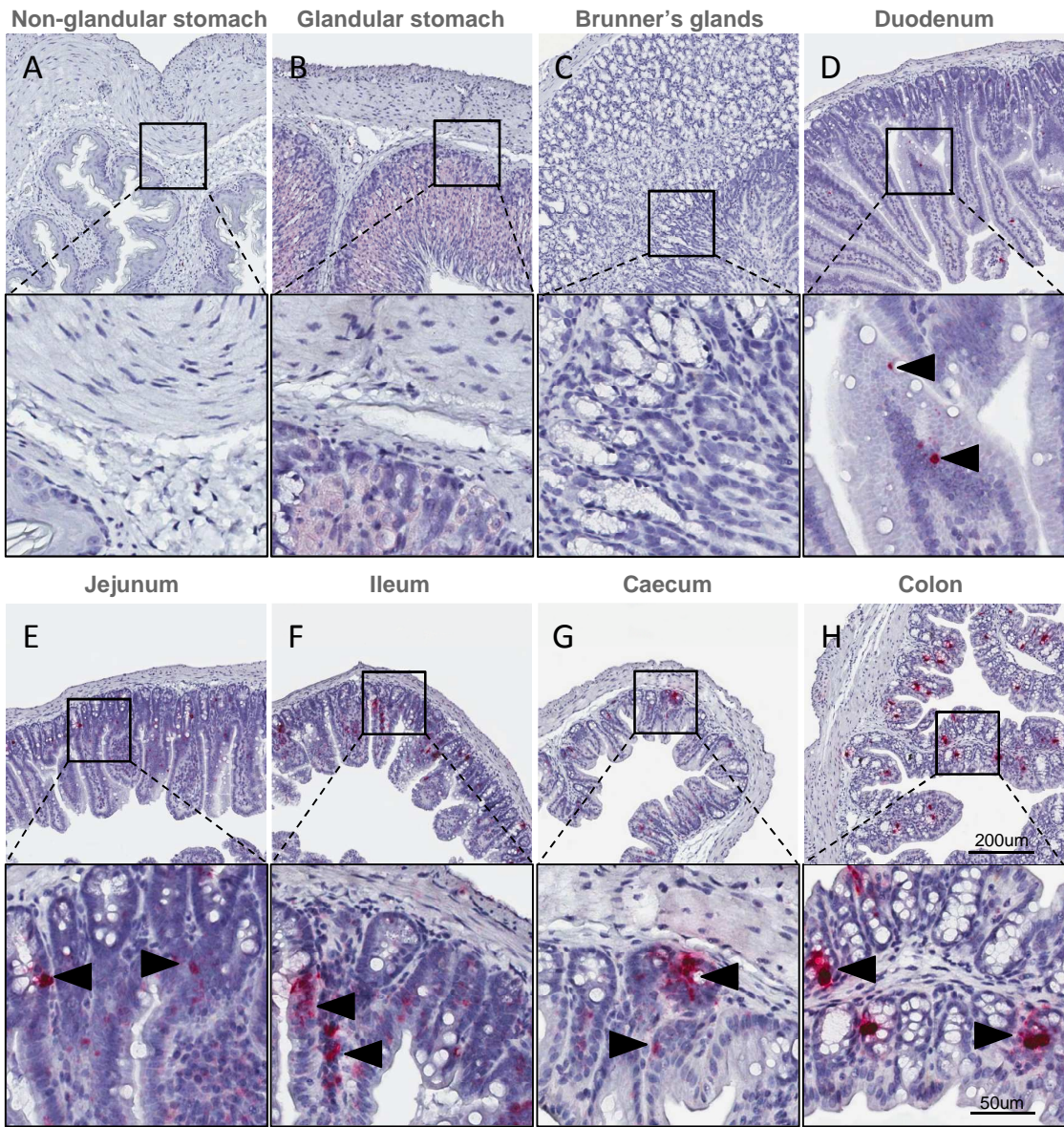
The effect of liraglutide, teduglutide and co-agonism on small intestine (A), colon (B) and total intestinal volume (C) as estimated by stereology. Volume of muscularis + submucosa (Musc.+ Subm.) and mucosa layers was measured separately.
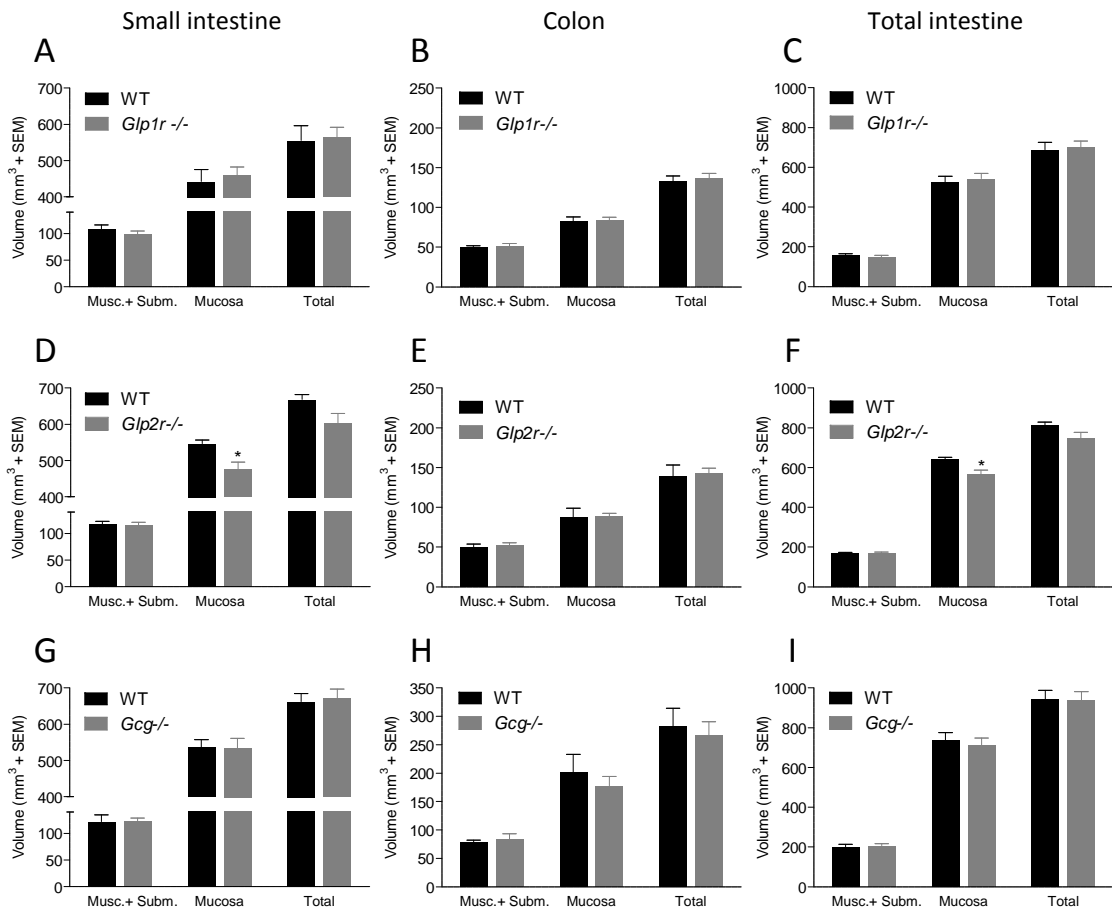
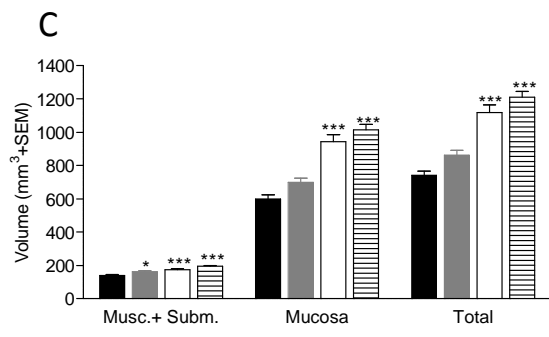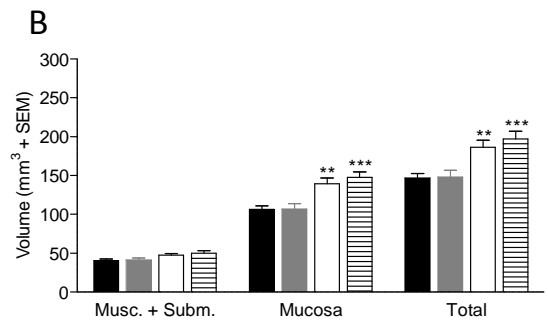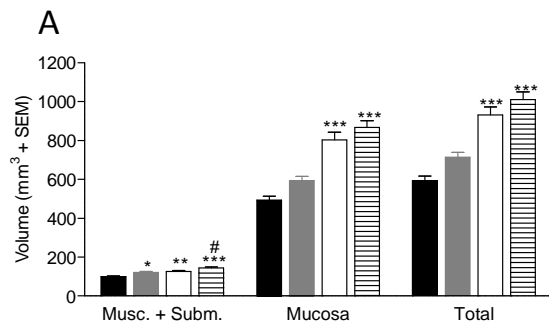*Figure 6: Mice subjected to VSG surgery do not display increased gut volume.*

The effect of VSG or sham surgery on duodenum (A), total small intestine (B), colon (C) and total intestine (D) volume as estimated by stereology. Volume of muscularis + submucosa (Musc.+ Subm.) and mucosa layers was measured separately.
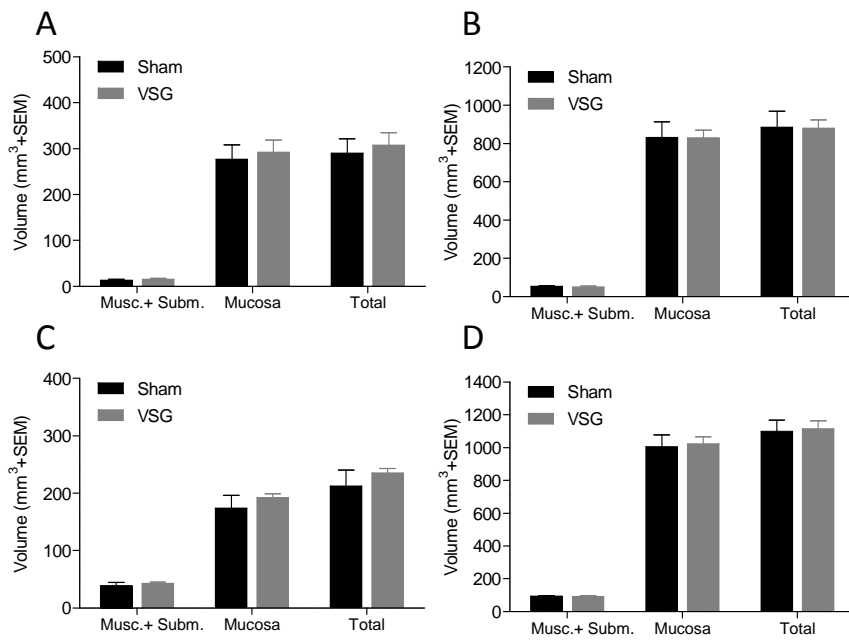
Non-glandular stomach   **Glandular stomach**   **Brunner's glands**   **Duodenum**

A   B   C   D

Jejunum   **Ileum**   **Caecum**   **Colon**

E   F   G   H

200um

50um

Non-glandular stomach **Glandular stomach** **Brunner's glands** **Duodenum**

A B C D

Jejunum **Ileum** **Caecum** **Colon**

E F G H

200um

50um

| Non-glandular stomach | Glandular stomach | Brunner's glands | Duodenum |
| Jejunum | Ileum | Caecum | Colon |

Small intestine

A

Colon

B

Total intestine

C

D

E

F

G

H

I

Highlights

- The preproglucagon system does not play an essential role in normal gut development in mice.
- Pharmacological treatment with GLP-1 and GLP-2 analogs show potent intestinotrophic effects.
- VSG-operation, in contrast to RYGB, display normal gut mucosal and submucosal tissue volume.