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Short Article

Microbial Modulation of Energy Availability in the Colon Regulates Intestinal Transit

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SUMMARY

Gut microbiota contribute to host metabolic efficiency by increasing energy availability through the fermentation of dietary fiber and production of short-chain fatty acids (SCFAs) in the colon. SCFAs are proposed to stimulate secretion of the proglucagon (*Gcg*)-derived incretin hormone GLP-1, which stimulates insulin secretion (incretin response) and inhibits gastric emptying. We find that germ-free (GF) and antibiotic-treated mice, which have severely reduced SCFA levels, have increased basal GLP-1 levels in the plasma and increased *Gcg* expression in the colon. Increasing energy supply, either through colonization with polysaccharide-fermenting bacteria or through diet, suppressed colonic *Gcg* expression in GF mice. Increased GLP-1 levels in GF mice did not improve the incretin response but instead slowed intestinal transit. Thus, microbiota regulate the basal levels of GLP-1, and increasing these levels may be an adaptive response to insufficient energy availability in the colon that slows intestinal transit and allows for greater nutrient absorption.

INTRODUCTION

The gut microbiota has coevolved with the host and contributes to efficient energy metabolism (Ley et al., 2008; Tremaroli and Bäckhed, 2012), which confers a selective advantage in conditions of food scarcity. Studies comparing mice that have a normal microbiota (conventionally raised [CONV-R]) with mice that lack a microbiota (germ-free [GF]) have demonstrated profound effects of the gut microbiota on host metabolism. Although CONV-R mice eat less, they have significantly more body fat and higher fasting glucose and insulin levels than GF mice (Bäckhed et al., 2004). The gut microbiota contributes to metabolic efficiency by increasing energy harvest from the diet as well as modulating the expression of host genes to promote energy storage (Bäckhed et al., 2004, 2005). Unlike the human genome, the gut microbiome encodes many enzymes for degrading plant polysaccharides, such as cellulose, xylan, pectin, and resistant starch (Gill et al., 2006). Gut microbes ferment these otherwise indigestible polysaccharides in the colon to produce short-chain fatty acids (SCFAs), a useable energy source for the host. For humans consuming a typical western diet, microbially produced SCFAs are estimated to contribute 6%–10% of total energy requirements, and the contribution is expected to be higher for humans consuming high-fiber diets and for herbivorous species (Bergman, 1990).

Efficient energy metabolism requires communication between the gut and peripheral organs such as the pancreas, liver, adipose tissue, and brain. Information about nutritional status in the gut is relayed by various signals, including gut-derived hormones such as glucagon-like peptide-1 (GLP-1). Transient postprandial increases in GLP-1 have many effects on metabolism, including the stimulation of insulin secretion (incretin effect), inhibition of gastric emptying, and an increased feeling of satiety (Holst, 2007). Secretion of GLP-1 from enteroendocrine L cells can be stimulated by sugars, amino acids, and long-chain fatty acids (Diakogiannaki et al., 2012). However, given that these nutrients typically do not reach high concentrations in the colon, where L cells are found at the highest density (Eissele et al., 1992), it is unclear how GLP-1 secretion is regulated in the colon. Dietary supplementation with fermentable fibers has been shown to increase GLP-1 levels in rodents and humans (Delzenne and Cani, 2005; Delzenne et al., 2005; Freeland et al., 2010; Zhou et al., 2008), and SCFAs can stimulate GLP-1 secretion in vitro (Tolhurst et al., 2012; Zhou et al., 2008). Thus, it has been suggested that the gut microbiota increases GLP-1 levels through the production of SCFAs.

Here, we investigate how the gut microbiota affects the production of GLP-1 by comparing GF and CONV-R mice. Surprisingly, we find that the absence of microbially produced SCFAs in GF colon results in significantly higher plasma GLP-1 levels. This colonic-derived GLP-1 has an important role in slowing small intestinal transit, which may be an adaptive response for promoting nutrient absorption.

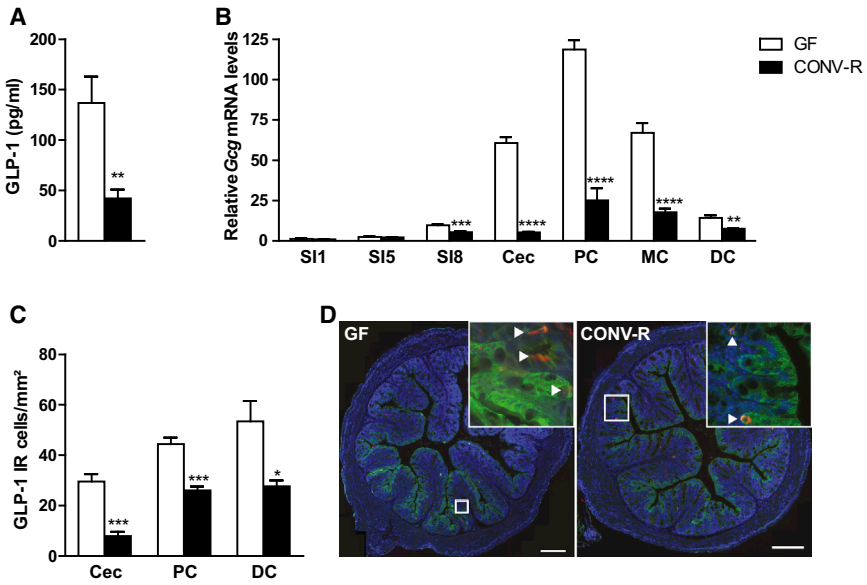

Figure 1. Plasma GLP-1 Levels, Colonic Proglucagon Expression, and L Cell Number Are Higher in GF Mice

(A) GLP-1 levels in portal vein plasma of GF (n = 8) and CONV-R (n = 10) mice that were fasted for 4 hr prior to blood collection.

(B) Relative proglucagon expression in the small intestine (SI), cecum (Cec), proximal colon (PC), mid colon (MC), and distal colon (DC). The small intestine was divided into eight equal segments, and gene expression was analyzed in segments 1, 5, and 8 (n = 5 mice per group).

(C) Quantification of GLP-1-immunoreactive (IR) cells in the cecum (Cec), proximal colon (PC), and distal colon (DC) of GF and CONV-R mice (n = 5 mice per group).

(D) Representative images of GF and CONV-R proximal colon stained for GLP-1 (red), cytokeratin 8 (green), and Hoechst (blue).

Scale bars represent 200 μm. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

RESULTS

Basal GLP-1 Levels Are Elevated in GF Mice

Analysis of plasma GLP-1 levels in fasting GF and CONV-R mice revealed that GLP-1 levels were 3-fold higher in the absence of a microbiota (Figure 1A). Increased basal GLP-1 levels could be due to one or more factors: decreased activity of dipeptidyl peptidase IV (DPPIV, the enzyme that inactivates GLP-1), increased expression of proglucagon (*Gcg*, the gene from which GLP-1 is derived), or increased numbers of L cells. We did not find any differences in DPPIV activity in plasma between GF and CONV-R mice (20.1 ± 0.8 nmol/ml/min [GF] versus 20.1 ± 0.5 nmol/ml/min [CONV-R]; n = 4; p = 0.98). We found that *Gcg* expression was similar in the proximal small intestine of GF and CONV-R mice but significantly higher in the cecum and colon of GF mice in comparison to CONV-R mice (Figure 1B). The greatest fold differences were found in the cecum and proximal colon, the regions where microbial density is the highest (Figure 1B). In addition, GF mice had approximately 4-fold more GLP-1-positive cells in the cecum and 2-fold more GLP-1-positive cells in the proximal and distal colon in comparison to CONV-R mice (Figures 1C and 1D). Thus, elevated plasma GLP-1 levels in GF mice appear to originate from the cecum and colon, and the gut microbiota affects both *Gcg* expression and L cell number.

SCFAs Increase and Proglucagon Expression Decreases upon Colonization

Energy metabolism in the colon is unique in that colonocytes use SCFAs, particularly butyrate, as a primary energy source (Roediger, 1980, 1982), whereas most other tissues in the body use glucose. Colonocytes from GF mice, which lack their preferred energy source, exhibit defects in energy metabolism, including reduced ATP levels and impaired mitochondrial respiration (Donohoe et al., 2011). These energy defects are specific for the proximal colon and are not observed in other parts of the gut or other organs (Donohoe et al., 2012). Thus, we hypothesized that energy availability in the colon affects *Gcg* expression in L cells.

To test our hypothesis, we modulated energy availability in the colon by manipulating the gut microbiota. We analyzed SCFA levels in cecal content as an indicator of energy availability and examined the corresponding effects on GLP-1 parameters after the colonization of GF mice with an unfractionated microbiota from a CONV-R donor. As expected, the total SCFA concentration in the cecal content of GF mice was low (Figure 2A). Low levels of acetate, which are thought to be derived from the diet (Høverstad and Midtvedt, 1986), were detected, whereas propionate and butyrate were barely detectable (Figures S1A–S1C available online). After colonization, the SCFA concentration increased 7-fold after only 24 hr, reaching a level similar to that of CONV-R mice (Figure 2A). This rapid increase in SCFA concentration was associated with significant decreases in colonic *Gcg* expression after 24 and 72 hr (Figure 2B). The number of GLP-1-positive cells in the proximal colon did not change significantly after 24 hr but decreased to a level similar to CONV-R 72 hr after colonization (Figure 2C). The average plasma GLP-1 levels decreased gradually after colonization, although the differences were not statistically significant because of large variation in the sample groups (Figure S1D).

To test the effect of microbial production of SCFAs on *Gcg* expression more specifically, we colonized GF mice with single bacterial strains that have different fermentation abilities. We chose two representative members of the gut microbiota: *Escherichia coli*, a Gram-negative bacterium that ferments simple sugars (Clark, 1989), and *Bacteroides thetaiotaomicron*, a Gram-negative bacterium that ferments a wide range of plant polysaccharides (Xu et al., 2003). Colonization with *E. coli* resulted in a small increase in acetate (Figure S1E) but did not significantly alter total SCFA levels, *Gcg* expression, or GLP-1-positive cell number in comparison to GF (Figures 2D–2F). In contrast, colonization with *B. thetaiotaomicron* produced significant increases in acetate and propionate (Figures S1E–S1G), resulting in a 4-fold increase in total SCFA levels, a 2.5-fold decrease in colonic *Gcg* expression, and a 1.7-fold decrease in GLP-1-positive cells (Figures 2D–2F). However, GLP-1 levels

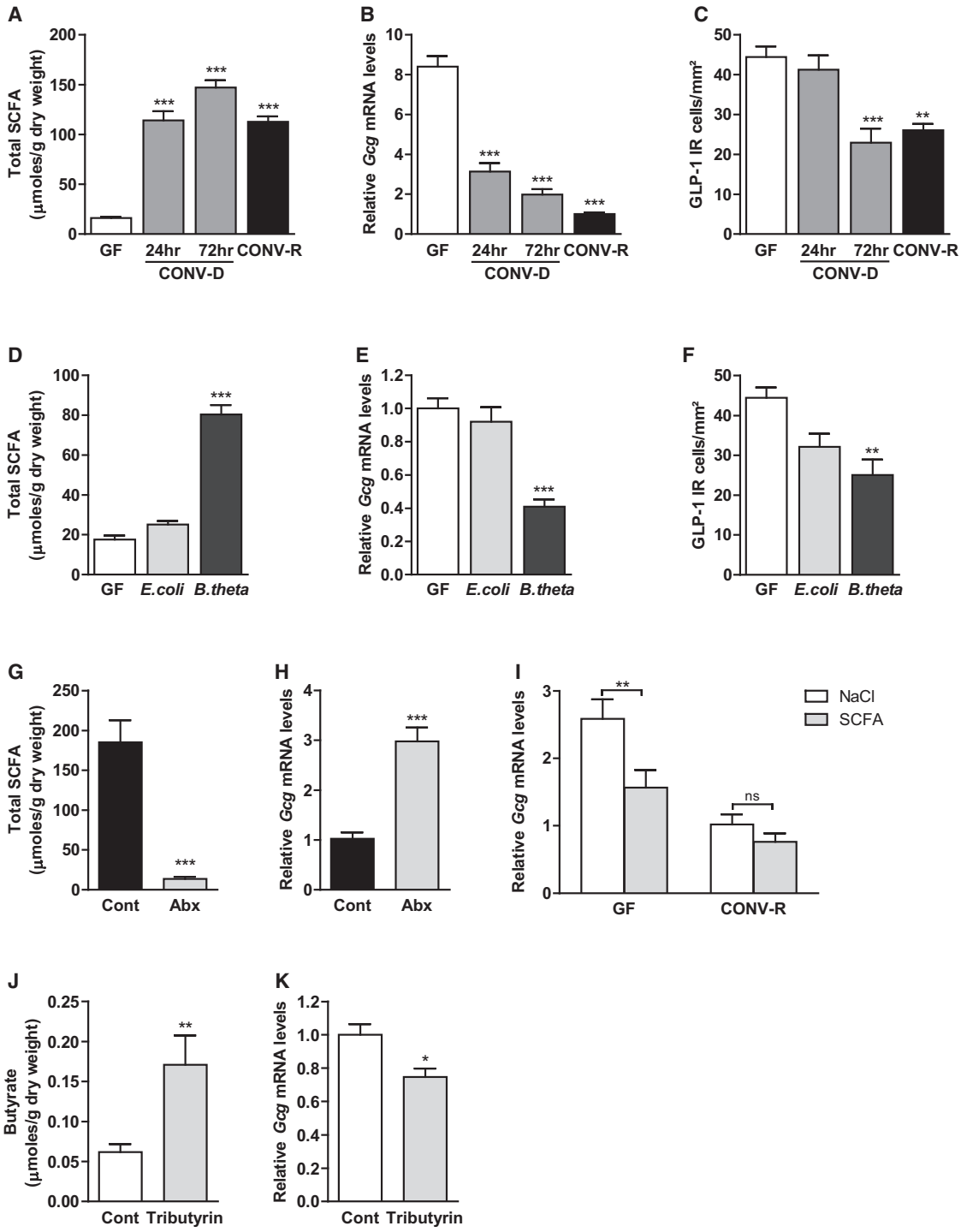

Figure 2. Corresponding Changes in SCFA Levels, Proglucagon Expression, and GLP-1-Positive Cells after Colonization or Antibiotic Treatment

(A–C) Total SCFA concentrations (acetate, propionate, and butyrate) in cecal content (A), relative proglucagon expression in proximal colon (B), and quantification of GLP-1-immunoreactive (IR) cells in proximal colon (C) of GF mice, GF mice that were conventionalized (CONV-D) with microbiota from a CONV-R donor for 24 or 72 hr, and CONV-R mice (n = 4–5 mice per group).

(D–F) Total SCFA concentrations in cecal content (D), relative proglucagon expression in proximal colon (E), and quantification of GLP-1-immunoreactive (IR) cells in proximal colon (F) of GF mice and mice that were monocolonized for 4 weeks with either *E. coli* or *B. thetaiotaomicron* (n = 5–10 mice per group).

(G and H) Total SCFA concentrations in cecal content (G) and relative proglucagon expression in proximal colon (H) of CONV-R mice that were orally administered antibiotics (Abx; 200 mg/kg each of bacitracin, neomycin, and streptomycin) or vehicle control (Cont; water) once daily for 3 days (n = 5–6 mice per group).

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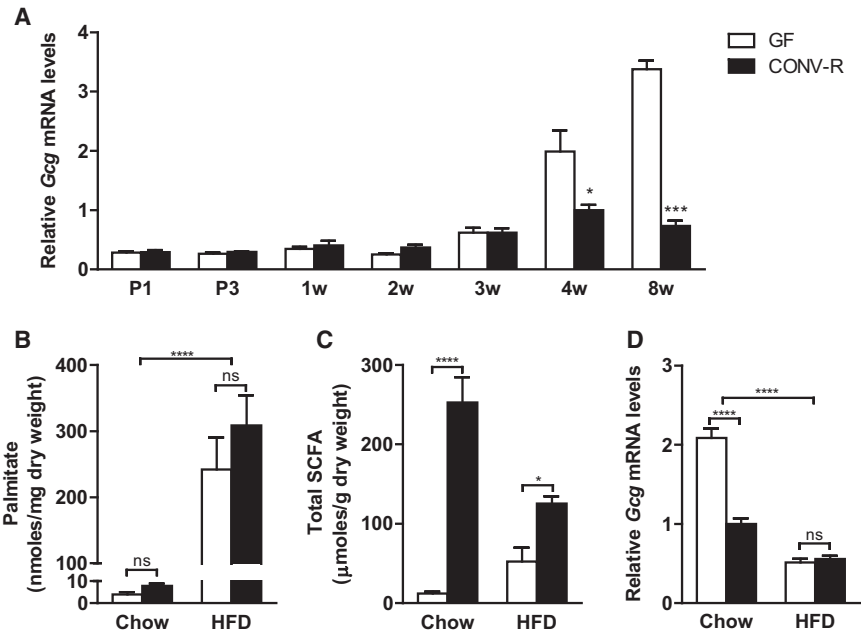

Figure 3. Proglucagon Expression Increases in GF Mice after Weaning onto Chow Diet

(A) Relative proglucagon expression in the proximal colon in GF and CONV-R mice on postnatal days 1 (P1) and 3 (P3) and during the first weeks of life (1w = 1 week old) (n = 5 mice per group). Pups were weaned onto standard chow diet at 3 weeks old.

(B–D) Palmitate levels in cecal content (B), total SCFA levels in cecal content (C), and relative proglucagon expression in the proximal colon (D) in 4-week-old GF and CONV-R mice that were weaned onto either standard chow or HFD (40% of calories from fat) at 3 weeks of age (n = 4–6 mice per group).

Data are presented as mean ± SEM. *p < 0.05, ***p < 0.001, ****p < 0.0001.

were not significantly different after *B. thetaiotaomicron* colonization (Figure S1H).

We also examined whether the depletion of the microbiota in CONV-R mice by antibiotic treatment would increase *Gcg* expression. Indeed, we found that 3-day treatment with a combination of antibiotics resulted in a 13-fold decrease in SCFA concentration and a 3-fold increase in *Gcg* expression (Figures 2G and 2H).

SCFAs Suppress Proglucagon Expression in GF Colon

To provide direct evidence that SCFAs affect *Gcg* expression in the colon, we incubated proximal colon tissue ex vivo with either a physiological concentration of SCFAs or an equimolar solution of sodium chloride. SCFA treatment resulted in significantly lower *Gcg* expression in GF colon but did not have a significant effect on *Gcg* expression in CONV-R colon, which would have been exposed to high SCFA concentrations in vivo (Figure 2I). In addition, we fed GF mice a diet containing 10% tributyrin, a triglyceride that is less readily absorbed in the small intestine than butyrate and is metabolized to butyrate in the colon (Donohoe et al., 2012). GF mice fed the tributyrin diet had a 2.8-fold increase in butyrate in the cecal content and a 1.3-fold decrease in colonic *Gcg* expression in comparison to GF mice fed an isocaloric control diet (Figures 2J and 2K). Altogether, these experiments show that SCFAs suppress *Gcg* expression in GF colon. This effect does not appear to be specific for a particular SCFA, given that increasing levels of acetate and propionate (*B. thetaiotaomicron* colonization) or butyrate (tributyrin diet) suppress *Gcg* expression.

Diet and Microbiota Affect Proglucagon Expression in the Colon

The contribution of the gut microbiota to energy harvest in the colon depends on the composition of the diet. Given that diet and gut microbiota change considerably at the suckling-to-weaning transition, we analyzed colonic *Gcg* expression in GF and CONV-R mice from birth through young adulthood. In neonatal mice, colonocytes obtain energy from milk lactose and lipids, which reach the colon because the absorptive ability of the small intestine is not yet fully developed (Pácha, 2000). We hypothesized that there would be little difference in dietary energy availability, and, thus, little difference in colonic *Gcg* expression, between GF and CONV-R mice during the suckling period. At 3 weeks of age, mice are weaned onto a standard chow diet, which is rich in plant polysaccharides. Given that bacterially produced SCFAs would not be available to replace milk lipids upon weaning in GF mice, we predicted that the resulting energy deficit would lead to increased *Gcg* expression in the weeks following weaning. In agreement with our hypothesis, we found that colonic *Gcg* expression did not differ between GF and CONV-R mice during the first 3 weeks of life but increased substantially in GF mice in comparison to CONV-R mice at 4 weeks of age (2-fold) and was even greater at 8 weeks of age (5-fold) (Figure 3A).

In an attempt to determine whether prolonged fasting reduces colonic energy supply and alters GLP-1 levels in the presence of a complex microbiota, we subjected CONV-R mice to an 18 hr fast. However, we found that total SCFA concentrations, colonic *Gcg* expression, and plasma GLP-1 levels were not significantly different in 18 hr fasted mice in comparison to fed mice (data not shown).

To test whether increasing energy supply from the diet could suppress the increase in *Gcg* expression in GF mice, we weaned

(I) Relative proglucagon expression in proximal colon from GF (n = 10) and CONV-R (n = 9) mice after 6 hr treatment ex vivo with 140 mM NaCl (osmotic control) or 140 mM mixed SCFAs (80 mM acetate + 40 mM propionate + 20 mM butyrate). Proximal colon segments were divided in half longitudinally; one half was treated with NaCl, and the other half was treated with SCFAs.

(J and K) Butyrate concentrations in cecal content (J) and relative proglucagon expression in proximal colon (K) of 4-week-old GF mice that were fed a diet containing 10% tributyrin oil or an isocaloric control diet for one week (n = 4–6 mice per group).

Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S1.

mice onto a high-fat diet (HFD, 40% of calories from fat) and analyzed *Gcg* expression in the proximal colon after 1 week on the diet. We analyzed cecal levels of the long-chain fatty acid palmitate because it is abundant in the diet and has previously been shown to rescue the defect in mitochondrial respiration in GF colonocytes (Donohoe et al., 2011). We found that cecal palmitate levels were much higher in HFD-fed mice in comparison to chow-fed mice and that colonization status did not have a significant effect on palmitate levels (Figure 3B). In addition, we found that colonization status had a smaller effect on SCFA levels for HFD-fed mice than for chow-fed mice (Figure 3C). Importantly, the increase in colonic *Gcg* expression observed in GF mice in comparison CONV-R mice on a chow diet was abolished in GF mice fed an HFD (Figure 3D). Altogether, these results support our initial hypothesis that energy availability in the colon affects *Gcg* expression.

Increased GLP-1 in GF Mice Results in Slower Intestinal Transit

Next, we examined the physiological consequences of increased GLP-1 levels. The role of colonic-derived GLP-1 is not well understood, but GLP-1 is well-characterized as an incretin hormone (Holst, 2007). Thus, we investigated whether increased GLP-1 levels in GF mice contribute to improved oral glucose tolerance. We found that GF mice have significantly better oral glucose tolerance than CONV-R mice (Figure 4A). However, blocking GLP-1 signaling with the GLP-1 receptor antagonist exendin 9-39 (Ex-9) shifted glucose tolerance curves to a similar extent for GF and CONV-R mice (fold difference $_{AUC}$ [Ex-9:saline] = 1.3 for both groups) (Figures 4A and 4B). Furthermore, although GF mice had lower fasting insulin levels, the fold increase in insulin after glucose gavage was similar in GF and CONV-R mice (Figures S2A–S2B). Thus, there are underlying differences in glucose metabolism in GF and CONV-R mice, but the relative incretin effect of GLP-1 appears to be similar. GLP-1 can also promote β cell proliferation and survival (Holst, 2007). However, there were no significant differences in β cell mass or in insulin content of islets in GF and CONV-R mice (Figures S2C–S2E). We conclude that enhancing glucose metabolism in GF mice, which are already lean and insulin sensitive, is not the primary function of increased basal GLP-1 levels. Furthermore, the rapid postprandial secretion of GLP-1, which accounts for the majority of the incretin effect, occurs before ingested nutrients reach the colon and remains intact in patients after ileal resection or colectomy (Nauck et al., 1996). These observations suggest that colonic-derived GLP-1 may be more important for late-phase secretion or other functions.

Next, we investigated whether increased GLP-1 levels in GF mice play a role in the modulation of gastric emptying and gastrointestinal transit, given that these processes are known to be regulated by GLP-1 (Marathe et al., 2011) and that the overexpression of GLP-1 from neuroendocrine tumors has been associated with severely reduced gastrointestinal transit in humans (Brubaker et al., 2002; Byrne et al., 2001). In agreement with previous findings (Kashyap et al., 2013; Samuel et al., 2008), we found that GF mice exhibited significantly slower small intestinal transit in comparison to CONV-R controls (Figure 4C). Given that the overall rate of gastric emptying was similar in GF and CONV-R mice (Figure S2F), the difference in transit most likely reflects a difference in small intestinal motility. To determine whether the slower intestinal transit in GF mice depends on GLP-1 signaling, we measured transit in mice that had been preadministered Ex-9. Strikingly, blocking GLP-1 signaling with Ex-9 completely rescued the transit phenotype in GF mice (Figure 4C). Although Ex-9 treatment had the expected effect on glucose tolerance in CONV-R mice (Figure 4A), it had no effect on transit in CONV-R mice (Figure 4C).

As additional support for the role of elevated GLP-1 levels in slowing intestinal transit, we investigated transit in *Glp-1r*−/− mice and C57Bl/6 controls after treatment with antibiotics. Antibiotic treatment resulted in significantly lower cecal SCFA levels, 3-fold higher colonic *Gcg* expression, and approximately 3-fold higher GLP-1 levels in both C57Bl/6 and *Glp-1r*−/− mice (Figures S2G–S2I). However, although intestinal transit was significantly slower after antibiotic treatment in C57Bl/6 mice, there was no significant difference in transit between control- and antibiotic-treated *Glp-1r*−/− mice (Figure 4D). These results demonstrate that functional GLP-1 receptor signaling is required to slow intestinal transit after antibiotic treatment.

Consistent with the trends observed for *Gcg* expression, L cell number, and GLP-1 levels, we found that intestinal transit increased significantly 72 hr after colonization with a complete microbiota (Figure 4E) and after monocolonization with *B. thetaiotaomicron* but not *E. coli* (Figure 4F). We also found that intestinal transit was normalized to the CONV-R rate in GF mice that were fed an HFD (Figure 4G).

DISCUSSION

Here, we show that colonic *Gcg* expression, L cell number, and basal GLP-1 levels are significantly elevated in GF mice, which lack microbially produced SCFAs. Given that colonocytes use SCFAs, particularly butyrate, as a primary energy source, colonocytes from GF mice are energy deprived (Donohoe et al., 2011). We find that increasing energy availability by colonizing with polysaccharide-fermenting bacteria or supplementing the diet with short- or long-chain fatty acids reduces colonic *Gcg* expression, suggesting that colonic L cells sense local energy availability and regulate basal GLP-1 secretion accordingly. In addition, perturbation of the microbiota in CONV-R mice by antibiotic treatment results in reduced SCFA levels and increased *Gcg* expression and GLP-1 levels. Thus, the continuous production of SCFAs by the gut microbiota under normal physiological conditions may play a role in establishing basal GLP-1 levels.

Although average plasma GLP-1 levels decreased after colonization, the differences were not statistically significant because of large variation within the groups. This may reflect normal variations in our mouse population and/or problems with sample degradation. Accurate measurement of active GLP-1 is difficult because of rapid degradation—the half-life of active GLP-1 in plasma is estimated to be 1–2 min (Holst, 2007). We attempted to minimize GLP-1 degradation with the use of DPPIV inhibitors and aprotinin and by processing samples at 4°C, but we cannot exclude the possibility that degradation contributed to variation in our sample groups.

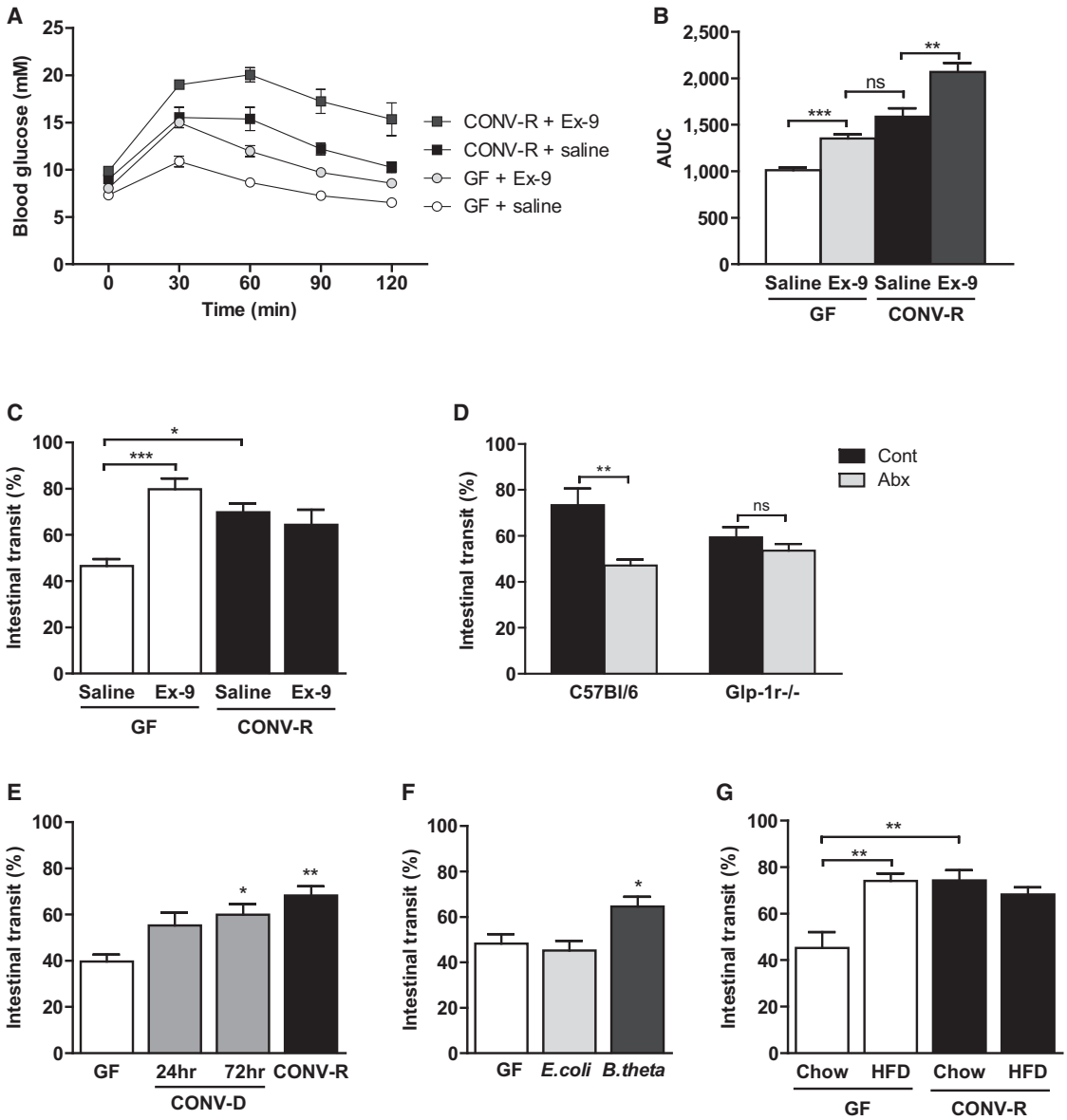

Figure 4. Slower Small Intestinal Transit in GF Mice Is Dependent on GLP-1 Signaling

(A and B) Oral glucose tolerance (A) and average area under the curve (AUC) (B) of GF and CONV-R mice that were pretreated with GLP-1 antagonist Ex-9 or saline control (A) (n = 5–7 mice per group).

(C) Small intestinal transit in GF and CONV-R mice that were pretreated with Ex-9 or saline control (n = 5 mice per group).

(D) Small intestinal transit in C57Bl/6 and *Glp-1r*−/− mice that were orally administered antibiotics (Abx; 200 mg/kg each of bacitracin, neomycin, and streptomycin) or vehicle control (Cont; water) once daily for 3 days (n = 6–7 mice per group).

(E) Small intestinal transit in GF mice, GF mice that were conventionalized (CONV-D) with microbiota from a CONV-R donor for 24 or 72 hr, and CONV-R mice (n = 5 mice per group).

(F) Small intestinal transit in GF mice and mice that were monocolonized for 4 weeks with either *E. coli* or *B. thetaiotaomicron* (n = 5 mice per group).

(G) Small intestinal transit in 4-week-old GF and CONV-R mice that have been fed standard chow or HFD for 1 week (n = 4–6 mice per group).

Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S2.

We show that intestinal transit in GF mice is accelerated by the GLP-1 receptor antagonist Ex-9 and that the antibiotic-induced effect on intestinal transit is abolished in *Glp-1r*−/− mice, demonstrating that functional GLP-1 receptor signaling is required to slow intestinal transit when the microbiota is absent or depleted. Ex-9 treatment had no effect on transit in CONV-R mice. Similarly, experiments in rats have shown that Ex-9 treatment had no effect on basal contractile motility in the small intestine but could reverse the inhibition in contractile motility caused by peptone infusion (Giralt and Vergara, 1999). These results suggest that basal GLP-1 levels have little effect on intestinal transit under normal physiological conditions,

whereas increases in GLP-1, either transient (e.g., in response to nutrients) or chronic (e.g., in response to energy deprivation in the colon), may slow intestinal transit and, thus, be reversible by Ex-9.

The elevated GLP-1 levels in GF mice do not appear to affect satiety, given that GF mice are known to eat more (Bäckhed et al., 2004). This might be explained by the fact that the anorectic effect of GLP-1 is dependent on glucose availability (Sandoval et al., 2012) and that GF mice have lower blood glucose levels. In addition, given that GLP-1 is rapidly degraded, it may have stronger effects locally, for example, on enteric neurons to regulate transit than centrally. Other hormones, such as leptin, which is lower in GF mice (Bäckhed et al., 2004), may have more important roles in regulating food intake.

We propose that colonic GLP-1 has an important function in slowing intestinal transit in response to insufficient energy availability in the colon. Given that intestinal transit rate affects both nutrient absorption and bacterial growth (Stephen et al., 1987), it needs to be tightly regulated in order to allow for optimal nutrition while protecting against bacterial overgrowth and pathogenic infection. We propose that, in the absence of a microbiota, colonic-derived GLP-1 increases in order to slow intestinal transit, allowing more time for nutrient absorption. Upon colonization and the resulting increase in energy availability, colonic GLP-1 is suppressed in order to speed up transit, thus preventing bacterial overgrowth. We attempted to reduce colonic energy supply by fasting mice for 18 hr but found no significant difference in SCFA concentrations. Therefore, a longer fasting period or long-term caloric restriction might be required to reduce SCFA concentrations to a level that would significantly alter GLP-1 levels. Elevated GLP-1 levels and slower gastrointestinal transit times have been reported in patients with anorexia nervosa (Germain et al., 2007; Kamal et al., 1991), suggesting that this function may be conserved in humans. Our findings provide an example of how the microbial contribution to energy supply affects host gene expression and physiology in the gut.

EXPERIMENTAL PROCEDURES

Mice and Diets

Unless otherwise indicated, experiments were performed with 12- to 15-week-old Swiss Webster mice that were fed an autoclaved low-fat, polysaccharide-rich chow diet (LabDiet 5021) ad libitum. GF Swiss Webster mice were maintained in flexible film isolators under a strict 12 hr light cycle. GF status was monitored regularly by anaerobic culturing and PCR for bacterial 16S ribosomal RNA. For the tributyrin diet experiment, 3-week-old mice were weaned onto either TestDiet 5W2G (LabDiet 5020 fortified with 10% tributyrin oil [Sigma-Aldrich] and irradiated) or isocaloric irradiated control LabDiet 5020 (a nonautoclavable version of LabDiet 5021). For the HFD experiment, 3-week-old mice were weaned onto an irradiated high-fat, high-sugar western diet with 40% of calories from fat (Adjusted Fat Diet TD.96132, Harlan Teklad). All mouse experiments were performed with protocols approved by the Research Animal Ethics Committee in Gothenburg, Sweden.

Colonization of GF Mice

For colonization with an unfractionated microbiota, the cecal content from an adult CONV-R mouse was resuspended in 5 ml sterile PBS, and 200 μl was given by oral gavage to GF mice. The resulting CONV-D mice were maintained in standard makrolon cages for 24 or 72 hr. For monocolonization experiments, sterile cotton swabs were dipped in liquid culture of either *E. coli* W3110 or *B. thetaiotaomicron* VPI-5482 (ATCC 29148) and fed to GF mice. Monocolonized mice were housed in separate sterile isolators for 4 weeks. At the end of the colonization period, mice were fasted for 4 hr before killing and tissue harvest. Colonization density was verified by culture.

Antibiotic Treatment

Mice were given oral gavage of bacitracin, neomycin, and streptomycin (200 mg/kg body weight of each antibiotic) or water (vehicle control) each morning for 3 days. On day 4, mice were fasted for 4 hr before analyzing small intestinal transit and organ harvest.

Measurement of SCFAs and Palmitate

SCFA and palmitate levels in cecal content were analyzed with a modification of the methods described previously (Moreau et al., 2003; Samuel et al., 2008). Approximately 100 mg of cecal contents and 100 μl of internal standards (16 mM acetate, 3.2 mM propionate, and 3.7 mM butyrate for SCFA or 1 mM for palmitate) were added to glass vials and freeze dried. Samples were acidified with 50 μl of 37% HCl, and SCFAs and palmitate were extracted with two rounds of diethyl ether extraction (2 ml diethyl ether, rotation shake for 15 min, and centrifugation for 5 min at 2,000 × *g*). The organic supernatant was collected, 50 μl of the derivatization agent *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (Sigma-Aldrich) was added, and samples were incubated overnight. SCFAs or palmitate were quantified with a gas chromatograph (Agilent Technologies 7890A) coupled to a mass spectrometer (Agilent Technologies 5975C).

Ex Vivo Experiments

One centimeter pieces of proximal colon were excised, divided in half longitudinally, and prepared for organ culture as described previously (Cima et al., 2004). One half was treated for 6 hr with 140 mM mixed SCFA (80 mM acetate + 40 mM propionate + 20 mM butyrate), a concentration representative of physiological conditions in rodent cecum (Hara et al., 1999; Mineo et al., 2006; Suzuki et al., 2008). The control half was treated with 140 mM NaCl as an osmotic control. Tissues were washed in PBS, frozen immediately in liquid nitrogen, and stored at −80°C until quantitative RT-PCR analysis.

Glucose Tolerance Tests

Mice were fasted for 4 hr and given oral gavage of 20% D-glucose (3 g/kg body weight). Blood was drawn from the tail vein at 0, 30, 60, 90, and 120 min, and blood glucose levels were measured with a HemoCue glucometer. Extra blood was collected from the tail vein at 0, 15, and 30 min for analysis of serum insulin levels with insulin ELISA assay (Crystal Chem). For Ex-9 experiments, mice received intraperitoneal injection of Ex-9 (250 nmol/kg body weight, Sigma-Aldrich) or 0.9% saline (vehicle control) 30 min before the start of the experiment.

Small Intestinal Transit

Mice were fasted for 4 hr and given oral gavage of 100 μl of 1.5% methylcellulose containing 5% Evans Blue dye (Sigma-Aldrich). Small intestinal transit was assessed 45 min after gavage in Swiss Webster mice and 30 min after gavage in C57Bl/6 and *Glp-1r*−/− mice. The total length of the small intestine and the length covered by Evans Blue were measured, and transit was expressed as the percent of small intestinal length covered by Evans Blue. For Ex-9 experiments, mice received intraperitoneal injections of Ex-9 (250 nmol/kg body weight) or 0.9% saline (vehicle control) at 2 hr and at 30 min prior to the start of the experiment.

Statistical Analysis

Data are presented as mean ± SEM. Statistical differences between groups of two were analyzed with a Student's t test, comparisons of three or more groups with one independent variable (e.g., colonization status) were analyzed by one-way ANOVA with ad hoc Bonferroni post tests, and comparisons of groups with two or more independent variables (e.g., colonization status and diet) were analyzed by two-way ANOVA with ad hoc Bonferroni post tests with the use of GraphPad Prism 5.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.09.012.

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