Original Article

MOLECULAR METABOLISM

Glucagon-like peptide-2 promotes gallbladder refilling via a TGR5-independent, GLP-2R-dependent pathway

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ABSTRACT

Objective: Glucagon-like peptides (GLPs) are secreted from enteroendocrine cells in response to nutrients and bile acids and control metabolism via actions on structurally-related yet distinct G protein coupled receptors. GLP-1 regulates gut motility, appetite, islet function, and glucose homeostasis, whereas GLP-2 enhances intestinal nutrient absorption. GLP-1R agonists are used to treat diabetes and obesity, and a GLP-2R agonist is approved to treat short bowel syndrome. Unexpectedly, reports of gallbladder disease have been associated with the use of both GLP-1R and GLP-2R agonists and after bariatric surgery, although the mechanisms remain unknown.

Methods: We investigated whether GLP-1 or GLP-2 acutely controls gallbladder (GB) volume and whether GLP-2 regulates GB muscle activity in mice. The expression of *Tgr5*, *Glp2r*, and *Glp1r* was assessed in mouse GB, and the effects of GLP-2 on hepatic bile acid (BA) flow, intestinal and liver BA uptake, and GB gene expression were determined. GLP-2 regulation of GB volume was assessed in wildtype, $Glp2r^{-/-}$ and $Tgr5^{-/-}$ mice. The effect of GLP-2 on GB smooth muscle (GBSM) calcium transients was characterized *ex vivo*.

Results: Acute administration of the GLP-1R agonist exendin-4 lowered glucose but had no effect on GB volume in mice. In contrast, GLP-2 rapidly enhanced GB filling in a dose-dependent manner, actions maintained in the presence of cholecystokinin, and mediated through the canonical GLP-2R. GLP-2 also rapidly induced immediate early gene expression in GB, consistent with detection of the endogenous *Glp2r* in GB RNA. The ability of GLP-2 to increase GB volume was not abrogated by systemic administration of hexamethonium, propranolol, a vasoactive peptide receptor antagonist or N-Nitroarginine methyl ester, and was maintained in $Tgr5^{-/-}$ mice. In contrast, lithocholic acid, a Tgr5 agonist, increased GB filling in $Glp2r^{-/-}$ but not in $Tgr5^{-/-}$ mice. GLP-2 had no effect on ileal uptake or hepatic clearance of taurocholic acid or on hepatic bile flow, yet reduced the frequency of spontaneous calcium transients in murine GBSM *ex vivo*, in a tetrodotoxin-sensitive manner.

Conclusions: Our data extend endocrine concepts of regulation of GB filling beyond FXR-FGF15/19 and the direct effects of BA via Tgr5, to encompass a novel BA-Tgr5-L cell GLP-2 axis providing nutrient-mediated feedback from BA to terminate meal-related GB contraction. These findings have implications for conditions characterized by elevated circulating levels of GLP-2 such as after bariatric surgery and the development and use of agents that promote Tgr5 activation, L cell secretion, or GLP-2R agonism for the treatment of metabolic disease.

Keywords Bile acids; Gallbladder; Enteroendocrine; Glucagon-like peptide; TGR5; GLP-1; GLP-2

1. INTRODUCTION

Following food ingestion, a complex series of events link the stomach, pancreas, gallbladder (GB), liver, and small intestine, facilitating the digestion, absorption, and, ultimately, assimilation of available nutrients. These processes are coordinated by neural relays linking the gut to the enteric, peripheral and central nervous system, as well as by signals initiated through release of enteroendocrine cell (EEC) hormones which transmit signals from the gastrointestinal tract to the nervous system, pancreas, GB, and vasculature. Both neural circuits and EEC-derived hormones also control gut motility and satiety, providing feedback communication to ensure coordinated regulation of the intake and assimilation of available energy.

The entrance of food into the stomach triggers a number of signals, driven by peptides liberated from EECs, to enable digestion of complex macromolecules. Gastrin lowers the pH of the stomach by directly promoting acid secretion and indirectly through histamine liberation, thereby activating enzyme zymogen precursors, to begin the process of digestion. Secretin in turn inhibits gastric acid and gastrin release, augments pancreatic bicarbonate secretion, and raises the pH in the duodenum. Cholecystokinin (CCK) liberated from EECs in the proximal small bowel stimulates digestive enzyme secretion from the exocrine

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pancreas and enhances bile discharge from the GB, facilitating the emulsification, breakdown and ingestion of fats.

The physiological cycle of GB filling and emptying dictates the flow of the bile into the intestine, enabling the enterohepatic circulation of bile acids (BA), which governs bile acid homeostasis. Although classic concepts of gut endocrinology defined the importance of meal-induced CCK secretion for GB contraction and bile discharge, more recent studies have demonstrated that BA themselves exhibit unique hormonal signaling properties regulating diverse functions, including GB relaxation and refilling. One pathway involves direct BA activation of the Tgr5 receptor expressed on smooth muscle cells of the gallbladder [1,2]. The second mechanism involves BA stimulation of the farnesoid X receptor (FXR) on enterocytes, leading to release of fibroblast growth factor (FGF) 15/19 and activation of FGF Receptor 4/β-Klotho to relax GB smooth muscle [3,4].

BA also control the release of gut hormones from multiple EEC populations, stimulating the liberation of proglucagon-derived peptides (PGDPs) from L cells situated predominantly in the distal small bowel and colon. Notably agonists for both FXR and Tgr5 modulate GLP-1 secretion and in turn, activation of the canonical GLP-1 receptor (GLP-1R) [5]. The translational importance of this emerging BA-L cell pathway has been further highlighted by recent observations from studies of the cardiovascular safety of GLP-1R agonists used in the treatment of type 2 diabetes (T2D). Notably, acute gallstone disease, encompassing cholelithiasis and cholecystitis, was reported more frequently in patients treated with liraglutide [6]. Furthermore, sporadic cases of GB disease have been reported in human subjects with short bowel syndrome treated with teduglutide, a GLP-2 analogue [7,8]. Moreover, obese human subjects experience more GB disease after some forms of bariatric surgery, an intervention frequently associated with elevated levels of both BA and PGDPs, including GLP-1 and GLP-2. To explore whether GLP-1 and/or GLP-2 influence GB physiology, we have now studied the acute actions of these peptides on GB activity in mice. Here, we delineate a Tgr5-independent, GLP-2R-dependent pathway that counteracts the actions of cholecystokinin and promotes GB filling.

2. MATERIALS AND METHODS

2.1. Animals

Wild-type (WT) C57Bl/6 male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Tgr5*$^{-/-}$ mice [9], obtained from Schering-Plough/Merck, and *Glp2r*$^{-/-}$ mice, generated in our lab [10], both on a C57Bl/6 background were bred at the Toronto Centre for Phenogenomics animal facility. *Tgr5*$^{+/+}$ *and Glp2r*$^{+/+}$ littermates were used as controls for all experiments involving the corresponding knockout mice. Studies were performed on mice aged 12–14 weeks that were fasted overnight in cages containing wire grid flooring to prevent ingestion of bedding or fecal material and had free access to water. All animal experiments were approved by the Animal Care Committee of the Mount Sinai Hospital and were consistent with ARRIVE Guidelines. C57BL/6 mice studied in Vermont were euthanized using protocols approved by the Institutional Animal Care and Use Committee of the University of Vermont.

2.2. Peptides, drugs and treatments

Custom synthesized human [Gly2]GLP-2, henceforth referred to as GLP-2, was from Pepceutical Ltd. (Nottingham, UK) and recombinant exendin-4 (#7-02177) from CHI Scientific (Maynard, MA). Vasoactive intestinal peptide (VIP, #H-3775), the VIP receptor antagonist [Lys1-Pro2,5-Arg3,4-Tyr6] VIP (VIP-hybrid, #H-9935) [11] and cholecystokinin octapeptide sulfated (CCK8, #H-2080) were purchased from Bachem (Torrance, CA). The NO synthase inhibitor N^G-Nitro-L-Arginine Methyl Ester (L-NAME, #N5751), the non-selective beta-adrenergic receptor blocker propranolol (Prop, #P0884), the nicotinic receptor antagonist hexamethonium bromide (HexBr, #H0879), lithocholic acid (LCA, #L6250), and sodium taurocholate (TCA, T4009) were from Sigma Aldrich (Oakville ON, Canada). Tetrodotoxin citrate (TTX, #1069) was from Tocris Biosciences (Minneapolis, MN). Peptides and drugs were dissolved in PBS, except lithocholic acid that was dissolved in dimethyl sulfoxide DMSO, and administered to mice by intraperitoneal injection.

2.3. Determination of gallbladder volume and blood glucose levels

GB volume was estimated from the weight of the bile collected from the organ (gravimetric method) or from the GB dimensions assuming an ellipsoidal geometry (geometric method). In brief, mice were euthanized by CO_2 inhalation, gallbladder was removed, and the bile drained into pre-weight microcentrifuge tubes. GB volume was calculated from the weight of the bile collected assuming a bile density of 1 mg/μl (gravimetric method [12]). Alternatively, following euthanasia and gallbladder removal, the dimensions of the organ were determined from images taken at 10× magnification. Gallbladder volume was calculated using the ellipsoid volume formula $V = \pi/6$ (length × width × height) (geometric method [13]). As shown in Supplementary Figure 1A, there was a good agreement between the GB volume values obtained using the gravimetric and geometric methods when they were compared side by side using the same set of gallbladders from vehicle- and GLP-2-treated mice. GB volume was normalized to body weight. Blood glucose levels were measured using Contour glucose meters (Bayer Inc., Mississauga, ON, Canada) in blood samples drawn from the tail vein.

2.4. Measurement of hepatic bile flow and ileal uptake of bile acids

To assess hepatic bile flow C57Bl/6 male mice were administered vehicle or GLP-2 10 min prior to anesthesia with ketamine/xylazine. 10 min following anesthesia a laparotomy was performed, the cystic duct was ligated and a polyethylene-10 catheter was inserted in the common bile duct. Bile was collected during a 30 min period and the volume estimated by gravimetry. Preliminary experiments confirmed that the ability of GLP-2 to stimulate gallbladder filling was preserved in mice anesthetized with ketamine/xylazine (Supplementary Figure 1B). To assess ileal uptake of bile acids, mice were treated with vehicle or GLP-2 10 min prior to anesthesia as described above. Five min after anesthesia, a laparotomy was performed. Next, a 5–6 cm long ileal segment immediately adjacent to the ileocecal junction was exposed, opened to remove fecal material from the lumen by flushing with PBS and ligated at both ends. 25 min following vehicle or GLP-2 treatment a 150 μL bolus of 0.2–15 mM taurocholic acid (TCA) supplemented with 1.5 μCi [^{3}H(G)]-TCA (#NET332, PerkinElmer Health Sciences Canada Inc, Woodbridge ON, Canada) was injected in the lumen of the ileal segment. 5 min after the bolus portal and cardiac blood samples were collected. Radioactivity determined in portal blood and in cardiac blood by scintillation counting was used as a measure of the ileal uptake and hepatic clearance phases, respectively, of the enterohepatic circulation of taurocholic acid. Preliminary experiments demonstrated that the appearance of [^{3}H]-TCA in the portal blood increased linearly for at least 10 min following intraileal injection of a 2 mM [^{3}H(G)]-TCA bolus.

2.5. Assessment of cardiovascular parameters by telemetry

Cardiovascular parameters were assessed in conscious, freely moving mice via implantation of PA-C10 radiotelemetry devices (Data Sciences

International, Saint Paul, MN) as described [14]. Mice were allowed 1 week to recover following device implantation prior to data collection.

2.6. RNA extraction, cDNA synthesis and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from mouse GB, jejunum, and liver was extracted by the guanidinium thiocyanate method. Reverse transcription (RT) was performed with 500 ng of total RNA treated with DNase I (#EN0521, Thermo-Fisher Scientific, Markham ON, Canada), using random hexamers (#58875) and SuperScript III (#18080-044) from Thermo-Fisher Scientific. The resultant cDNA was used to amplify by PCR the murine GLP-2R using Platinum Taq DNA polymerase (#10966018, Thermo-Fisher Scientific) and the primer pair 5′-GCC CAG TAG ATG CAG AGA GG-3′ and 5′-AGT TGC CAA GCT GTG GTG AT-3′ at an annealing temperature of 60 °C giving rise to a 1,660 base pair product spanning the entire GLP-2R open reading frame. PCR products were analyzed by agarose gel electrophoresis followed by Southern blot and hybridization using an internal mouse *Glp2r*-specific ^{32}P-labeled oligonucleotide probe. Autoradiography was performed on a storage phosphor screen, and signals were visualized on a Personal Molecular Imager (Bio-Rad Laboratories, Mississauga ON, Canada). *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) PCR amplification was performed as previously described [15]. Real-time quantitative PCR was performed on a QuantStudio 5 System (Thermo-Fisher Scientific) with TaqMan Fast Advanced Master Mix (#4444557, Thermo-Fisher Scientific) and TaqMan Gene Expression Assays (Thermo-Fisher Scientific) for *egr-1* (Mm00656724_m1), c-*fos* (Mm00487425_m1), *Nr4a1* (Mm01300401m_1), *JunB* (Mm00492781_m1), *Ptgs2* (*Cox2*, Mm00478374_m1), *Rasd1* (Mm00842185_g1), *glp2r* (Mm01329473_m1), *Gcg* (Mm00-801712_m1), *Tgr5* (Mm04212121_s1) and *Glp1r* (Mm00-445292_m1). Relative quantification of transcript levels was performed by the $2^{-\Delta Ct}$ method using 18S rRNA, *Ppia*, *Tbp*, or *Actb* for normalization.

2.7. Imaging and analysis of Ca^{2+} events in gallbladder whole mounts

Imaging of Ca^{2+} events in GB whole mount preparations was performed as previously described [2]. Briefly, the GB was removed and opened from fundus to the cystic duct in ice-cold modified Krebs solution. The full-thickness whole mounts were stretched open and pinned serosa side up between two pieces of Sylgard connected by metal pins. Tissue was then loaded for 1 h at room temperature in a HEPES buffer containing 10 μM fluo-4AM (F14201,Thermo-Fisher Scientific, Waltham, MA) and 2.5 μg/ml pluronic acid (#24040032, Thermo-Fisher Scientific). Following washing and incubation for at least 30 min at room temperature to allow de-esterification, the preparations were placed in a Ca^{2+} imaging chamber and superfused with aerated physiological saline solution at 35–37 °C. After a 15–20 min equilibration, Ca^{2+} events were visualized using an Andor iXon^{EM} + 897 back-illuminated EMCCD camera attached to an inverted fluorescent Olympus IX70 microscope equipped with a 40X objective. Movies were acquired over periods of 20–30 s (30 frames per second) and analyzed using SparkAN, a custom software written at the University of Vermont (A. D. Bonev).

2.8. Statistics

Data are presented as Tukey boxplots or as mean ± SD. Statistical significance was assessed by ANOVA followed by the Bonferroni *post hoc* test and, where appropriate, by unpaired Student's *t* test using GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

3. RESULTS

3.1. The GLP-1 receptor agonist exendin-4 does not regulate gallbladder volume in mice

As BA increase the secretion of GLP-1 and GLP-2, we assessed whether one or both of these EEC peptides act on the GB to regulate bile discharge or refilling. Acute administration of exendin-4 at doses sufficient to lower plasma glucose had no effect on GB volume *in vivo* (Figure 1A and B). In contrast, intraperitoneal GLP-2 administration produced a rapid, dose-dependent increase in bile volume in fasted mice, with a two-fold increase seen with 2 μg/kg of GLP-2, and an estimated EC_{50} of 0.1 μg/kg (Figure 1C). These actions of GLP-2 were rapid, detectable by 10 min, and sustained for at least 60 min (Figure 1D). Moreover, the increase in bile volume was observed using 0.1 mg/kg GLP-2, a dose that stimulates nutrient absorption and expands intestinal mucosal surface area in both preclinical and clinical studies [8,16]. CCK, a potent regulator of GB activity, induced robust GB contraction and reduced bile volume in fasted wildtype mice; however, bile volume remained unchanged following co-administration of CCK and GLP-2 (Figure 1E). The actions of GLP-2 to promote GB filling were mediated by the canonical GLP-2R, as they were detected in *Glp2r*$^{+/+}$ but not in *Glp2r*$^{-/-}$ mice (Figure 1F).

3.2. The gallbladder expresses a full length canonical *Glp2r* mRNA transcript

We next examined whether the actions of GLP-2 to control GB filling might be direct, via expression of the endogenous GLP-2R in GB. Conventional RT-PCR followed by Southern blotting identified a *bona fide* full length *Glp2r* mRNA transcript in mouse jejunum, a recognized target of GLP-2 action [17,18] as well as in RNA from liver and gallbladder (Figure 2A). Consistent with the lack of effects of GLP-1R agonism (exendin-4) on GB volume (Figure 1A), levels of *Glp1r* mRNA transcripts in the mouse GB were just above the limit of detection, at least 50-fold lower than corresponding levels of *Glp2r* expression (Figure 2B); expression of the proglucagon (*Gcg*) gene was undetectable in GB, ruling out a potential local paracrine effect for GLP-2 (Figure 2B). Importantly, expression of the *GLP2R* has also been described in an independent transcriptional analysis of RNA isolated from the human GB [19] and in human transcriptome analyses (http://www.proteinatlas.org/search/Glp2r). The relative level of *Glp2r* expression in GB (Figure 2B) approximated that of *Tgr5*, a BA receptor highly expressed in the GB [9] and known to mediate BA stimulation of GB filling.

As GLP-2 rapidly induces immediate early (IE) gene expression in the intestine [20], we examined the expression of several IE mRNA transcripts in GB 60 min following a single intraperitoneal injection of vehicle or GLP-2 (5 μg/kg) to fasted *Glp2r*$^{+/+}$ and *Glp2r*$^{-/-}$ littermate mice. Consistent with findings in the murine gut, mRNA levels of *cFos*, *Egr1*, *Nr4a1*, *Cox2*, *Rasd1*, and *JunB*, among many other IE transcripts, were robustly increased after GLP-2 administration in the GB of wildtype mice but not in the GB of *Glp2r*$^{-/-}$ mice (Figure 2C). Together, these results raise the possibility that EEC-derived GLP-2 may act directly via the canonical GLP-2R to regulate GB gene expression and stimulate accumulation of bile within the GB.

3.3. GLP-2 controls gallbladder filling independent of Tgr5

Intestinal luminal bile acids activate Tgr5 expressed in mucosal enteroendocrine L cells and cooperate with luminal nutrients to stimulate postprandial GLP-1 and GLP-2 secretion [21–23]. The finding that not only Tgr5, but also GLP-2R agonism promotes gallbladder filling raised the possibility of functional crosstalk between the

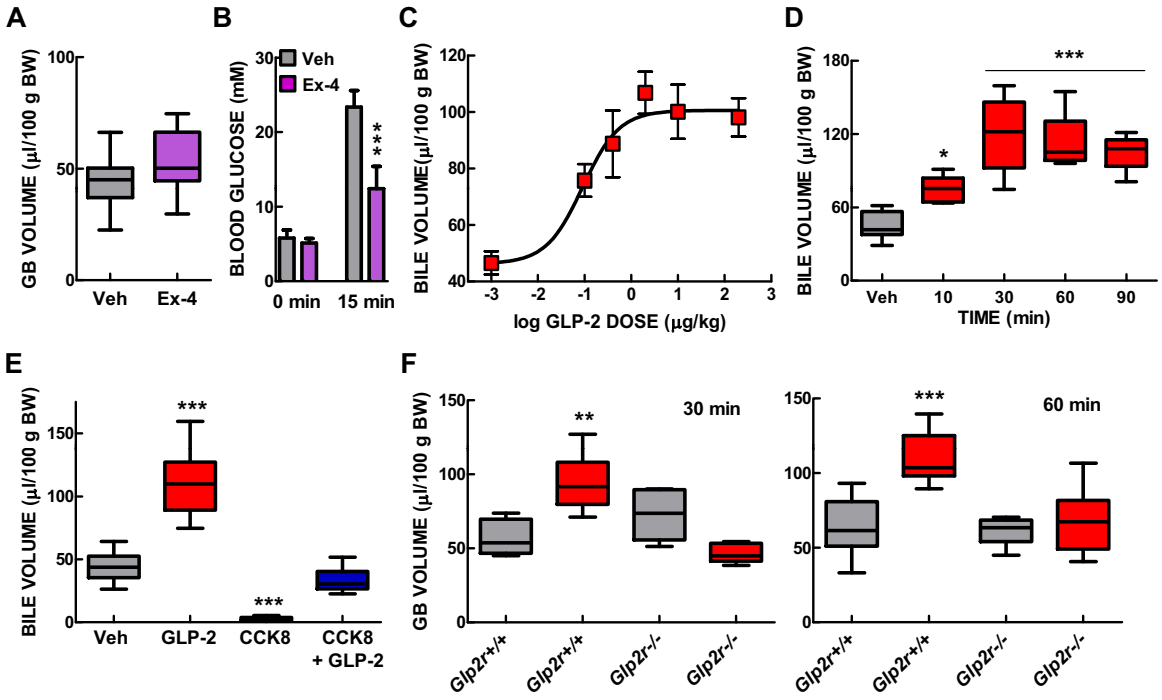

Figure 1: GLP-2 treatment acutely stimulates gallbladder filling in the mouse through the canonical GLP-2R. After an overnight fasting of C57Bl/6 male mice, (A and B) vehicle, or exendin-4 (10 nmol/kg) was administered ip followed 15 min later by ip glucose (2 g/kg). Gallbladder volume was assessed 30 min subsequent to vehicle or exendin-4 ($n = 14–16$ mice per treatment, combined from 4 independent experiments) (A) and blood glucose levels (B) monitored immediately before the glucose challenge and again 15 min after, at the time of euthanasia. Data in panel (B) are mean ± SD ($n = 10–13$ mice per condition, combined from 3 independent experiments). (C) GLP-2 was administered at the indicated doses 30 min prior to assessment of bile volume. Data are mean ± SD ($n = 5$ mice per dose). (D) GLP-2 (5 µg/kg) was administered and bile volume determined at the indicated time points ($n = 5–12$ mice per time point, combined from 2 independent experiments). (E) Vehicle, GLP-2 (5 µg/kg), CCK8 (100 ng/kg), or GLP-2 (5 µg/kg) + CCK8 (100 ng/kg) were administered 30 min prior to bile volume measurement ($n = 9–12$ mice per treatment, combined from 2 independent experiments). (F) Male mice of the indicated genotype were fasted overnight. Vehicle (gray boxplots) or GLP-2 (5 µg/kg, red boxplots) was administered 30 (left) or 60 min (right) prior to assessment of the gallbladder volume ($n = 5–11$ mice per condition, combined from 2 to 3 independent mouse cohorts). In panels (A), (D), (E), and (F) data are presented as Tukey boxplots. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs vehicle-treated either C57Bl/6 or *Glp2r*$^{+/+}$ mice.

BA/Tgr5 and GLP-2/GLP-2R signaling axes in the control of GB volume. To ascertain i) whether GLP-2 requires concomitant basal activation of Tgr5 signaling to regulate GB filling and ii) whether the GLP-2R contributes to the actions of Tgr5 agonists, we assessed GB filling in response to a) GLP-2 and b) the natural Tgr5 agonist lithocholic acid (LCA) in *Tgr5*$^{-/-}$ and *Glp2r*$^{-/-}$ mice, respectively. GLP-2 robustly increased GB volume in *Tgr5*$^{+/+}$ mice (Figure 3A). Although basal GB volume trended to be smaller in *Tgr5*$^{-/-}$ vs. *Tgr5*$^{+/+}$ mice as previously reported [1], exogenous GLP-2 administration increased GB volume in the absence of *Tgr5* (Figure 3A). In contrast LCA increased GB volume in *Tgr5*$^{+/+}$ but not in *Tgr5*$^{-/-}$ mice (Figure 3B). Moreover, the LCA-mediated increase in GB volume was preserved in both *Glp2r*$^{+/+}$ and *Glp2r*$^{-/-}$ mice (Figure 3C). These observations demonstrate that although Tgr5 agonists directly increase EEC glucagon-like peptide secretion [24,25], both GLP-2R and Tgr5 agonists stimulate gallbladder filling through independent mechanisms. Furthermore, these findings imply that administration of Tgr5 agonists may promote GB filling directly through Tgr5, and indirectly, through stimulation of GLP-2 secretion and GLP-2R-dependent GB filling.

3.4. GLP-2 does not regulate hepatic bile flow or intestinal BA uptake in mice

To identify potential mechanisms through which GLP-2 enhances bile accumulation within the GB, we explored the contributions of i) hepatic bile flow and ii) intestinal vs. liver BA uptake and BA clearance. As shown in Figure 4A, the bile flow rate, assessed following cannulation of the common bile duct, was similar in vehicle- vs. GLP-2-treated mice, indicating that a major surge in bile flow does not contribute to the acute increase in GB volume secondary to activation of GLP-2R signaling. Moreover, although GLP-2 rapidly increases intestinal blood flow [26], GLP-2 treatment prior to the intraileal injection of a bolus of radiolabeled taurocholic acid (TCA) did not alter the appearance of TCA in portal blood nor in systemic blood, which report the ileal uptake and first-pass hepatic clearance phases, respectively, of the enterohepatic circulation of TCA (Figure 4B). Furthermore, basal GLP-2R signaling is not essential for the ileal uptake of BA as no differences in the levels of [^{3}H]-TCA in portal blood or in systemic blood were observed in *Glp2r*$^{+/+}$ vs *Glp2r*$^{-/-}$ mice following intraileal [^{3}H]-TCA injection (Figure 4C).

3.5. Systemic attenuation of neural transmission does not abrogate GLP-2-mediated GB filling

The motility of the GB is regulated by the interplay of spontaneous smooth muscle electrical activity, hormones, and neurotransmitters released locally from intrinsic postganglionic neurons and extrinsic sympathetic nerves [27]. Cholinergic vagal efferents provide the major driving force to the GB ganglia, activating nicotinic receptors on intrinsic neurons to elicit excitatory output. Conversely, sympathetic fibers comprise the main source of inhibitory input to the GB suppressing vagal transmission but also cause direct relaxation and filling of the gallbladder via activation of beta-adrenergic receptors on the GB

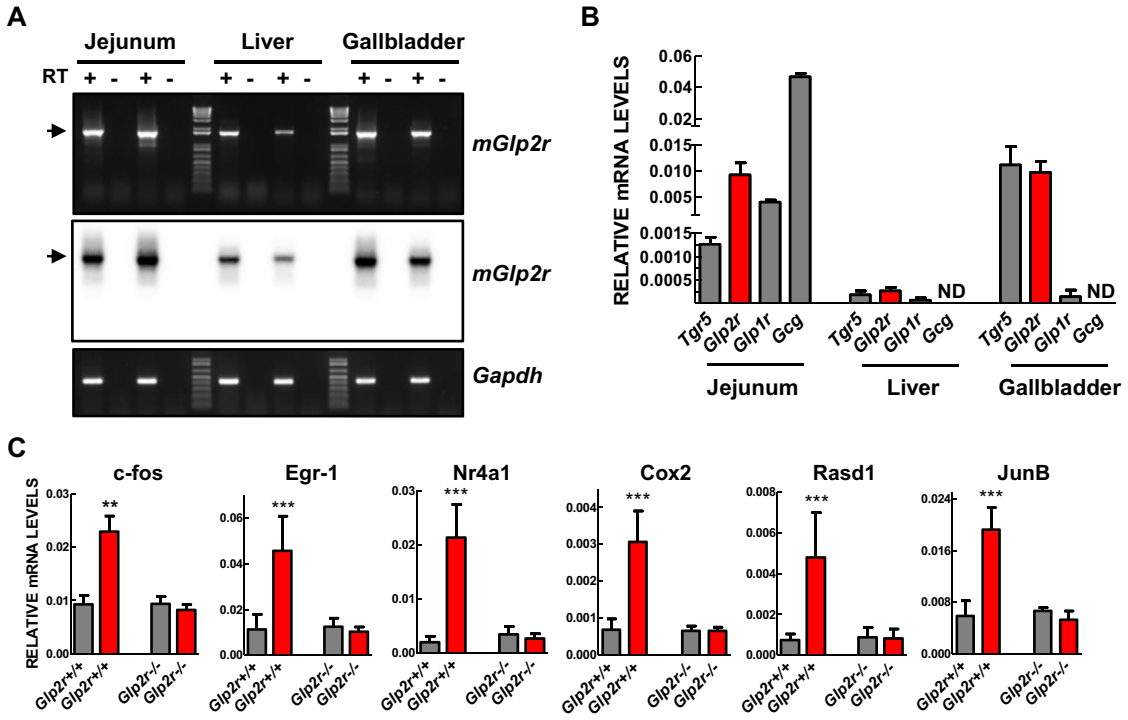

Figure 2: A canonical full-length *Glp2r* transcript is expressed in the murine gallbladder and mediates immediate early gene (IEG) induction. (A) The expression of mouse *Glp2r (mGlp2r)* and *Gapdh* transcripts was assessed by RT-PCR in total RNA from mouse jejunum, liver, and gallbladder. PCR products were analyzed by agarose gel electrophoresis followed by SYBR Green staining (top and bottom panels) or by Southern blot (middle panel) using an internal *mGlp2r*-specific ^{32}P-labeled oligonucleotide probe. The specificity of each reverse transcription reaction (RT+) was monitored by control reactions using samples in which the reverse transcriptase was omitted from the RT reaction mixture (RT−). The arrow points to a single PCR product (1660 b.p. long) spanning the entire *mGlp2r* ORF. (B) Levels for the indicated transcripts were assessed by qPCR in total RNA from the specified mouse tissues. Data are mean ± SD (n = 12 independent tissue samples for gallbladder and liver and 3 for jejunum). ND, not detectable. (C) Overnight fasted Glp2r KO male mice and wildtype littermates were treated with vehicle (gray bars) or GLP-2 (5 μg/kg, red bars). Gallbladders were collected 60 min after treatment. mRNA levels of the indicated IEG transcripts were assessed by qPCR. Data are mean ± SD (n = 5 mice per genotype, combined from 2 independent experiments). **p < 0.01 and ***p < 0.001 vs vehicle-treated *Glp2r*$^{+/+}$ mice.

smooth muscle [27]. We used pharmacological tools to test whether extrinsic innervation played a role in the effect of GLP-2 on gallbladder volume. Blocking nicotinic receptors with the antagonist hexamethonium was associated, as expected [28], with a significant increase in GB volume, which was further augmented in GLP-2 treated animals (Figure 5A). Furthermore, the non-selective beta-adrenergic receptor blocker propranolol did not prevent GLP-2-induced gallbladder filling (Figure 5B), yet markedly reduced heart rate, consistent with its known mechanism of action (Supplementary Figure 2A).

Nitric oxide (NO) and vasoactive intestinal peptide (VIP) are known to mediate actions of GLP-2 in the intestine [26,29]. NO synthase and VIP are detected in the neuronal bodies and nerve terminals of the

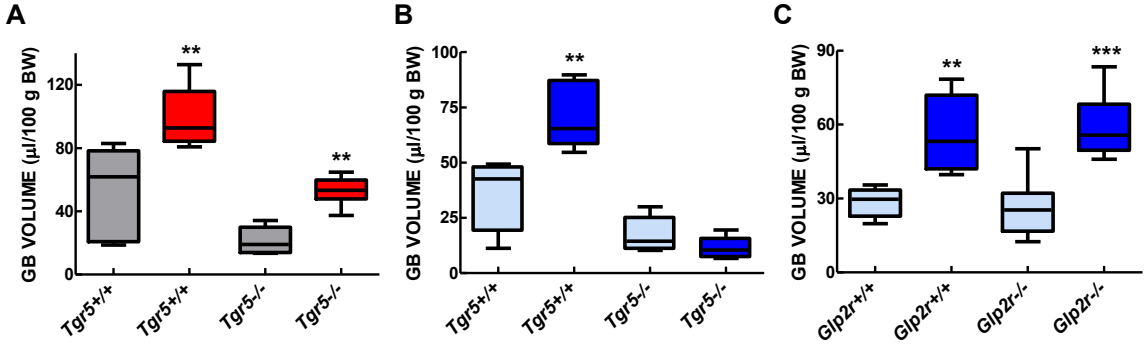

Figure 3: Both GLP-2R and Tgr5 signaling stimulate gallbladder filling but independently of one another. Male mice of the indicated genotype were fasted overnight. Vehicle (gray and light blue boxplots), GLP-2 (5 μg/kg, red boxplots) (A) or lithocholic acid (60 mg/kg, dark blue boxplots) (B and C) were administered ip 30 min prior to assessment of gallbladder volume. Data are presented as Tukey boxplots (n = 5–8 mice per condition, combined from 2 independent mouse cohorts). **p < 0.01 and ***p < 0.001 vs the corresponding vehicle-treated mice. The vehicle was PBS in panel A and DMSO in panels B and C.

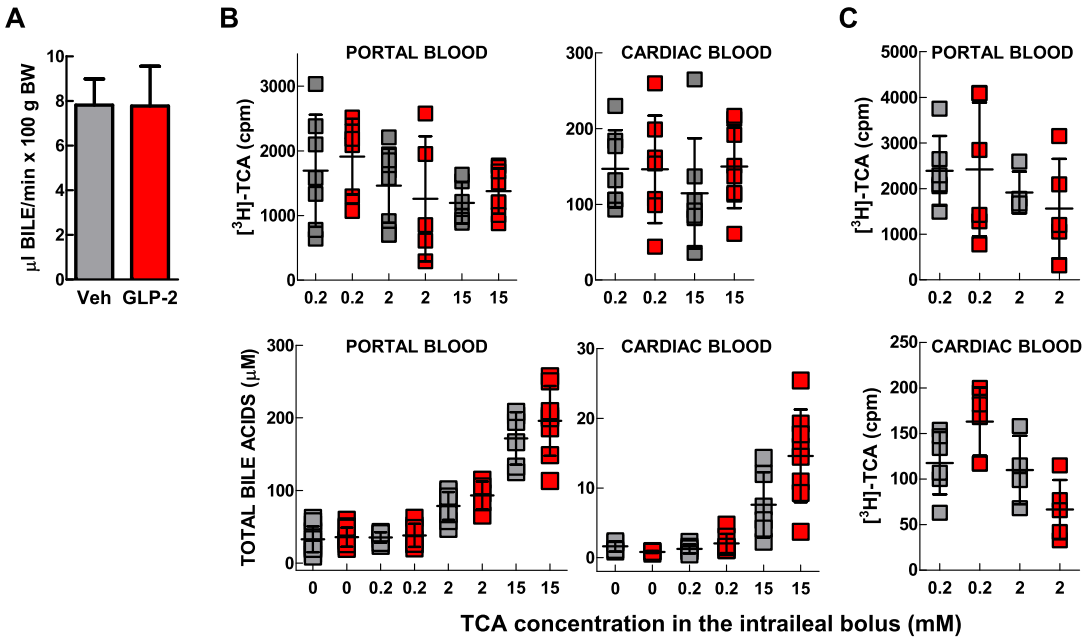

Figure 4: GLP-2R signaling does not modify the hepatic bile flow or the ileal uptake and hepatic clearance of taurocholic acid (TCA). Overnight fasted C57Bl/6 male mice were administered ip vehicle or GLP-2 10 min prior to anesthesia with ketamine/xylazine. (A) 10 min subsequent to anesthesia, a laparotomy was performed. Next, the cystic duct was ligated, and a catheter was inserted in the common bile duct. Bile was collected during a 30 min period and the volume measured. Data are mean ± SD (n = 6 and 4, for Veh- and 5 µg/kg GLP-2-treated mice, respectively) combined from 2 independent experiments. (B) 5 min after anesthesia, a laparotomy was performed, a 5–6 cm long ileal segment was isolated, cleaned of fecal material, and tied at both ends. 25 min following vehicle (gray filled squares) or GLP-2 (200 µg/kg, red filled squares) treatment, a 150 µL bolus of [^{3}H]-TCA was injected into the lumen of the ileal segment. 5 min after the bolus, portal and cardiac blood samples were collected. Radioactivity determined in portal blood (top left plot) and in cardiac blood (top right plot) was used as readout of the ileal uptake and hepatic clearance phases, respectively, of the enterohepatic circulation of TCA. Plots on the bottom of panel B illustrate total bile acid content in the portal and cardiac blood samples where [^{3}H]-TCA was determined. Bile acid levels at 0 mM TCA correspond to mice receiving an intraileal bolus of PBS. Data are mean ± SD combined from 4 independent experiments. Each data point corresponds to a mouse. (C) The ileal uptake (top plot) and hepatic clearance (bottom plot) of TCA were assessed in overnight fasted Glp2r KO (red filled squares) male mice and wildtype (gray filled squares) littermates as described above. Data are mean ± SD combined from 3 independent experiments. Each data point corresponds to a mouse.

gallbladder and both NO and VIP are released from the gallbladder wall after vagal stimulation to act directly on the GB smooth muscle causing relaxation [30] [31]. Pre-treatment with the VIP receptor antagonist VIP-hybrid or with the NO synthase inhibitor L-NAME failed to prevent the increase in gallbladder volume elicited by GLP-2 (Figure 5C). Of note, at the doses utilized, L-NAME significantly increased blood pressure whereas VIP-hybrid inhibited the hypotensive action of exogenous VIP (Supplementary Figures 2B and C). Taken together, these *in vivo* studies suggest that GLP-2-mediated regulation of GB volume does not involve suppression of vagal cholinergic excitatory input and is not strictly dependent on nitrergic, VIP-ergic, or adrenergic inhibitory neurotransmission.

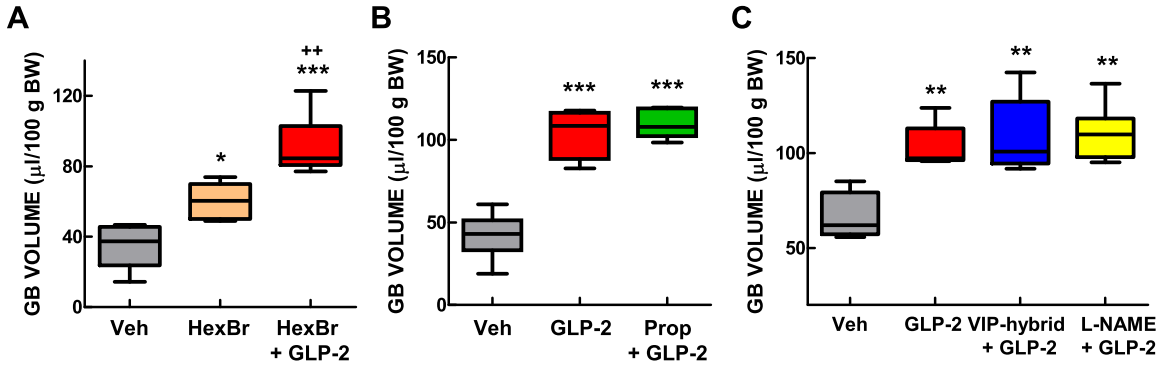

Figure 5: GLP-2 regulates gallbladder volume acting downstream of the gallbladder ganglionic relay and independently of the direct adrenergic, nitrergic, or VIP-ergic inhibitory input to the gallbladder smooth muscle. C57Bl/6 male mice fasted overnight were treated ip with vehicle, hexamethonium bromide (HexBr, 40 mg/kg) (A), propranolol (Prop, 3 mg/kg) (B), VIP-hybrid (0.5 mg/kg), or L-NAME (40 mg/kg) (C), 20 min prior to ip administration of either vehicle or GLP-2 (5 µg/kg). Gallbladder volume was assessed 30 min after. Data are presented as Tukey boxplots (for panels A and C n = 5–8 mice per condition, combined from 2 independent experiments; for panel B n = 5 mice per condition). *p < 0.05, **p < 0.01 and ***p < 0.001 vs vehicle; ++p < 0.01 vs HexBr.

3.6. GLP-2 reduces spontaneous activity in the mouse GB smooth muscle (GBSM) *ex vivo*

As neither extrinsic neural inputs nor ganglionic inhibitory output were essential for GLP-2-regulated GB filling, we assessed whether GLP-2, like CCK and Tgr5 agonists, exerts an inhibitory effect on the GBSM [1,32]. GB smooth muscle electrical activity is characterized by the generation of rhythmic spontaneous action potentials that cause synchronized increases of intracellular calcium (calcium flashes), which are associated with contractions of smooth muscle bundles [27]. We utilized calcium imaging to evaluate the effect of GLP-2 on the basal rhythmic activity of the GBSM in whole mount preparations of mouse GB *ex vivo*. As shown in Figure 6A, GLP-2 markedly reduced the frequency of GBSM calcium flashes within minutes. Treatment with tetrodotoxin alone had no effect on basal calcium flashes (Figure 6B); however, tetrodotoxin blocked the GLP-2-regulated calcium flashes (Figure 6B), consistent with a neurally-mediated action of GLP-2 on GB smooth muscle.

4. DISCUSSION

Emerging data from preclinical and clinical studies support a functional connection between luminal and/or circulating BA and the stimulation of glucagon-like peptide secretion from EECs. Moreover, the clinical use of incretin mimetics that potentiate GLP-1R signaling for the treatment of diabetes and obesity and the use of GLP-2R agonists for the treatment of short bowel syndrome has heightened the potential interest into whether and how these agents in turn might regulate the synthesis, storage, and release of BA. Our data demonstrate that acute administration of exendin-4 (chemically identical to exenatide used in the treatment of diabetes) had no effect on GB volume in fasted mice. These findings are consistent with the available human data demonstrating that acute administration of exenatide had no effect on GB volume in healthy human subjects [33], although exenatide did reduce GB ejection fraction in the presence of co-administered CCK. Furthermore, administration of liragutide once daily for 12 weeks to human subjects with type 2 diabetes had no effect on GB volume or emptying in response to a high fat meal [34]. Taken together with the very low level of *Glp1r* mRNA transcripts in the mouse GB, the biological importance of direct or indirect actions of GLP-1 on the GB remain uncertain.

In contrast, our current data clearly establish a functional link between EEC-derived GLP-2 and the control of GB filling, through the canonical GLP-2R. Our findings do not address the importance of endogenous GLP-2 for physiological control of GB emptying; rather they highlight

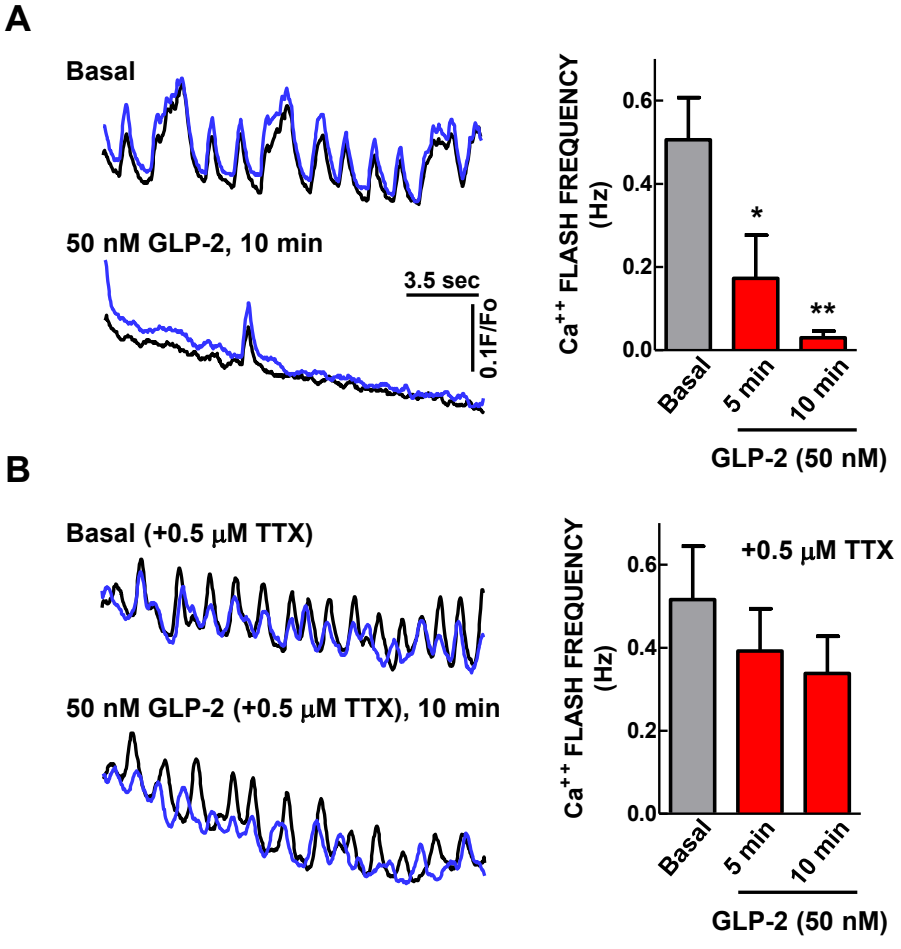

Figure 6: GLP-2 decreases the frequency of Ca^{2+} flashes in whole mount preparations of murine gallbladder. Full-thickness gallbladder tissue from C57Bl/6 mice was loaded with the calcium sensitive dye Fluo-4AM. Following wash and equilibration, spontaneous calcium events were visualized with an EMCCD camera attached to an inverted fluorescent microscope. (A) Traces showing rhythmic and synchronous calcium flashes of two GM smooth muscle cells (blue and black) from a single muscle bundle. Application of GLP-2 to the bath rapidly reduced the frequency of the spontaneous calcium flashes in the GB smooth muscle. (B) Rhythmic and synchronous calcium flashes persist when GLP-2 is applied in the presence of tetrodotoxin (TTX). Data are mean ± SE from 4 to 6 GB preparations per group. *$p < 0.05$ and **$p < 0.01$ vs basal. Fluorescence intensity is expressed as F/Fo, ratio of measured fluorescence (F) to baseline fluorescence (Fo).

the potential for elevated levels of GLP-2 to reduce GB emptying, even in the face of a hormone that robustly promotes GB contraction, exemplified by CCK. Our findings raise the possibility that the GLP-2/GLP-2R axis is a component of the postprandial feedback loop that promotes GB relaxation and refilling following CCK-induced contraction and release of bile into the duodenum. Hence, it seems reasonable to view the expression of the BA acid receptor Tgr5 on GLP-2-secreting EECs as a component of a proximal-distal gut endocrine system that attenuates the acute GB contractile response to food ingestion and facilitates refilling of the GB for the next meal (Figure 7).

The delineation of a GLP-2-GLP-2R GB axis (Figure 7) that controls GB filling raises new considerations for understanding the pathophysiology and treatment of metabolic disease. Plasma levels of GLP-2 and BA are increased in obese subjects following bariatric surgery, a population at significantly increased risk for gallstones and acute GB disease [35,36]. Although the association of bariatric surgery and biliary disease has been generally ascribed to weight loss, it is notable that elevated levels of BA may promote i) FXR-dependent secretion of FGF15/19 and ii) Tgr5 activation, leading to indirect and direct mechanisms that attenuate GB emptying. Our current findings reveal a third mechanism, namely BA-mediated activated of Tgr5 on EECs, leading to GLP-2 secretion and GLP-2R activation that further impedes GB emptying. Collectively, these observations have potential implications for increased retention of BA in the GB, the development of lithogenic sludge and gallstone formation.

Our findings also imply that both synthetic and naturally occurring Tgr5 agonists likely promote GB filling through both direct Tgr5-dependent actions on the GB [1] and, indirectly, through the EEC, GLP-2 secretion, and GLP-2R activation. Although most studies of Tgr5-mediated EEC secretion have focused on analysis of GLP-1 [24], GLP-2 is co-secreted, together with GLP-1, following Tgr5 activation [25], and circulating GLP-2 is likely to be increased following sustained increases in BA and Tgr5 activation *in vivo* [21]. Despite the apparent basolateral localization of Tgr5 on EECs [37], increased GB filling has been detected with intestinally-restricted Tgr5 agonists, consistent with the ability of some gut-restricted Tgr5 agonists to modestly enhance glucagon-like peptide secretion [38].

In summary, we define a new pharmacological action for GLP-2, namely the enhancement of GB filling via a decrease in GB smooth muscle activity. These findings include actions of GLP-2 on the GB to attenuate electrical activity and promote smooth muscle relaxation and require the canonical GLP-2R. These observations have implications for therapeutic strategies designed to increase L cell secretion, or GLP-2R signaling, for the treatment of metabolic disease.

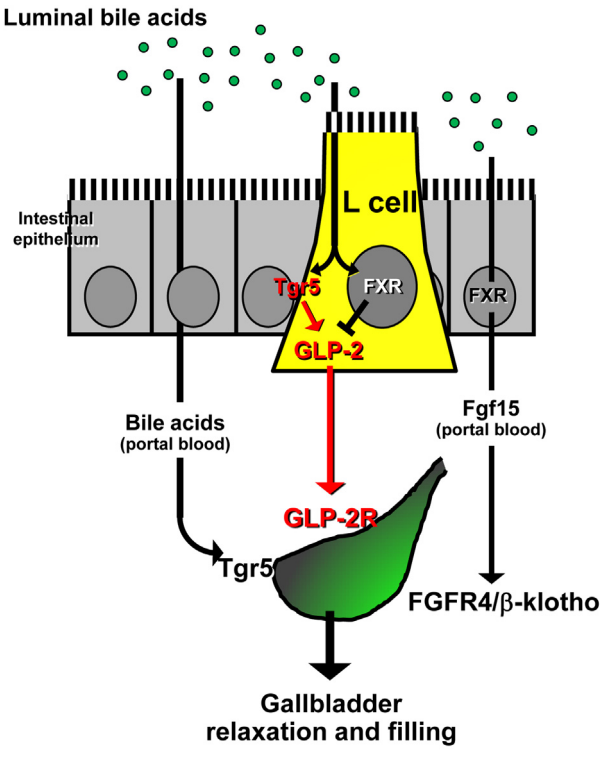

Figure 7: Bile acid-dependent pathways that promote GB refilling following bile discharge into the intestine. We propose that the GLP-2/GLP-2R axis is a component of the postprandial feedback loop that promotes GB relaxation and refilling following CCK-induced contraction and release of bile into the duodenum. Bile acids may promote GB filling directly through Tgr5-dependent actions on the GB, indirectly through FXR-FGF15/19 or through Tgr5-dependent stimulation of GLP-2 secretion from the L cell and activation of the GB GLP-2R.

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CONFLICT OF INTEREST

DJD is a party to a GLP-2 licensing agreement, together with the University Health Network, the University of Toronto, and NPS Pharmaceuticals, Lexington MA, a wholly owned subsidiary of Shire Pharmaceuticals Inc.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.03.006.

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