The aromatic amino acid sensor GPR142 controls metabolism through balanced regulation of pancreatic and gut hormones

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ABSTRACT

Objectives: GPR142, which is highly expressed in pancreatic islets, has recently been deorphanized as a receptor for aromatic amino acids; however, its physiological role and pharmacological potential is unclear.

Methods and results: We find that GPR142 is expressed not only in β- but also in α-cells of the islets as well as in enteroendocrine cells, and we confirm that GPR142 is a highly selective sensor of essential aromatic amino acids, in particular Trp and oligopeptides with N-terminal Trp. GPR142 knock-out mice displayed a very limited metabolic phenotype but demonstrated that L-Trp induced secretion of pancreatic and gut hormones is mediated through GPR142 but that the receptor is not required for protein-induced hormone secretion. A synthetic GPR142 agonist stimulated insulin and glucagon as well as GIP, CCK, and GLP-1 secretion. In particular, GIP secretion was sensitive to oral administration of the GPR142 agonist an effect which in contrast to the other hormones was blocked by protein load. Oral administration of the GPR142 agonist increased [³H]-2-deoxyglucose uptake in muscle and fat depots mediated through insulin action while it lowered liver glycogen conceivably mediated through glucagon, and, consequently, it did not lower total blood glucose. Nevertheless, acute administration of the GPR142 agonist strongly improved oral glucose tolerance in both lean and obese mice as well as Zucker fatty rat. Six weeks in-feed chronic treatment with the GPR142 agonist did not affect body weight in DIO mice, but increased energy expenditure and carbohydrate utilization, lowered basal glucose, and improved insulin sensitivity.

Conclusions: GPR142 functions as a sensor of aromatic amino acids, controlling GIP but also CCK and GLP-1 as well as insulin and glucagon in the pancreas. GPR142 agonists could have novel interesting potential in modifying metabolism through a balanced action of gut hormones as well as both insulin and glucagon.

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1. INTRODUCTION

In the academic world, GPR142 has remained a rather overlooked orphan receptor since it was cloned in 2006 [1], although a comprehensive profiling of G protein coupled receptor (GPCR) expression [2] by Regard and coworkers identified early on that it was highly and almost exclusively expressed in the islets of the pancreas, and it was also shown to be associated with an obese phenotype upon knock down in zebrafish [3]. In the pharmaceutical industry, these observations placed GPR142 on the shortlist of potentially interesting metabolic targets, which led to the discovery of potent, efficacious, synthetic GPR142 agonists that could stimulate insulin secretion and thereby improve glucose tolerance in mice and even monkeys [4—7]. Nevertheless, in view of the success of highly efficacious GLP-1 mimetics, which display a series of additional beneficial effects including improved cardiovascular outcome, which also is the case for SGLT-2 inhibitors, the pharmaceutical industry lost interest in targets such as GPR142, which apparently only provided glucose-dependent insulin secretagogue activities.

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Nevertheless, our knowledge about GPR142, its physiological role and its pharmacological potential, is very limited. In 2012, the Amgen group described phenylalanine-related synthetic agonists for GPR142 as pharmacological tool compounds, and noted that aromatic amino acids acted as agonist for the receptor [4]. In 2016, Lin and co-workers confirmed this and demonstrated that GPR142 agonists could stimulate not only insulin but also the secretion of intestinal incretin hormones [8]. Very recently, the same group from Eli Lilly proposed that the effect of GPR142 on insulin secretion is mediated by the α-cells of the pancreatic islets and that treatment of islets with GPR142 agonists for several days increased the ability of α-cells to store and secrete GLP-1 [9]. Importantly, the GPR142 agonist also increased β-cell proliferation, which they interpreted to be an indirect effect mediated through local production of GLP-1 in the islets.

Originally [10], we decided to study GPR142 expression and function rather broadly in an unbiased manner mainly using the synthetic GPR142 agonist C-22 as a pharmacologic tool [5]. This compound was shown to have good oral bioavailability although a relatively fast plasma clearance [7]. We focused on effects on gut and pancreatic hormones and studied positive interactions of GPR142 agonism with dietary intake of glucose and protein. We discovered that GPR142 efficiently stimulated pancreatic glucagon secretion, as also recently described [9], and we found that the effect of GPR142 agonism on glucose disposal is a balance between insulin-mediated glucose uptake in fat and muscle and glucagon-mediated glucose production in the liver. Here we present the results from those acute studies using the synthetic GPR142 agonist, and we describe the metabolic phenotype of GPR142 deficient mice as well as the effects of chronic treatment of diet induced obese (DIO) mice with the GPR142 agonist. We find that, surprisingly, GPR142 agonism also increases energy expenditure and improves insulin sensitivity.

2. MATERIAL AND METHODS

2.1. Compounds
Amino acids were purchased from Sigma—Aldrich (St. Louis, MO). The GPR142 agonists C-I and C-22 [5] were synthesized by Wuxi AppTec Inc (Shanghai, China).

2.2. Animals
All animal studies were approved by the Danish Animal Experiments Inspectorate. Male C57BL/6NTac wild type and GIP—receptor knockout (GIPRKO), GPR142 knockout (GPR142KO) and GIP-Venus, Glu-Venus transgenic mice were used in this study. In addition, several separate cohorts of C57BL/6NTac mice that were used for compound testing were purchased from Taconic (Denmark). GIP-Venus and Glu-Venus reporter mice have been previously described [11,12]. All mice were kept and bred at the Department of Experimental Medicine at Copenhagen University (except for GIP-Venus reporter mice that were bred at Cambridge Institute for Metabolic Science). All mice were provided with food and water ad libitum and maintained on a 12-h light/dark cycle. DIO mice were generated by placing C57BL/6NTac (Taconic, Denmark) mice on high fat diet (D12492, Research Diets, NJ, USA) including 60 kcal% from fat, 20 kcal% from carbohydrates starting at 8 weeks of age. The DIO mice were 35—40 weeks of age at the time of the experiment.

7-week-old male Zucker fatty rats (Charles River Laboratories, MA) were fed on chow diet (D5008, Research Diets, NJ, USA) and water ad libitum and maintained on a 12-h light/dark cycle.

2.3. GPR142 knockout mouse generation
The gene-targeting vector for generation of a GPR142 knockout line was created through replacement of a 73 base-pair fragment (5′ TGG CAC TGG CCC GTC TTG CTG CCA GGA CCA GGA AAC CCT CCT ATC ACT ATC TCC TGG CAC TCA CGG CTT CAG AT-3′) of the coding sequence of mouse GPR142 within exon 3 with a lacZ-Neo-stop codon cassette, resulting in the deletion of transmembrane domains 2—7 of the GPR142 protein. The germ knockout line was backcrossed to C57BL/6NTac for 8 generations. Mouse genotyping was performed by PCR of genomic DNA with the following oligonucleotides: 5′- AAC GTC CTG ACT TCA GTG GCA CTG G-3′; 5′- AAC GTC CTG ACT TCA GTG GCA CTG G-3′; 5′- GGG CCA GCT CAT TCC TCC CAC TCA T-3′. The PCR products consisted in fragments of 249 bp (wild type allele) and 449 bp (knockout allele).

2.4. Generation of GPR142 stable cell lines and inositol phosphate accumulation assay
Mouse GPR142 was RACE cloned from mouse cDNA libraries. Inducible stable cell line was generated in Chinese hamster ovary (CHO) cells using the GeneSwitch mifepristone-regulated expression system according to the manufacturer's instruction (Life Technologies, NY, USA). Cells were plated into poly-D-lysine-coated 96-well plates (20,000 cells/well) and incubated overnight at 37 °C with 2 nM Mifepristone to induce GPR142 expression. The following day cells were incubated with 0.5 μCi/ml myo[^{3}H]inositol (Perkin Elmer) in 100 μL growth medium. 24 h after, IP accumulation assay were performed as described earlier [13].

2.5. Gene expression analysis
Human total RNA was obtained from Invitrogen (CA, USA). Mouse total RNA was isolated from tissues collected from C57Bl/6NTac mice (Taconic, NY, USA). Fluorogenic Taqman probes for GPR142 were

purchased from Applied BioSystems (CA, USA). Relative expression levels of GPR142 were determined by real-time quantitative PCR using the ABI PRISM 7900 Sequence Detection System from Applied BioSystems (CA, USA) and normalized to beta-actin mRNA levels.

2.6. Single-cell suspension and FACS purification

Mouse α- and δ-cells were FACsorted from GLU-Venus and Sst-CrexEYFP derived islets, respectively, while β-cells were co-isolated based on their bigger size identified by the forward and side scatter signal, as described previously [14]. RNA was isolated and processed using the Ovation Rapid DR Library System (NuGEN) and sequenced using an Illumina HiSeq 2500 system at the Genomics Core Facility, Cancer Research UK Cambridge Institute (Cambridge, UK), as described previously [14].

Single cell suspensions from the small intestine of GIP-Venus or Glu-Venus mice were prepared and separated into fluorescence-positive and fluorescence-negative pools of cells using fluorescence-activated cell sorting (FACS) as described [15]. The cells were sorted directly into lysis buffer (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) and the RNA was purified using the RNAqueous-Micro RNA isolation kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). cDNA was prepared using Superscript III (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The expression of 379 G protein-coupled receptors was tested using custom-designed 384 well qPCR plates from Bar Harbor BioTechnology. Primer target sequences have been published before [16]. A genomic DNA sample was used as a calibrator and relative copy numbers were calculated as described [16].

2.7. Insulin and glucagon secretion assay in isolated islets

Islets were isolated from C57Bl/6NTac mice (Taconic, Denmark) with liberase digestion and allowed to recover overnight in RPMI 1640 medium with 10% FBS, penicillin—streptomycin, and 11 mmol/L glucose. Islet perifusion method was described previously [17]. Human islets were acquired from the Islet Transplant Center (University of Chicago, Chicago, IL). After culturing overnight in RPMI 1640 medium with 11 mM glucose, isolated islets were pre-incubated at 37 °C in freshly prepared Krebs—Ringer Bicarbonate (KRB) buffer without glucose for 2 h followed by another 2 h of incubation in KRB buffer with 2 or 16 mM glucose. For glucagon secretion, batches of 10 isolated islets were equilibrated in KRB buffer at 2 mmol/L glucose for 45 min at 37 °C and then transitioned in medium with presence or absence of C22. Glucose was increased in a stepwise manner. Supernatants were collected and assayed for hormone content.

2.8. GLP-1 secretion from primary ileal organoids

Mouse ileal organoids were established as previously described [18,19]. For GLP-1 secretion experiments, 15 μL of Matrigel/organoid suspension was transferred into each well of 96 well plates. 48 h following seeding, organoids cultures were washed with DMEM/F12 supplemented with 2 mM L-glutamine and 10 mM HEPES and incubated in 30 μL of the same medium with either 1% DMSO (vehicle) or with test compounds during 2 h at 37 °C. Medium was collected and spun at 300 g for 2 min at 4 °C, transferred to fresh tubes, and assayed for hormone content. After the experiment, the DNA content in the wells was measured using DNA Quantitation Kit Fluorescence Assay (Sigma) according to the manufacturer's instructions. Experiment was repeated 3 times with 3—6 replicates per series. GLP-1 levels were measured with Total GLP-1 kit (MesoScale Discovery®, MD, USA, cat. no. K150JVC). GLP-1 values from each well were normalized to the DNA content. The mean value obtained from the vehicle wells in each experiment was taken as a

reference and compound-stimulated secretion was calculated as fold to the vehicle.

2.9. Treatment and hormone measurements

Mice were gavaged with indicated doses of compounds or vehicle (sterile water) in 10 ml/kg volumes, according to body weight. Blood was collected from mice at indicated times either by orbital bleeding in conscious mice or from the necks of mice immediately after decapitation. Blood samples were collected in tubes containing EDTA, DPPIV and Aprotinin (Sigma). Samples were centrifuged at 3,500 g for 10 min at 4 °C; then, supernatants were transferred into separate tubes. Enzymatic assay kits were used for determination of plasma insulin and total GLP-1 (MesoScale Discovery®, MD, USA; cat. no. K152BZC and K150JVC), glucagon (Mercodia, Sweden; cat. no. 10-1281-01), total GIP (Millipore, MA, USA; cat. no. EZRMGIP-55K), respectively. CCK was measured using specific radioimmunoassays as described previously [20].

To measure tissues hormone content, a fragment of tissue was homogenized in acid-ethanol (1.5% HCl in 70% EtOH) and extracted overnight at −20 °C. The solution was centrifuged to remove unhomogenized tissue and neutralized. Hormone concentrations in extracts were determined as described above.

2.10. Gastric emptying

Gastric emptying was assessed by the acetaminophen absorption. Vehicle, 2 g/kg whey protein alone, C-22 (30 mg/kg) alone or in combination was co-administered orally with 100 mg/kg acetaminophen (Sigma—Aldrich) to overnight fasted mice. Blood samples were drawn from the retro-orbital sinus at 15, 30, and 60 min after administration. Plasma was prepared as described above and acetaminophen concentrations were determined using an acetaminophen kit with colorimetric detection (MULTIGENT, B2K996; Abbott Laboratories). Gastric emptying by phenol red method was performed as described previously [21].

2.11. Metabolic cages

Whole-body metabolic states were tested by indirect calorimetry in TSE metabolic cages (LabMaster, TSE Systems, Germany). After a 4-day habituation, oxygen consumption, CO_2 production, energy expenditure, food and water intake, and spontaneous locomotor activity were measured simultaneously and continuously for 3 days in housing cages utilizing an indirect open circuit calorimetry system with an infrared photo beams. Glucose oxidation in g/min/kg = $[(4.545 \times VCO_2) - (3.205 \times VO_2)]/1000$ was calculated as described [22].

2.12. Glucose and insulin tolerance tests

Oral glucose tolerance tests (OGTT) were performed as described before [17]. For test, mice were fasted overnight (DIO mice for 6 h), after which 2 g/kg (for DIO mice 1 g/kg) glucose solution was administered orally at a volume of 10 ml/kg. For compound testing, mice received either vehicle or compound C-22 30 min prior glucose loading by oral gavage. Blood glucose was measured from tail blood with a OneTouch Ultra glucometer (LifeScan, CA, USA) at 15, 30, 60, and 120 min. For OGTT in male Zucker fatty rats, glucose was administered at 3 g/kg. Insulin tolerance tests (ITT) were conducted as described before [17], 6 h fasted mice, were given an i.p. injection of 1 U/kg regular human insulin (Novo Nordisk, Denmark). Blood glucose levels were measured as described above.

2.13. Glucose uptake

Mice were fasted for four hours starting at the beginning of light phase. The mice were then gavaged with C-22 (30 mg/kg) and subsequently

anesthetized with pentobarbital (80 mg/kg body weight). 15 min later, before the assay was initiated, the pain reflex was tested and tail tip was cut off. Blood glucose levels were measured with a OneTouch Ultra glucometer (LifeScan, CA, USA) and a blood sample (40 μl) was taken as baseline. At time 0, the mice were injected retro-orbitally with a bolus of [3H] 2-deoxyglucose ([3H]2-DG; 0.3335 mCi/kg). Samples were again taken at 5, 10, 15, 20, 30, and 40 min, after which mice were euthanized by cervical dislocation and various tissues, were dissected and instantly frozen in liquid nitrogen and stored at $-80\ °C$ until future analysis. Blood and tissue samples processing were performed as described earlier [23,24].

2.14. Western-blot analysis

Western blot analyses were performed as previously described [23] using the following antibodies (all were from Cell Signaling Technology): phospho- Akt (Ser^{473}) (#9271, 1:1,000), phospho- GSK-3β(Ser^9) (#9336, 1:1,000), phospho-GS(Ser^{641}) (#3891, 1:1,000). Total protein content, AKT (#9272, 1:1,000), GSK3β (#9315, 1:1,000), GS (#3886, 1:1,000) were used to normalize phosphorylated proteins. GAPDH (#3683, 1:1,000) was used as an internal loading control.

2.15. Chronic treatment with C-22 mixed into high fat diet

The experiment was performed in single-housed DIO mice (27 weeks of HFD) habituated to single housing for two weeks. Vehicle or treatment (C-22, at dose 600 or 2,000 mg/kg food) were homogeneously blended into high fat diet (D12492, Research Diets, NJ, USA) and grounded into pellets, which were stored at -20 until use. To insure absence of aversive effects the animals, food intake and body weight were monitored daily and compared to individual baselines measured prior to the experiment (Suppl. Figure 7a,b). During initial food habituation, the animal group with C22 at dose 2000 mg/kg food demonstrated transiently, significantly reduced food intake (Suppl. Figure 6a), probably as result of taste aversion. The weight loss observed in this group (Suppl. Figure 7b) could confound further result interpretation; therefore, this group was terminated. C-22 in dose 600 mg/kg food was well tolerated with no visible aversive effect on food intake. All mice continued on the treatment diet for the 6-week study. After 2.5 weeks of treatment, mice were habituated to metabolic cages for 4 days. Whole-body metabolic states were then tested in TSE metabolic cages (LabMaster, TSE Systems, Germany) continuously for 3 days. OGTT was conducted after 4.5 weeks of treatment. Prior to OGTT mice were fasted for 6 h and fasting glucose and fasting insulin were measured. ITT were performed after 5.5 weeks treatment in 6 h fasted animals. Mice were given an i.p. injection of 1U/kg regular human insulin (Novo Nordisk, Denmark). Blood glucose levels during OGTT and ITT were measured as described above.

2.16. Statistical analysis

Statistical analyses were conducted in Prism (Version 4.0.3, Graphpad, CA, USA) either by one- or two-way analysis of variance (ANOVA) followed by Sidak's *post hoc* test and Student's t-test as appropriate. Statistical significance was defined as two-tailed $p < 0.05$.

3. RESULTS

3.1. GPR142 is expressed mainly in pancreatic islet and enteroendocrine cells

In agreement with the original report [2], we observed that, in both humans and mice, GPR142 is expressed orders of magnitude higher in pancreatic islets as compared to any other tissue tested (Figure 1A; and Supp. Figure 1). Originally, it was believed that GPR142 mainly was involved in β-cell function [5,7]; however, as shown in Figure 1B, GPR142 expression is higher in glucagon storing α-cell and in somatostatin storing δ-cells than in the β-cells, which is in agreement with recent single cell analysis of islets cells [25] and recent *in situ* hybridization data [9]. Low level GPR142 expression could be detected also in the GI-tract (Figure 1A), and qPCR analysis of FACS-purified GIP-Venus positive cells from Gip-Venus reporter mice revealed that although GPR142 was not that highly expressed it was one of the most highly enriched (68—fold) GPCRs in GIP cells (Figure 1C). Similar analysis of GLP-1 cells in different segments of the small intestine using the proglucagon (GCG), Glu-Venus reporter mice demonstrated that GPR142 was only enriched in the jejunum, not in the duodenum or the ileum (Figure 1D). In fact, GPR142 was expressed at close to background levels in GLP-1 cells of the ileum. In contrast, the expression of GPR142 was 50—100-fold higher in pancreatic Glu-Venus positive cells, i.e. α-cells, compared with Glu-Venus positive cells (i.e. L-cells) of the GI-tract (Figure 1D).

3.2. GPR142 is a highly selective receptor for aromatic amino acids — in particular Trp

When tested against all 20 natural L-amino acids, GPR142 proved to be extremely selective for the aromatic amino acids, as judged by the ability of L-Trp and L-Phe and with lower efficacy L-Tyr but none of the other natural amino acids to stimulate IP3 accumulation in GPR142 transfected CHO cells at 20 mM concentrations (Figure 2A). Dose response experiments revealed that L-Trp had a potency of 0.18 mM on GPR142, which was approximately 10-fold more potent than L-Phe (Figure 2B). Interestingly, certain di- and tri-peptides with N-terminal Trp also acted as agonists on GPR142 (Figure 2C). Thus, the di-peptide Trp-Tyr apparently had a potency like free L-Trp, whereas, for example Trp-Val and Trp-His were either less potent or inactive, respectively. We tested a series of tri-peptides with a Ser residue in position two to improve solubility. As shown in Figure 2C (right panel), Trp-Ser-Ser had a potency like the free L-Trp, but a maximal efficacy of only 25% whereas Ser-Ser-Trp showed limited activity, indicating the importance of the free a-amino group of Trp for GPR142 binding. Tri-peptides with Phe or Tyr at the N-terminus were totally inactive. We also tested a number of derivatives of tryptophan such as serotonin and melatonin as well as tryptophan metabolites of the kynurenine pathway, many of which also are biologically active but all of which failed to activate GPR142 (Supp. Table 1). Similarly, a number of microbial aromatic metabolites did not activate GPR142. Only 5-hydroxytryptophan stimulated GPR142, but with low efficacy and with more than ten-fold lower potency than L-Trp (Supp. Table 1).

3.3. GPR142 is required for the stimulatory effects of L-Trp but not protein on hormone secretion

L-Trp is known to stimulate the secretion of not only insulin but also glucagon and gut hormones [26—28]. In fact, L-Trp potentiated glucose stimulated insulin secretion from isolated murine islets with an efficacy almost similar to that of GLP-1 (Figure 2D). *In vivo*, 500 mg/kg of L-Trp administered by oral gavage increased plasma levels of insulin, glucagon, GIP and GLP-1 in overnight fasted wild type mice, although the increase in GLP-1 did not reach statistical significance in these particular experiments (Figure 2E). The very high dose was based on pilot dose-response experiments and doses used in the
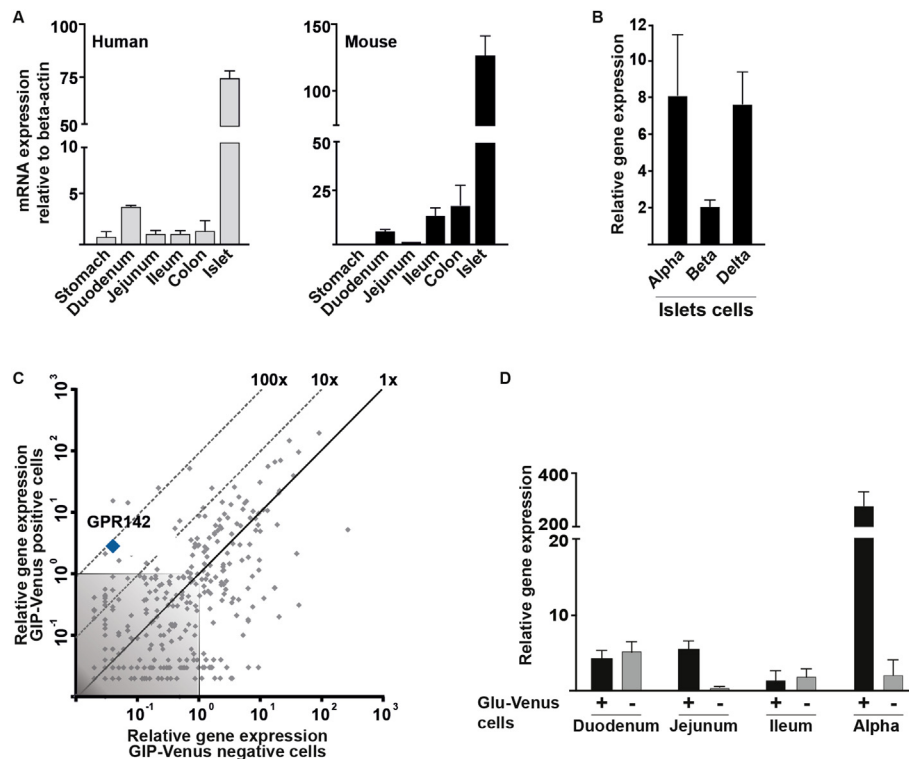
Figure 1: Expression of GPR142 in human and mouse tissues. **(A)** Expression of GPR142 mRNA in human (n = 3) and mouse (n = 3). GI-tract and pancreas assessed by RT-PCR and normalized to beta-actin. **(B)** GPR142 expression in FACS-purified α-, β-, and δ-cells from mouse pancreas (see Materials and Methods for details). The data were derived from n = 5, n = 4 and n = 6 independent α-, β-, and δ-cell isolations respectively. **(C)** Scattergram of the expression of 379 GPCRs in relative copy numbers in GIP-Venus-positive cells (y-axis) vs expression in GIP-Venus-negative cells (x-axis). The dotted lines indicate 10- and 100-fold enrichment of expression in GIP-positive vs neighboring cells. In gray is indicated the noise area for the analysis (∼ cycle threshold = 35). The data were derived from n = 3 independent cell isolations. **(D)** qPCR analysis (n = 3) of the GPR142 expression in Glu-Venus positive vs. Glu-Venus negative fraction of sorted cells from segments of the small intestine and from the pancreas. The results are presented as relative expression to reference genes (average of HPRT, Rn18s and Ywhaz).

literature [26]. Importantly, the fact that no hormone response to L-Trp was observed in GPR142 deficient mice indicate that L-Trp's effect on gastrointestinal and pancreatic hormones is mediated through GPR142 (Figure 2E).

Because GPR142 is a sensor of essential aromatic amino acids and certain oligo-peptides, the receptor could very well be involved also in the hormone responses to dietary protein intake. However, as shown in Figure 2F, GPR142 is not required, as the response in both insulin and glucagon as well as GIP and GLP-1 to an oral protein challenge was identical in GPR142 deficient mice as in littermate control animals.

### 3.4. GPR142 deficient mice display no major metabolic phenotype
Regarding overall phenotype, the GPR142 knockout mice exhibited normal growth with no significant difference from littermate controls in body composition, apart from a trend towards increased lean mass in the GPR142 knockout mice (Supp. Figure 2a). Indirect calorimetry did not reveal any difference in energy expenditure and respiratory exchange ratio (RER) and no changes in spontaneous locomotor activity and food intake (Supp. Figure 2b—e). GPR142 knockout mice also had normal response to fasting/re-feeding challenge in terms of basal metabolic parameters (Supp. Figure 2b—e) and displayed normal insulin responses during oral glucose tolerance test (OGTT) (Supp. Figure 2f,g). Importantly, exposure to prolonged high-fat diet did not induce any significant difference in the metabolic phenotype including response in OGTT and ITT (Supp. Figure 3a—h).

3.5. GPR142 activation stimulates insulin secretion in a glucose-dependent manner and is not blocked by protein intake
To try to understand the role of GPR142 in metabolism in more detail we turned to pharmacological studies and among the high potency, efficacious, synthetic agonists for GPR142 [4—7] we chose the potent (Kd = 22 nM, Figure 2B), orally active compound 22 [5] to initially focus on effects on pancreatic hormone secretion.

As shown in Figure 3A, the synthetic GPR142 selective agonist stimulated insulin secretion from mouse islets in a highly glucose-dependent fashion. This was also the case in human islets as here shown using another GPR142 agonist, C1 (Figure 3B). C22 also induced insulin secretion from the Min6 mouse β-cell line, which express endogenous GPR142 receptors (Supp. Figure 4).

In fasting lean mice, oral administration of C-22 increased plasma levels of insulin in a dose-dependent manner (Figure 3C), which was not observed in GPR142 deficient mice (Figure 3D). The *in vivo* insulin response to the GPR142 agonists was strongly increased when co-administered with glucose (2 mg/kg) (Figure 3E). As GPR142 is an amino acid receptor it was possible that an attenuated response to the synthetic agonist could be observed in the presence of increased levels of amino acids following an oral protein challenge. However, as shown in Figure 3F, the incremental insulin response to the GPR142 agonist was not diminished but in fact significantly enhanced when given in combination with a high oral protein load, which, importantly in these experiments, by itself increased plasma insulin to the same degree as C-22 alone (Figure 3F).
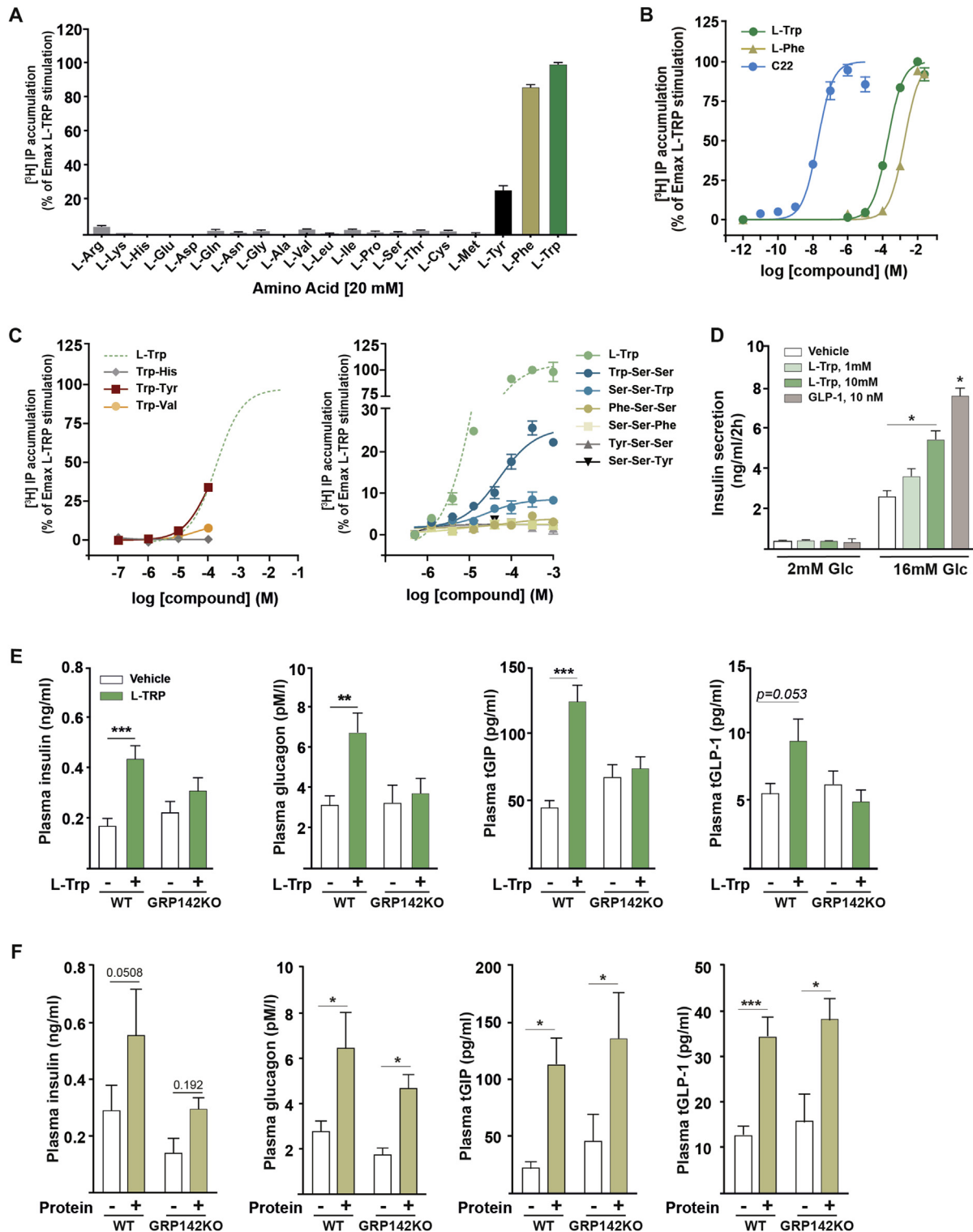
Figure 2: Identification of GPR142 as a sensor of aromatic amino acids and oligopeptides and requirement of GPR142 for the effect of L-Trp but not protein on hormone secretion in mice. (A) Relative [³H] IP accumulation in mouse GPR142 transfected CHO cells after stimulation with different amino acids (n = 3). Accumulated [³H] IP was detected with poly-L-lysine-coated Ysi SPA beads. Data are normalized to Emax for TRP (100%) in IP-accumulation. (B) Dose response for C-22, L-Trp and L-Phe in terms of [³H] IP accumulation in mouse GPR142 transfected CHO cells (n = 3). (C) Relative [³H] IP accumulation after stimulation with different di- and tri-peptides (n = 3). (D) Insulin secretion from isolated islets (n = 3). Islets were cultured overnight and batches of 5 isolated islets were incubated for 2 h with 2 or 16 mmol/l glucose in the absence or presence of L-Trp. (E,F) Plasma insulin, glucagon, GIP, GLP-1 levels in overnight fasted wild type and GPR142 deficient mice (n = 6—9 per group) after an oral Trp (500 mg/kg) (E) or protein (2 g/kg whey protein) (n = 6—8 per group) (F) challenges. Data are presented as mean values ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005 vehicle vs C-22 determined by Student's unpaired t-test or one-way ANOVA, as appropriate.
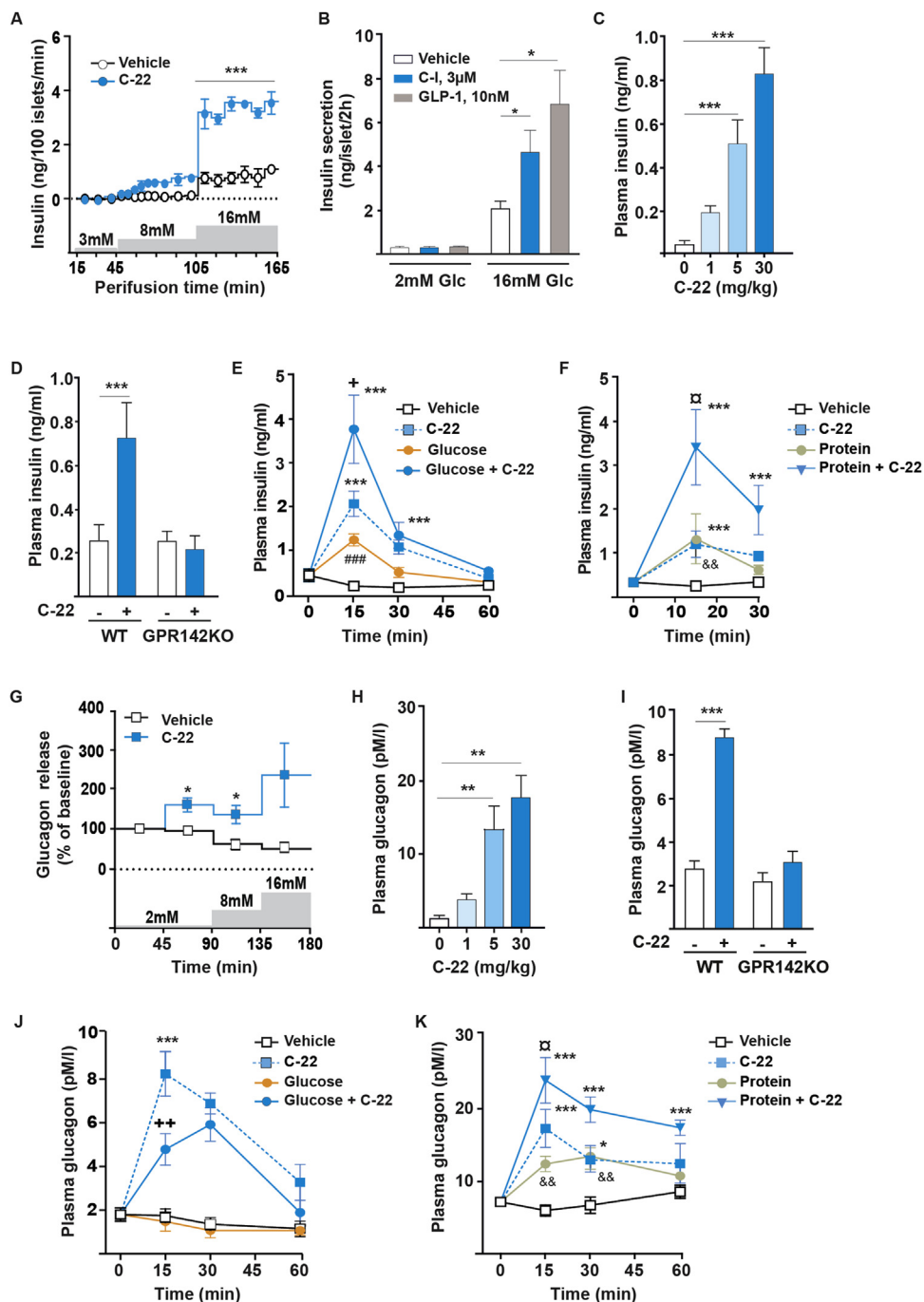
Figure 3: Acute effects of synthetic GPR142 agonist on insulin and glucagon secretion in mice. (A) Representative insulin secretion profile from perifused mouse islets exposed to step-wise increasing concentrations of glucose in the absence or presence C-22. Automated multichannel perifusion apparatus used (25 islets per channel; n = 3 channels per group; 0.26 ml/min flow rate). The figure is representative of 3 independent experiments. (B) Insulin secretion from human islets with 2 or 16 mmol/l glucose in the absence or presence of synthetic GPR142 agonist (C—I) or GLP-1 at indicated concentration. N = 3. (C) Plasma level of insulin in mice (n = 8 in each group) 15 min after an oral administration of vehicle or C-22 in dose 1, 5 or 30 mg/kg. (D) Plasma level of insulin in wild type and GPR142KO mice (n = 7—8 in each group) orally administered with vehicle or 30 mg/kg C-22; 15 min after administration. (E) Time course of changes in plasma level of insulin in mice (n = 8 in each group) after an oral administration of vehicle, 2 g/kg glucose alone, 30 mg/kg C-22 alone, or in combination. (F) Plasma level of insulin in mice (n = 6—9 in each group) after an oral administration of vehicle, 2 g/kg whey protein alone, C-22 (30 mg/kg) alone or in combination. (G) Glucagon secretion from mouse islets exposed to step-wise increasing concentrations of glucose in the absence or presence of C22. N = 3. (H) Plasma level of glucagon in mice (n = 6 in each group) 15 min after an oral administration of vehicle or C-22 in dose 1, 5 or 30 mg/kg. (I) Plasma level of glucagon in wild type and GPR142 deficient mice (n = 7—9 in each group) 15 min after an oral administration of vehicle or 30 mg/kg C-22. (J) Time course of changes in plasma glucagon level in mice (n = 8 in each group) after an oral administration of vehicle, 2 mg/kg glucose alone, C-22 alone or in combination. (K) Plasma level of glucagon in mice (n = 6—9 in each group) after an oral administration of vehicle, 2 g/kg whey protein alone, C-22 (30 mg/kg) alone or in combination. All data are presented as mean values ± SEM. Data in (A—D, G—I) *p < 0.05, **p < 0.01, ***p < 0.005 vehicle vs C-22 determined by Student's unpaired t-test or one-way ANOVA, as appropriate. Data in (E,F,J,K) ***p < 0.005 vehicle vs C-22; ##p < 0.001 vehicle vs glucose; + p < 0.05, ++ p < 0.01 C-22 vs C-22 in combination with glucose; && p < 0.01 vehicle vs protein; ¤ p < 0.05 C-22 vs C-22 in combination with protein determined as two-way ANOVA with Sidak's post-hoc test.

3.6. GPR142 stimulates glucagon secretion, an effect which is not blocked by protein intake

C-22 stimulated glucagon secretion from isolated murine islets at 2 and 8 mM glucose and numerically also at 16 mM, which did not reach statistical significance (Figure 3G). In lean, fasting mice, oral administration of the synthetic GPR142 agonist increased plasma glucagon levels in a dose-dependent manner (Figure 3H), which was not observed in GPR142 deficient animals (Figure 3I). The rapid, robust glucagon response to C-22 was diminished significantly when co-administered with oral glucose (Figure 3J). As observed for insulin, the incremental glucagon response to C-22 was not attenuated but enhanced by an oral protein load (Figure 3K).

3.7. GIP secretion is very sensitive to oral GPR142 agonist stimulation which is blocked both by glucose and by protein intake

In the enteroendocrine system, we observed that plasma levels of total GIP (tGip) were increased strongly in a dose-dependent manner by oral administration of C-22 (Figure 4A), which was not the case in GPR142 deficient mice (Figure 4B). Interestingly, tGIP was particularly sensitive to the GPR142 agonist as a dose of only 1 mg/kg induced a significant, large increase in plasma tGIP levels (Figure 4A), which was not observed for insulin and glucagon, where 5 or 30 mg/kg doses of C-22 were required for significant stimulation (Figure 3C,H). This is likely due to the fact that GIP is expressed mainly in the proximal small intestine where the orally administered C-22 compound is being absorbed and that the GIP cells consequently are exposed to relatively high concentrations of the compound as compared to cells in the pancreatic islet. GIP was very strongly stimulated by oral glucose, and, in contrast to insulin, we did not observe any further increase in GIP levels when C-22 was administered on top of the glucose load (Figure 4C). This lack of response was not because GIP was maximally stimulated as we also observed lack of stimulatory effect of C-22 when the GPR142 agonist was administered on top of a smaller glucose load (Supp. Figure 5).

As expected plasma levels of tGIP were increased significantly in response to the oral protein load (Figure 4D). In these experiments, the tGIP response to C-22 alone was similar to the response to protein, but, in contrast to insulin and glucagon, C-22 was not able to stimulate tGIP further when administered on top of the protein challenge (Figure 4D). GIP released from the proximal intestine could theoretically be involved in the GPR142-induced responses of insulin and glucagon as GIP is known to stimulate the secretion of both of these hormones [29,30]. We addressed this issue by measuring the hormone responses in GIP receptor (GIPR) deficient mice. As shown in Figure 4E, both insulin and glucagon responses to the GPR142 agonist were numerically lower in the GIPR deficient mice as compared to littermate control mice; however, the differences did not reach statistical significance as clear responses in insulin and glucagon were observed also in the GIP receptor deficient mice (Figure 4E). Thus, GIP could possibly be responsible for part of the response of the pancreatic hormones, but clearly the GPR142 agonist stimulates insulin and glucagon secretion also independently of GIP, which likely involves direct action on pancreatic cells.

3.8. GPR142 also stimulates CCK secretion

Like GIP, CCK is mainly expressed in the upper part of the small intestine and as shown in Figure 4F, oral administration of the GPR142 agonist C-22 also increased plasma levels of CCK and in a GPR142 dependent manner (Figure 4G). However, in contrast to what was observed for GIP, C-22 was able to further stimulate CCK in the presence of protein intake (Figure 4H). Due to the fact that large amounts of sample material are required for the CCK measurement we did not prioritize further the characterization of GPR142 effects on CCK secretion.

3.9. GPR142 increases plasma GLP-1 levels independent on GIP

Although some GLP-1 is expressed in the upper small intestine [31], the main site of GLP-1 production in the GI-tract is the lower small intestine and colon [32,33]. In agreement with this, we observed that C-22 could stimulate tGLP-1 secretion from ileal organoids (Figure 4I) Oral administration of C-22 increased plasma tGLP-1 levels in a dose- and GPR142-dependent manner (Figure 4J,K), but the stimulation only reached significance at the highest dose tested, i.e. 30 mg/kg (Figure 4J), meaning that GLP-1 is less sensitive than insulin and glucagon in respect of GPR142 stimulation and much less sensitive than GIP (Figure 4A). When administered on top of the oral glucose challenge, C-22 was not able to stimulate tGLP-1 more than glucose alone, whereas the response to C-22 was still observed after 30—45 min where no response to glucose alone was observed (Figure 4L). However, when administered on top of the protein challenge the response to C-22, which alone stimulated tGLP-1 to a similar degree as the protein, was not blocked by the protein as an almost additive stimulatory effect was observed (Figure 4M), as also was observed for CCK, glucagon, and insulin (Figure 3F,K) but not GIP (Figure 4D).

In mice, the stimulatory effect on GLP-1 secretion could in principle be mediated through GIP [34]; however, as shown in Figure 4N, C-22 stimulated tGLP-1 to the same degree in GIP receptor deficient mice.

3.10. GPR142 delays gastric emptying

As determined both by the acetaminophen absorption method and by the phenol red method, C-22 decreased gastric emptying in a GPR142-dependent manner (Figure 5A,B). The effect of the GPR142 agonist was not blocked by oral protein, which in itself delayed gastric emptying (Figure 5A).

3.11. GPR142 improves glucose tolerance and increases carbohydrate oxidation in lean rodents

At this stage it was clear that stimulation of GPR142 resulted in an increase in plasma levels of a number of pancreatic and gut hormones with potential complex co-actions on glucose homeostasis and metabolism in general including gastric emptying. Importantly, as shown in Figure 5C, the net result of an acute oral administration of the GPR142 agonist was a substantial GPR142-dependent improvement of glucose tolerance even in fasting lean animals (Figure 5C).

When tested acutely in lean mice habituated to metabolic cages, oral administration of the GPR142 agonist did not affect energy expenditure, food intake (Figure 5D,E), or locomotor activity (Supp. Figure 6). However, during the first hour after the oral administration, C-22 induced a significant increase in the respiratory exchange ratio (RER) in wild type mice but not in the littermate GPR142 knockout mice (Figure 5F). This translated into an increase in glucose oxidation (Figure 5G), indicating a shift in substrate preference toward carbohydrate oxidation. In these acute experiments, the effect of C-22 was transient (Figure 5F) conceivably due to the short pharmacokinetic half-life of the compound. Thus, GPR142 activation apparently leads to an increase in whole body carbohydrate oxidation.

3.12. GPR142 stimulates glucose uptake in muscle and adipose tissue and reduces liver glycogen stores in both lean and obese animals

Although the GPR142 agonist clearly had a beneficial effect on overall glucose tolerance, the fact that several different 'counteracting' glu-coregulatory hormones potentially could be involved justified a more
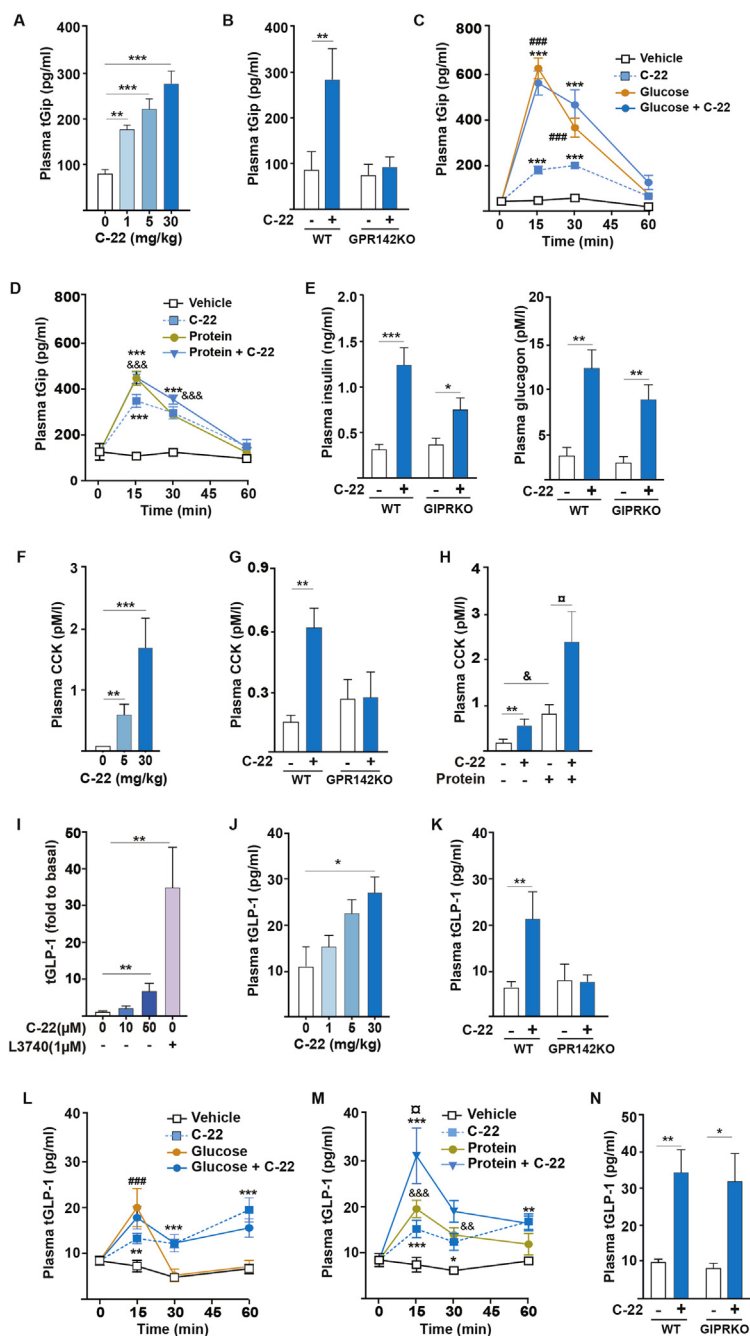
Figure 4: Acute effects of synthetic GPR142 agonist C-22 on enteroendocrine hormones in mice. **(A)** Plasma level of tGIP in mice (n = 6−8 in each group) 15 min after an oral administration of vehicle or C-22 in dose 1, 5 or 30 mg/kg. **(B)** Plasma level of tGIP in mice (n = 7−8 in each group) 15 min after an oral administration of vehicle or C-22 (30 mg/kg) in wild type and GPR142 deficient mice. **(C)** Time course of changes in plasma level of tGIP in mice (n = 8 in each group) after an oral administration of vehicle, 2 g/kg glucose alone, 30 mg/kg C-22 alone or combination. **(D)** Time course of changes in level of tGip in mice (n = 6−9 in each group) after an oral administration of vehicle, 2 g/kg whey protein alone, C-22 (30 mg/kg) alone or in combination. **(E)** Plasma level of insulin and glucagon in wild type and GipR deficient mice (n = 6−7 per group) orally administered with vehicle or 30 mg/kg C-22; 15 min after administration. **(F)** Plasma level of CCK in mice (n = 6−7 in each group) 15 min after an oral administration of vehicle or C-22 in dose 5 or 30 mg/kg. **(G)** Plasma level of CCK in mice (n = 8 in each group) 15 min after an oral administration of vehicle or C-22 (30 mg/kg) in wild type and GPR142 deficient mice. **(H)** Plasma level of CCK in mice (n = 6−8 in each group) 15 min after an oral administration of vehicle, 2 g/kg whey protein alone, C-22 (30 mg/kg) alone or in combination. **(I)** tGlp-1 secretion (fold to vehicle) in ileal organoids cultures (n = 4) in presence of C22 or vehicle. L-3740, TGR5 receptor agonist was used as positive control. **(J)** Plasma level of tGLP-1 in mice (n = 6−8 in each group) 15 min after an oral administration of vehicle or C-22 in dose 1, 5, or 30 mg/kg. **(K)** Plasma level of tGLP-1 in mice (n = 7−8 in each group) 15 min after an oral administration of vehicle or C-22 (30 mg/kg) in wild type and GPR142 deficient mice. **(L)** Time course of changes in plasma level of tGLP-1 i mice (n = 8 in each group) after an oral administration of vehicle, 2 g/kg glucose alone, 30 mg/kg C-22 alone or combination. **(M)** Time course of changes in plasma level of tGLP-1 in mice (n = 6−9 in each group) after an oral administration of vehicle, 2 g/kg whey protein alone, C-22 (30 mg/kg) alone or in combination. **(N)** Plasma level of tGLP-1 in wild type and GipR deficient mice (n = 6−7 per group) orally administered with vehicle or 30 mg/kg C-22; 15 min after administration. Data in **(A,B,E-K,N)** *p < 0.05, **p < 0.01, ***p < 0.005 vehicle vs C-22, determined by Student's unpaired t-test or one-way ANOVA, as appropriate. Data in **(C,D,L,M)** *p < 0.05, **p < 0.01, ***p < 0.005 vehicle vs C-22; ##p < 0.01, ###p < 0.005 vehicle vs glucose; && p < 0.01, &&& p < 0.005 vehicle vs protein; ¤ p < 0.05 C-22 vs C-22 in combination with protein determined as two-way ANOVA with Sidak's post-hoc test.
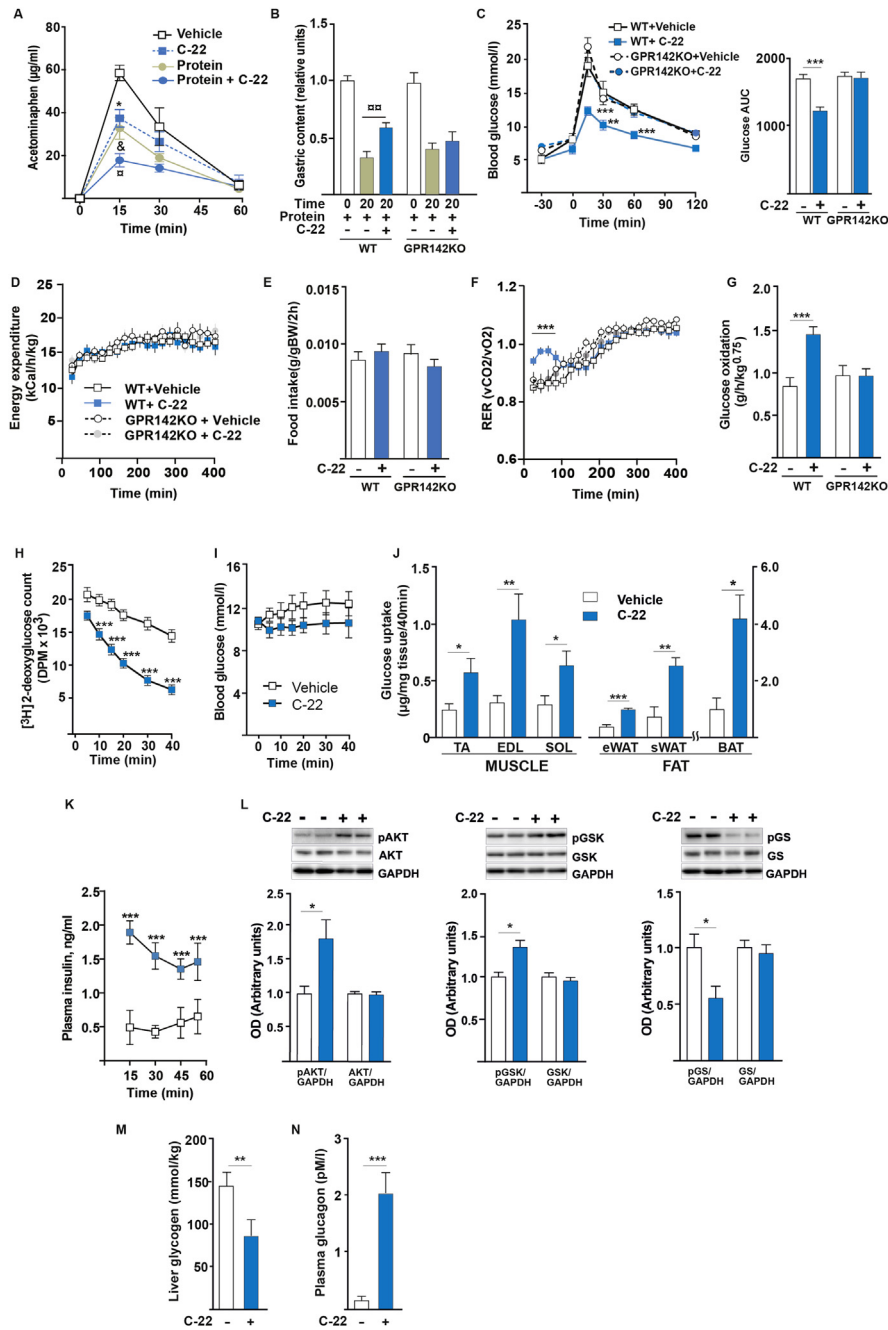
Figure 5: Acute effects of synthetic GPR142 agonist on metabolic functions in lean mice. **(A, B)** Gastric emptying. **(A)** Time courses of plasma acetaminophen in mice (n = 7−8 in each group) orally administered with vehicle, 2 g/kg whey protein alone, C-22 (30 mg/kg) alone or in combination. All treatments were mixed with acetaminophen. **(B)** Gastric content of phenol red recovered from the stomach of wild type and GPR142 KO mice (n = 5−7 in each group) sacrificed 0 or 20 min after oral administration 2 g/kg whey protein alone or in combination with C22 (30 mg/kg). 100% is mean absorbance of phenol red recovered from the stomachs of wild type mice at 0 min following 2 g/kg whey protein load. **(C)** Blood glucose level during OGTT and AUC in wild type and GPR142 deficient mice (n = 8 mice in each group) after an oral administration of vehicle or C-22 (30 mg/kg) followed by 2 g/kg oral glucose challenge. **(D−F)** Indirect calorimetry measurements of energy expenditure **(D)**, food intake **(E)**, and RER **(F)** in non-fasted wild type and GPR142 deficient (n = 8 in each group) mice after oral administration of 30 mg/kg C-22 or vehicle. Significant difference indicated on graph was calculated as mean value for 1 h after treatment. **(G)** Glucose oxidation was calculated as described [22] for 1 h after treatment. **(H)** Time course profile of [^{3}H] 2-deoxyglucose specific blood activity; and **(I)** blood glucose concentration after vehicle or C-22 treatment in 4 h fasted anesthetized mice (n = 7−8 in each group). **(J)** Tissue-specific glucose uptake in tibialis anterior (TA), extensor digitorum longus (EDL), soleus (SOL), epididymal (eWAT) and subcutaneous (sWAT) white adipose tissue and brown fat (BAT) in mice (n = 7−8 in each group) over a 40-min period starting 15 min after C-22 (30 mg/kg) administration. The accumulation of [^{3}H] 2-deoxyglucose-6-phosphate was normalized to tissue weight. **(K)** Time course profile of plasma insulin after vehicle or C-22 treatment in 4 h fasted anesthetized mice. **(L)** Representative western-blot analysis and quantification of expression and phosphorylation of AKT, GSK and GS in TA muscle 55 min after vehicle or C-22 treatment (n = 8 in each group). Data expressed as level of phosphorylated or total form to GAPDH. **(M)** Liver glycogen content over a 55-min period after vehicle or C-22 treatment in 4 h fasted anesthetized mice (n = 7−8 in each group). **(N)** Plasma glucagon concentration 55 min after vehicle or C-22 treatment in 4 h fasted anesthetized mice (n = 7−8 in each group). All data are presented as mean values ± SEM. Data in **(A,C,D,F,H,I,K)** **$p < 0.01$, ***$p < 0.005$ vehicle vs C-22 determined as two-way ANOVA with Sidaks's post-hoc test. Data in **(B)** ¤¤ $p < 0.01$ protein vs protein in combination with C22; **(E,G,J,L−N)** *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vehicle vs C-22 determined by Student's unpaired t-test or one-way ANOVA, as appropriate.

detailed analysis of the GPR142-mediated changes in glucose metabolism. As shown in Figure 5H, C-22 potently enhanced the whole-body clearance of [^{3}H]-2-deoxyglucose while surprisingly not affecting the blood glucose levels as such (Figure 5I), which would have been expected if the effect was mediated solely by an insulin action. The GPR142 agonist induced a considerable accumulation of [^{3}H]-2DG6P in different types of muscles (Figure 5J). Similarly, C-22 induced increased glucose uptake in both epididymal and subcutaneous white fat depots and in inter-scapular brown adipose tissue (Figure 5J). As the GPR142 agonist in these experiments strongly stimulated insulin secretion (Figure 5K), it is most likely that the increased glucose uptake in muscle and adipose is mediated through insulin, which was supported by the fact that we observed increased activities in classical insulin signaling pathways in skeletal muscle, i.e. increased phosphorylation of AKT(Ser^{473}) and increased phosphorylation of the AKT substrate glycogen synthase kinase-3β GSK(Ser^9), which decreases its activity and in accordance with the decreased phosphorylation (increased activity) of glycogen synthase (Figure 5L). The fact that total blood glucose did not decrease in response to the GPR142 agonist while the tracer data clearly indicated that glucose uptake was strongly increased (Figure 5H,I), suggests that glucose production was increased at the same time. This notion was supported by the fact that liver glycogen content was decreased by approx. 40% compared with vehicle-treated mice (Figure 5M); an effect most likely attributed to the very strong increase in plasma glucagon following the C-22 treatment (Figure 5N).

3.13. Obese rodents are equally or more susceptible to GPR142 effects on endocrine and metabolic functions

In DIO mice, which as expected had high circulating levels of insulin, C-22 strongly increased plasma insulin even further in both fasted and glucose-stimulated animals (Figure 6A). In the fasting DIO mice, the GPR142 agonist also stimulated glucagon secretion to a similar degree as in the lean mice (Figure 6A). However after glucose stimulation, where the glucagon levels in the DIO mice paradoxically were increased, which has been reported previously in diabetic animals [35], C-22 administration did not increase glucagon levels further, i.e. the apparent increase did not reach statistical significance (Figure 6A). Concerning the incretins, acute treatment of DIO mice with C-22 resulted in a significant elevation of both tGLP-1 and tGIP (Figure 6A), i.e. as observed in the lean mice (Figure 4A,J).

In DIO mice, oral administration of the GPR142 agonist lowered basal blood glucose (from 10.1 ± 0.4 to 7.5 ± 0.3) and improved glucose tolerance highly significantly in a dose-dependent manner with a maximal effect reached with 5 mg/kg (Figure 6B). Similarly, in Zucker fatty rats, oral administration of C-22 improved glucose tolerance dramatically (Figure 6C) while as expected stimulated insulin secretion (Figure 6D). As observed in the lean mice, C-22 increased the clearance of [^{3}H]-2-deoxyglucose tracer from plasma of DIO mice without lowering total blood glucose (Figure 6E,F). In fact, blood glucose levels of C-22 treated DIO mice were slightly but not significantly increased (Figure 6F). As observed in the lean animals, glucose uptake was increased in various muscle and fat depots of the DIO mice (Figure 6G); however, the basal glucose uptake was rather high in the EDL muscle and was not further increased, and the increase in glucose uptake in the epididymal white fat did not reach statistical significance. Importantly, in the DIO animals we observed a statistically significant decrease in liver glycogen similar to the decrease observed in the lean animals conceivably due to glucagon-induced glycogenolysis explaining the lack of drop in blood glucose despite the increased glucose uptake in muscle and fat (Figure 6H).

3.14. Chronic treatment of obese mice with synthetic GPR142 agonist increases energy expenditure, decreases basal glucose and increases insulin sensitivity

The effect of chronic exposure to the GPR142 agonist was studied in DIO mice which after reaching a body weight of approx. 50 g (27 weeks of HFD) were either continued on the 60% HFD or switched to HFD containing C-22 at a dose of ∼30 mg/kg/day for six weeks. No effect on body weight (Figure 7A) or body composition (data not shown) was observed, although the GPR142 treated group did show a non-significant nominal increase in food intake in particular during the light phase (Figure 7B). However, as revealed by indirect calorimetry, the C-22-treated mice displayed a clear, significant increase in energy expenditure (Figure 7C), which closely corresponded to the increase in calorie intake (Figure 7D), which could explain the lack of effect on body weight. In addition, C-22-treated mice had increased activity (Figure 7E). Furthermore, as observed in the acutely treated lean animals (Figure 5F), the GPR142 agonist treated mice had — in this case continued — an increased respiratory exchange ratio during light phase (Figure 7F), corresponding to an increased carbohydrate utilization, calculated as an increase in glucose oxidation (Figure 7G).

OGTT and insulin tolerance tests (ITT) were both performed after six hours fasting, which, due to the relatively short T^1/$_2$ of the C-22 compound, reflects the effect of the long-term treatment with C-22 on the organism but does not reflect direct effects of the compound as it would no longer be present in therapeutic concentrations in plasma when these tests were performed (Supp. Figure 7c). As shown in Figure 7H, six weeks treatment with C-22 significantly decreased fasting glucose (8.77 ± 0.25 versus 7.03 ± 0.28) and there was a trend toward reduction in fasting insulin level (Figure 7I). However, the incremental glucose excursions after the oral glucose load were only marginally decreased in the C-22 treated animals as compared to the control animals (Figure 7H), and there was no significant difference in glucose AUC (p = 0.056). Importantly, the ITT showed (Figure 7J) that, whereas the DIO mice that were still on normal HFD clearly were very insulin intolerant, displaying basically no decrease in blood glucose in response to 1 U/kg insulin i.p., the C-22 treated animals displayed a clear increase in insulin sensitivity with a significant decrease in blood glucose from 6.8 ± 0.2 to 4.9 ± 0.4 after insulin treatment.

It has recently been reported that GPR142 increased β-cell proliferation and that 96 h treatment with a GPR142 agonist increased insulin, glucagon and GLP-1 content of isolated islets [9]. However, we did not observe any increase in pancreatic content of insulin, glucagon, and total GLP-1 or duodenal content of total GLP-1 in the DIO mice treated with C-22 for six weeks (Figure 7K,L). On the contrary, we observed a small insignificant decrease in the content of all four hormones.

4. DISCUSSION

In the present study, we confirm that the recently deorphanized receptor GPR142 is almost exclusively expressed in endocrine cells of the GI-tract and pancreas and that it functions as a highly selective receptor for aromatic amino acids, in particular Trp. Moreover, we find that GPR142 also recognizes small oligopeptides with an N-terminal Trp residue. By use of the highly selective and potent GPR142 agonist, C-22 we observe that GPR142 affects a number of metabolic functions in mice conceivably through its stimulatory effects on enteropancreatic hormones. Importantly, chronic treatment of DIO mice with the GPR142 agonist improves metabolic function including insulin sensitivity and energy expenditure. However, despite these clear indications of a pharmacotherapeutic potential, the actual physiological role of GPR142 is still rather unclear.
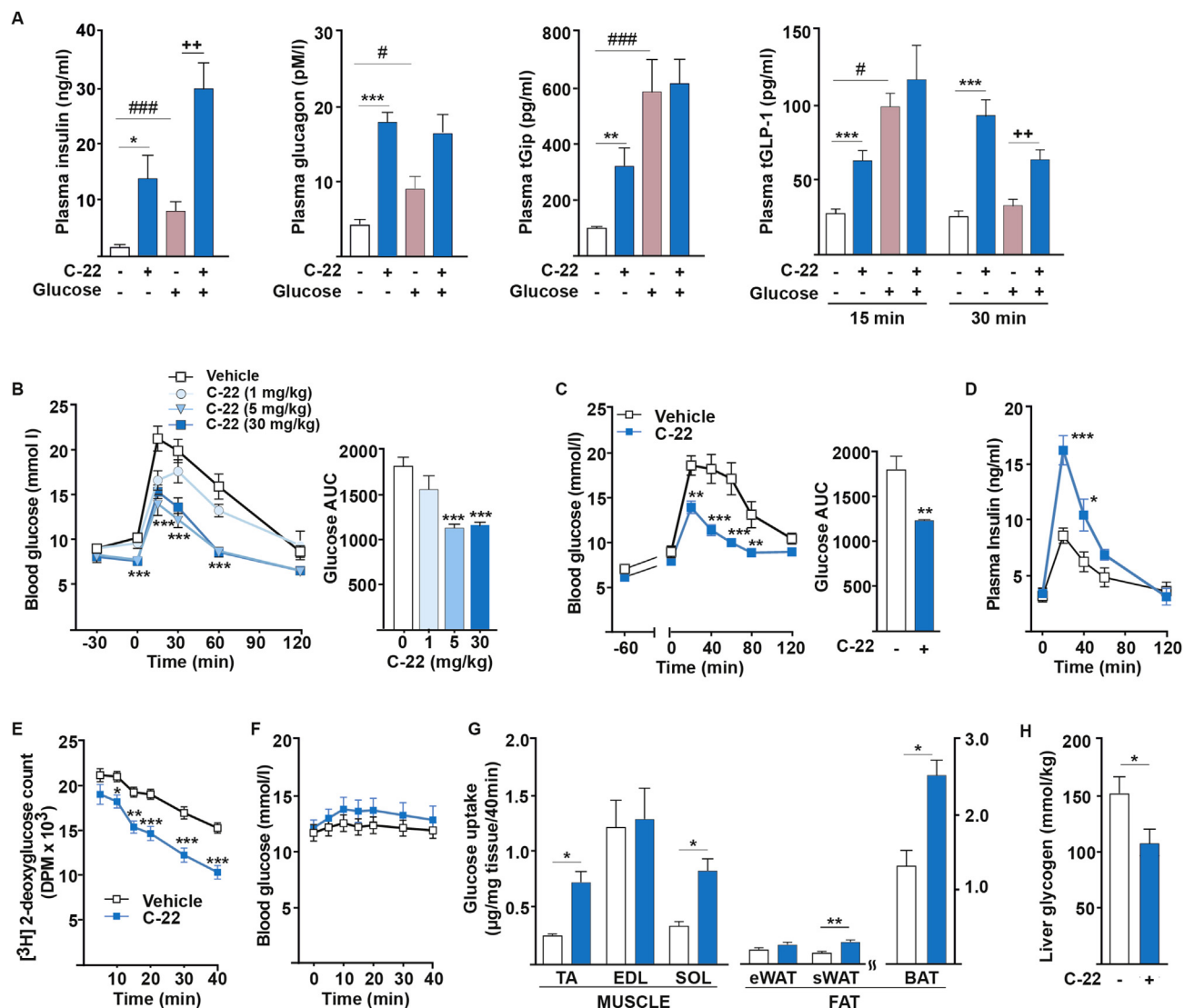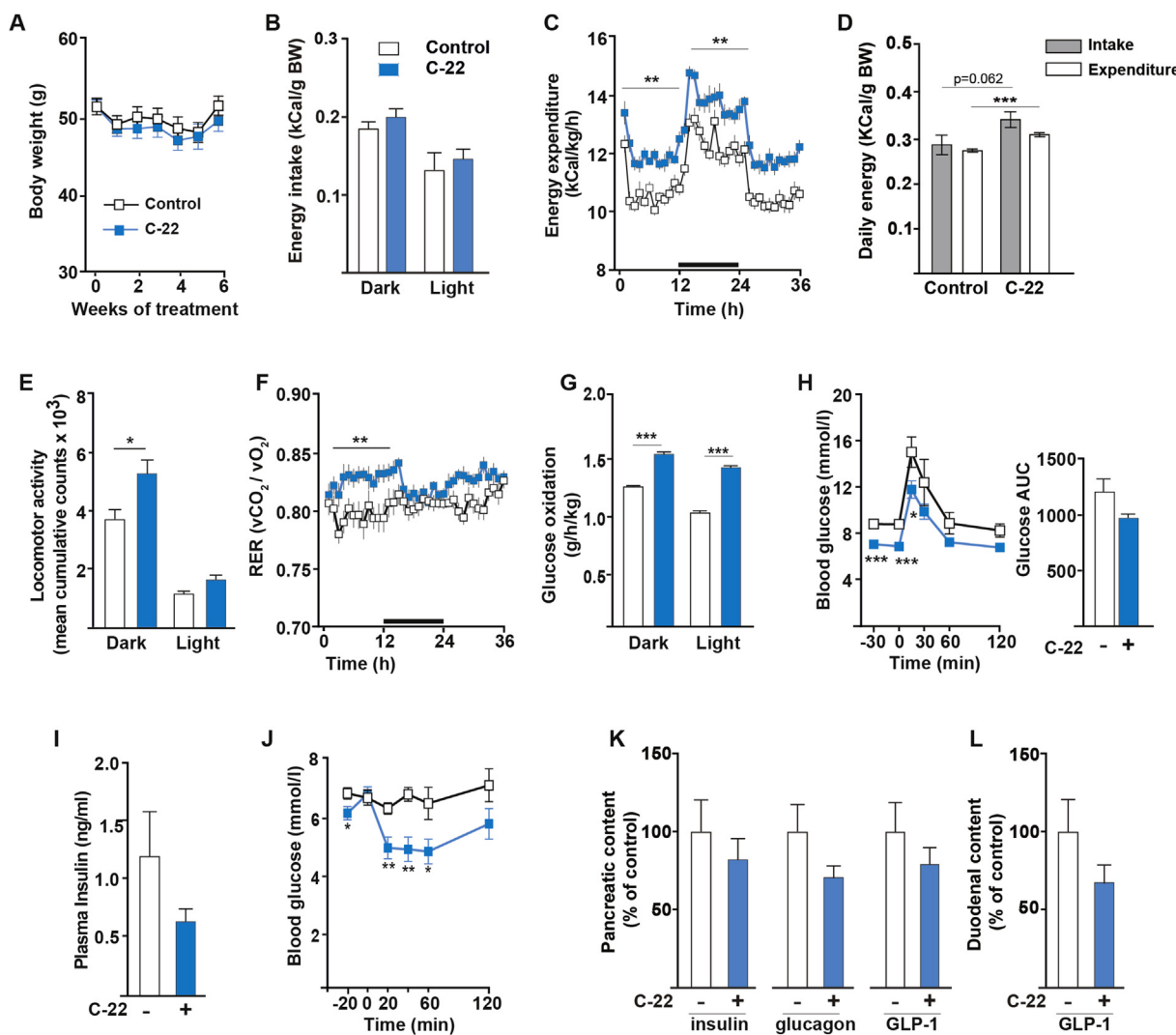
Figure 6: Acute effects of synthetic GPR142 agonist on hormone secretion, glucose tolerance and glucose uptake in DIO mice and Zucker rats. (A) Plasma insulin, glucagon, tGip, tGLP-1 levels in DIO mice (n = 7−8 in each group) 15 min after an oral administration of vehicle, 2 g/kg glucose alone, C-22 alone or in combination with 2 g/kg glucose. Plasma tGLP-1 additionally measured at 30 min after administration. (B) Blood glucose level during OGTT and AUC in mice (n = 7−8 in each group) after an oral administration of vehicle or C-22 in dose 1, 5 or 30 mg/kg followed by oral glucose challenge (2 g/kg). (C) Plasma glucose and (D) insulin level during OGTT in Zucker fatty rats (n = 6 in each group) after an oral administration of vehicle or C-22 in dose 30 mg/kg followed by oral glucose challenge (3 g/kg). (E) Time course profile of [³H]2-deoxyglucose specific blood activity and (F) blood glucose concentration after vehicle or C-22 treatment in 4 h fasted anesthetized mice (n = 13−14 in each group). (G) Tissue-specific glucose uptake in tibialis anterior (TA), extensor digitorum longus (EDL), soleus (SOL), epididymal (eWAT) and subcutaneous (sWAT) white adipose tissue, liver, and brown fat (BAT) in mice (n = 13−14 in each group) over a 40-min period started 15 min after C-22 (30 mg/kg) administration. The accumulation of [³H] 2-deoxyglucose-6-phosphate was normalized to tissue weight. (H) Liver glycogen content over a 55-min period after vehicle or C-22 treatment in 4 h fasted anesthetized mice. All data are presented as mean values ± SEM. Data in (**A,G,H**) *p < 0.05, **p < 0.01, ***p < 0.005 vehicle vs C-22; #p < 0.05, ###p < 0.005 vehicle vs glucose; ++ p < 0.01 C-22 vs C-22 in combination with glucose determined by Student's unpaired t test or one-way ANOVA, as appropriate. Data in (**B−F**) *p < 0.05, **p < 0.01, ***p < 0.005 vehicle vs C-22 determined as two-way ANOVA with Sidak's post-hoc test.

4.1. Effects of GPR142 agonists on entero-pancreatic hormone secretion

C-22 has good bioavailability [5,7] and was administered orally both in the acute and chronic studies. We find enteroendocrine GIP cells to be particularly sensitive to GPR142 agonist stimulation as plasma levels of GIP increased even in response to the lowest dose tested as opposed to the other gut and pancreatic hormones. This is probably due to the fact that GIP is stored and released from cells located in the duodenum and upper small intestine where C-22 likely is absorbed and that GIP cells consequently are exposed to higher local concentrations of the

compound. Another interesting observation was that both an oral glucose challenge and an oral protein challenge blocked the ability of the GPR142 agonist to stimulate GIP secretion further. That was not the case for the other hormones, as C-22 instead acted in an additive manner, i.e. it stimulated CCK, GLP-1, insulin and glucagon secretion even further in combination with protein (discussed below in relation to the physiological role of GPR142 as a dietary protein sensor). The inhibitory effect of oral protein on C-22 stimulation of GIP secretion is likely caused by receptor desensitization caused by exposure of GIP cells in the proximal small intestine to high concentrations of absorbed aromatic amino acid

Figure 7: Effects of chronic in-feed treatment with synthetic GPR142 agonist on metabolic functions of DIO mice. Effects on body weight (**A**), food intake (**B**), energy expenditure (**C**), daily energy balance (**D**), locomotor activity (**E**), RER (**F**), glucose oxidation (**G**), OGTT (tested at week 4 from the start of treatment) (**H**), fasting insulin (**I**) and insulin tolerance (**J**) (tested at weeks 5 from the start of treatment) of DIO mice after feed administration of vehicle or C-22 (~30 mg/kg/day). N = 7−8 in each group. (**K**) Pancreatic content of insulin, glucagon, tGLP-1 (**n = 7−8** in each group) and (**L**) duodenal tGLP-1 (n = 6−7 in each group) from acid-ethanol extraction after treatment intervention for 6 weeks. In (**B,E,G**) data was averaged for light and dark phases. (**C,F**) represent 36 h profile for energy expenditure (**C**) and RER (**F**). Black horizontal bar represents lights-off (active) period during a 12:12 h light/dark cycle. Significant difference indicated on graph was calculated as mean for light and dark phases. Data in (**H,J**) *p < 0.05, **p < 0.01, ***p < 0.005 vehicle vs C-22; determined as two-way ANOVA with Sidak's post-hoc test.

and oligopeptide metabolites from the protein. However, the fact that glucose also blocks the ability of the GPR142 agonist to further stimulate GIP secretion is more difficult to explain, since it was also observed with lower glucose doses, which only stimulated GIP partly. Perhaps the GIP cell is specially tuned in respect of dietary metabolite sensing?

In relation to pancreatic islets, it was generally assumed that GPR142 would be a β-cell receptor as the receptor originally was discovered to be almost exclusively expressed in islets [2]. This notion was underscored by the glucose-dependent strong stimulatory effect on insulin secretion of the first GPR142 agonists, including C-22 [5,6], which we could confirm in the present study. However, as also demonstrated here and as recently shown also by *in situ* hybridization [8], GPR142 is in fact even more highly expressed in α-cells, which also is evident from single cell data analysis [25]. Importantly, the GPR142 agonist is a very powerful stimulator of glucagon secretion which even works at high glucose level that normally suppresses glucagon secretion (Figure 3G,J).

Very recently, it was proposed that GPR142-induced increase in circulating levels of GLP-1 also originated from the α-cells because of an increase in expression of the peptide precursor converting enzyme PC1/3 [9]. The amount of GLP-1 stored in islet cells increased to one third of the amount of glucagon upon a four-day treatment of islet cells with the GPR142 agonist. It was also proposed that α-cell-derived GLP-1 through a paracrine mechanism could be responsible for an observed β-cell proliferative effect of the GPR142 agonist [9]. This would implicate that chronic treatment with a GPR142 agonist should increase production of GLP-1 in the islets, which would have a beneficial insulinotropic effect. However, in the present study we did not observe any increase in the amount of GLP-1 or insulin stored in the pancreas from DIO mice treated chronically with C-22.

Concerning GPR142-induced increase in circulating GLP-1, it is still possible that intestinally derived GLP-1 is in play as the GPR142 agonist did stimulate GLP-1 secretion at least from intestinal organoids.

However, the expression level of GPR142 in intestinal GLP-1 cells is relatively low and the efficacy of the GPR142 agonist was also relatively low as compared to for example a TGR5 receptor agonist.

4.2. Acute metabolic effects of GPR142 agonists

Despite stimulating CCK and GLP-1, C-22 did not decrease food intake in the present study but did decrease gastric emptying rather strongly even on top of the inhibitory effect of an oral protein challenge. This inhibitory effect of the GPR142 agonist on gastric emptying, which probably mainly is mediated by CCK, could relate to a possible physiological role of GPR142 being one of several sensors of dietary protein ingestion as gastric emptying plays an important role in controlling delivery of protein for further digestion and absorption in the small intestine. Interestingly, gastric emptying is reduced by oral tryptophan in humans [36,37], which very well could be mediated through GPR142. The effect on gastric emptying should be taken into account when evaluating the therapeutic potential of GPR142 agonist. The GPR142 agonist improved glucose tolerance rather dramatically even in lean, normal animals. Thus, although GPR142 stimulates both insulin and glucagon efficiently it is the insulin effect which dominates at least in the OGTT situation. There were no signs of GPR142 agonist resistance in DIO mice, as the agonist stimulated hormone secretion and improved glucose tolerance very efficiently also in these animals. This was also observed in Zucker Fatty rats. However, when the glucose uptake was directly studied the two glucoregulatory hormones leveled out each other's effect in respect of balancing blood glucose, i.e. the strong insulin mediated increase in glucose uptake in muscle and fat as determined by labeled 2-deoxyglucose was counteracted by a conceivably glucagon-mediated glycogenolytic effect on the liver resulting in no change in total blood glucose levels. All of these effects were also observed in DIO mice. In respect of overall substrate preference, the GPR142 agonist induced an increase in carbohydrate burning as indicated in an increase in RER observed both in the acute and the chronic setting.

4.3. Effects of chronic treatment with a GPR142 agonist on metabolic functions

Although C-22 has a great bioavailability and consequently can be administered as part of the diet, its plasma clearance is, unfortunately, rather quick [5,7], which means that the compound is no longer present in therapeutic concentrations in experiments, which are performed after fasting, for example OGTT; this should be considered when interpreting results from the chronically treated animals. Thus, we only observe a rather limited effect on the incremental glucose excursions in the chronically GPR142 treated DIO animals as compared to the large improvements in glucose tolerance observed after acute administration of C-22 in the DIO animals. Importantly however, we do observe a significant decrease in fasting basal glucose in the chronically treated animals, which according to the PK of the compound is not a result of a direct stimulatory effect of the compound on insulin secretion, because the compound is no longer present when the samples are taken. Conceivably the decrease in basal glucose is instead a result of the increase in insulin sensitivity, which is induced in these animals upon chronic treatment with the GPR142 agonist. The increase in insulin sensitivity is also observed under fasting conditions in which the compound is no longer present (Figure 7J). The basis for the surprising increase in insulin sensitivity in these highly insulin resistant animals is still unclear and requires further studies. It may be noted that GPR142 is not expressed consistently on immune cells (unpublished data), so it is unlikely that an anti-inflammatory effect is involved.

Energy expenditure was increased in DIO animals upon chronic treatment with the GPR142 agonist. This was also rather surprising, as no effect on energy expenditure was observed in the acute setting at least not in lean animals. This stimulatory effect on energy expenditure in DIO animals, which was most prominent in the dark phase, was measured in the TSE system, in which the animals were freely eating the C-22-containing food and therefore exposed to the compound during the measurements. Consequently the GPR142 effect on energy expenditure could be a result of the increased glucagon levels [38]. Importantly, there was no effect on body weight in these very obese DIO animals despite the observed increase in energy expenditure, which possibly could be related to the nominal, but not significant increase in food intake.

It could be speculated that a GPR142 agonist with a better PK, allowing for constant drug exposure, would provide both the observed improved insulin sensitivity and the robust increase in glucose tolerance observed in the acute setting combined with an increase in energy expenditure. Such a compound could be combined with a GLP-1 mimetic, which on top of its other beneficial effects would provide an additional robust anorectic effect.

4.4. Is GPR142 a physiological sensor of dietary protein?

Amino acid homeostasis is tightly regulated through a balance between protein synthesis and degradation. The pool of systemic free amino acids is derived from dietary protein, de novo synthesized amino acids, and amino acids generated from autophagy (reviewed in [39]). Dietary intake of protein will affect a series of processes throughout the body, including increased amino acid uptake, increased protein synthesis, decreased protein degradation, and increased ureagenesis. Hormonal and neuronal mechanisms including a balance between insulin and glucagon effects are critical in controlling these processes. Accordingly, dietary protein and free amino acids are known to act as robust stimulators of insulin and glucagon as well as most gut hormones.

Several GPCRs are already known to function as sensors of amino acids, e.g., the calcium sensing receptor (CasR), taste receptors T1R1/T1R3, and metabotropic glutamate receptors. However, among these only CasR is expressed in a manner which would implicate it as a sensor of dietary protein in the GI-tract, which is supported by a number of functional studies [40—45]. The expression pattern and functional properties of GPR142 would indicate that also this receptor functions as a sensor of dietary proteins through aromatic amino acid and oligopeptide metabolites. The observation in the present study that GPR142 is responsible for the stimulatory effect of orally administered L-Trp on gut and pancreatic hormones would support this notion. However, the amount L-Trp administered was far from physiological and our results with an oral protein challenge clearly indicate that GPR142 is not required for sensing of dietary proteins by the enteropancreatic endocrine cells as normal hormone response to the protein was observed in GPR142 KO animals (Figure 2F). As at least CasR and possibly other receptors are also likely involved in sensing of dietary proteins it is expected that compensatory mechanisms could have arisen in the GPR142 deficient mice. Importantly, GPR142 has both the expression pattern and functional properties to function as a physiological sensor of dietary proteins, including the ability to stimulate both insulin and glucagon and strongly stimulate GIP, which, in contrast to GLP-1, stimulates both insulin and glucagon. The balance between insulin and glucagon is important both for postprandial amino acid metabolism and to avoid hypoglycemia due to insulin stimulation [46]. More studies including use of pharmacological antagonists and inducible knock out animal models will be required to determine the possible physiological role of GPR142 as a sensor of dietary proteins.

4.5. Concluding remarks

Based on the present and recently published studies, it is clear that GPR142 is not just another insulin secretagogue receptor. GPR142 appears to have a role as a sensor of aromatic essential amino acids controlling the secretion of in particular GIP but also CCK and GLP-1 in the GI-tract and both insulin and glucagon and perhaps GLP-1 in the pancreas. In the present study, we have focused on effects on glucose metabolism; however, future studies should probably focus more on the potential physiological role of GPR142 in amino acid metabolism, i.e. indirectly through its balanced control of insulin and glucagon secretion. The pharmacological potential of GPR142 is also much more interesting than just increasing insulin secretion and improving glucose tolerance. Our study indicates that chronic GPR142 agonist treatment may increase insulin sensitivity and energy expenditure through mechanisms which needs to be further determined.

CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2018.10.012.

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