Genetic variation in *Glp1r* expression influences the rate of gastric emptying in mice

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ABSTRACT

We demonstrated previously that food intake traits map to a quantitative trait locus (QTL) on proximal Chromosome 17 which encompasses Glp1r (glucagon-like peptide 1 receptor), encoding an important modulator of gastric emptying. We then confirmed this QTL in a B6.CAST-17 congenic strain that consumed 27% more carbohydrate and 17% more total calories, yet similar fat calories, per body weight, compared with the recipient C57BL/6J. The congenic strain also consumed greater food volume. The current aims were to: 1) identify genetic linkage for total food volume in F2 mice, 2) perform gene expression profiling in stomach of B6.CAST-17 congenic mice using oligonucleotide arrays, 3) test for allelic imbalance in Glp1r expression, 4) evaluate gastric emptying rate in parental and congenic mice, and 5) investigate a possible effect of genetic variation in Glp1r on gastric emptying. A genome scan revealed a single QTL for total food volume (Tfv1) (LOD=7.6) which was confirmed in B6.CAST-17 congenic mice. Glp1r exhibited allelic imbalance in stomach which correlated with accelerated gastric emptying in parental CAST and congenic B6.CAST-17 mice. Moreover, congenic mice displayed an impaired gastric emptying response to Exendin-(9-39). These results suggest that genetic variation in Glp1r contributes to the strain differences in gastric emptying rate.
INTRODUCTION

The gut hormone glucagon-like peptide 1 (GLP-1) inhibits gastric emptying, reduces postprandial gastric secretion, and may play a physiological role in regulating appetite and energy intake. Systemic administration of GLP-1 in physiological amounts in both humans and animals potently inhibits gastric emptying (9, 13, 15, 31, 59, 61), and this effect can be blocked by administration of the GLP-1 receptor antagonist exendin(9-39) (21). The mechanism by which GLP-1 inhibits gastric emptying is thought to involve receptors located either in the central nervous system or associated with afferent pathways to the brain stem (9, 21, 60). In addition to the deceleration of gastric emptying, GLP-1 is involved in regulating satiety, as shown in normal-weight and obese men infused with GLP-1 (17, 33-34). GLP-1 also decreases food intake; by contrast, central administration of the GLP-1 receptor antagonist exendin-(9-39) stimulates food intake (14, 54).

The glucagon-like peptide 1 receptor (GLP-1R) is a seven-transmembrane domain receptor belonging to the B family of G-protein-coupled receptors (6, 52). The GLP-1R recognizes GLP-1 specifically and does not show demonstrable binding by related peptides (52; for review, see ref. 6). In rodents, GLP-1 receptor mRNA and high-affinity GLP-1 binding sites have been identified in pancreatic islets, gastrointestinal tract, lung, kidney, heart, and throughout the brain (7-8, 16). The satiety-promoting effects of GLP-1 implicate Glp1r as a physiologic gene candidate for ingestive behavior phenotypes (1).

Glp1r maps to the peak of a genetic region containing overlapping quantitative trait loci (QTL) for carbohydrate intake (Mnic1) and total calorie intake (Kcal2) on proximal Chromosome 17 (46), mapped using a C57BL/6J (B6) x CAST/Ei (CAST) intercross. These coincident QTL suggest that a common gene may be contributing to both traits. QTLs require
confirmation in mice possessing a uniform genetic background. Thus the next step toward identifying complex trait gene(s) was to develop a congenic strain in which a CAST donor segment approximating the QTL region was introgressed onto the B6 genome. Phenotypic comparisons between congenic mice and controls homozygous for the recipient strain background were used to determine whether or not the differential locus retained its effect (26). We found that the B6.CAST-17 congenic strain, when compared with littermate B6 controls, selected 27% more calories from carbohydrate and 17% more total calories per body weight while consuming equivalent amounts of fat. These results verified the QTL, indicating that a gene(s) on CAST chromosome 17 is responsible for modifying nutrient intake.

Based on its genetic position and known physiological role in glucose homeostasis and stomach emptying, we therefore investigated Glp1r as a gene candidate for these nutrient intake QTL (26). We previously reported sequence differences at this locus including a non-conservative C416Y substitution in the CAST allele, located in the COOH terminal intracellular domain, which could have functional implications in the downstream regulation of protein activity. Moreover, expression studies revealed complex, tissue-specific differences in Glp1r mRNA levels between B6 and CAST in stomach, pancreas, and hypothalamus, tissues implicated in the physiologic effects of GLP-1. Thus we hypothesized that variation in Glp1r could influence nutrient intake through changes in either the regulatory or protein coding regions (26). However, a possible mechanism for its effects on ingestive behavior in this model has not yet been determined. In the present study we sought to determine whether differences in Glp1r expression between the B6.CAST-17 congenic and recipient strains is due to cis- or trans- acting effects, by performing an allelic imbalance test in congenic F1 mice.
Because GLP-1 appears to play an important role in the inhibition of gastric emptying, we investigated a possible functional effect of the CAST donor segment by evaluating stomach emptying rate in both the parental and congenic strains. A putative functional effect of the candidate Glp1r variant was then tested using the GLP-1 receptor antagonist exendin-(9-39). Glp1r is implicated as a physiological candidate for nutrient intake QTL based on the higher volume/weight and lower calorie density of carbohydrate relative to dietary fat. In this regard, the parental CAST and B6.CAST-17 congenic mice select/consume more carbohydrate and total energy per body weight (26), and thus higher food volume, when compared with B6. To uncover the genetic loci involved in total food volume, we conducted a genome scan for 10-d consumption (g) in an existing mapping population (46). Finally, although Glp1r is an obvious positional candidate, the congenic interval on chromosome 17 contains a large number of genes. To provide an unbiased approach to candidate gene identification, we therefore performed gene expression profiling in stomach of the B6.CAST-17 congenic strain using oligonucleotide arrays.

METHODS

Animals. Animals, breeding, and phenotyping protocols used in the genetic mapping study are described in detail elsewhere (46). Microsatellite-assisted selection at every generation was used to develop a speed congenic line, B6.CAST<sup>D17Mit19-D17Mit91</sup> (B6.CAST-17) (26). B6.CAST-17 S6 mice, heterozygous for the donor region, were intercrossed to generate mice homozygous for a ~38-cM CAST donor segment on the B6 background. This CAST segment extends at least from proximal marker D17Mit19 at 4.7Mb to distal marker D17Mit50 at 44.8Mb, containing 40.1 Mb. Littermate F<sub>2</sub> mice were then used to generate separate homozygous congenic and homozygous B6 lines for producing experimental cohorts. GLP-1 receptor-
deficient mice (44) at N6 on the C57BL/6J background were obtained from Dr. Daniel J. Drucker and bred locally. Mice were housed in polycarbonate cages with sterilized corncob bedding, kept at 22-23° C on a 14:10 h light/dark cycle, and initially fed no. 5001 chow (LabDiet, Richmond, IN). All animal protocols were approved by the PBRC Institutional Animal Care and Use Committee.

**Phenotyping.** At 7-10 wk of age, mice were transferred to the diet selection paradigm and given a choice between a fat/protein (F/P) and a carbohydrate/protein (C/P) diet for 10 d. The composition of the F/P and C/P diets was equivalent for protein (22% of energy) with the balance of calories contributed by fat or carbohydrate (78%) (see Reference 46 for diet composition).

**Genotyping and linkage analyses.** A genome scan with 98 Mit SSLP markers at ~20 cM intervals was performed in 502 mice (46) using the method of Sen and Churchill (45) with Pseudomarker release version 1.06, available at [http://www.jax.org/staff/churchill/labsite](http://www.jax.org/staff/churchill/labsite). Significance was assessed by permutation analysis (10); the LOD threshold for significant linkage was 3.5.

**Microarray experiment.** Genome-wide gene expression levels were compared between B6.CAST-17 congenic and littermate B6 mice. Mice were food deprived at 1300 (during the light period) and euthanized 4 h later. The whole stomach was quickly removed, rinsed with cold PBS, frozen in liquid nitrogen, and stored at –80 deg. C. RNA was isolated using TRI reagent (Molecular Research Center). Each strain was represented by two pools, each containing equal amounts of RNA from three animals, for a total of four samples. Thus, two biological replicates were analyzed for each strain.
**Microarray analysis.** The Applied Biosystems (AB) Mouse Genome Survey Microarray was used which contains ~34,000 features including a set of ~1000 controls. Each microarray uses 32,996 probes targeted to 32,381 curated genes representing 44,498 transcripts. Total RNA was isolated from stomach and the quality was determined using an Agilent Bioanalyzer 2100. RNA (1 µg) was used to transcribe DIG-labeled cRNA using AB Chemiluminescent RT-IVT Kit v2.0. Microarray hybridization (using 10 µg of fragmented, DIG-labeled cRNA), processing, chemiluminescence detection, imaging, auto-gridding, and image analysis were performed according to AB protocols, using the 1700 Chemiluminescent Microarray Analyzer Software v. 1.0.3. The AB Expression system software was used to extract assay signal and signal-to-noise ratio values from the images. Signal intensities across microarrays were normalized using the quantile-quantile method based on R-script (5; [http://www.bioconductor.org](http://www.bioconductor.org)). Features with a signal/noise value of \( \geq 3.0 \) and quality flag value of <5000 were considered “detected” and subjected to analysis. The transformed data were analyzed by ANOVA using Spotfire DecisionSite Software v16.0 (Spotfire, Somerville, MA). Differential expression was defined as a fold change \( \geq 1.5 \) and a \( P \)-value of < 0.05. The data were submitted to the NCBI Gene Expression Omnibus (GSE7756).

**Functional analysis of Glp1r coding SNP.** The complete coding region for C57BL/6J and CAST/Ei Glp1r was subcloned into an expression plasmid pcDNA 3.1 (Invitrogen) and transfected into CHO-K1 cells (ATCC CCL-61; 56), graciously provided by Dr. Tom Gettys, using lipofectin (Invitrogen). Stable transfectants were obtained after selection for zeocin (zeo) resistance at a concentration of 0.5 mg/ml (Invitrogen) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. Zeo\(^R\) CHO cells expressing the empty vector pcDNA3.1 were used as a negative control. Two independent populations of Glp1r\(^{CAST}\) and
Glp1r\textsuperscript{B6} transfected CHO cells were selected based on equivalent levels of transgene expression as measured by qRT-PCR, and assayed for cAMP production in response to GLP-1. The three transfected cell lines were passaged into 6-well plates and at 60% confluence, the cells were washed with PBS followed by assay buffer (DMEM containing 1% BSA). Cells were stimulated with GLP-1 (Genescript, NJ) at two different concentrations (100 nM and 500 nM) in the presence of 3-isobutyl-1-methylxanthine (IBMX, 1\(\mu\)M) for 8 minutes. cAMP levels were measured with the cAMP Biovision direct immunoassay kit according to manufacturer’s instructions. Data were normalized with respect to total protein measured from the same extracts.

*Screen for allelic imbalance in transcript levels.* A sequence-based assay was employed to measure allelic-specific transcript levels using cDNA derived from the whole stomach, pancreas and hypothalamus of adult male (B6 x B6.CAST-17)\(F_1\) mice (11, 28). A 247-bp amplicon spanning the previously identified SNP at position 1247 (26) was generated using the oligonucleotide primers: forward, 5’-GTCCAGATGGAGTTTCGGAA-3’ and reverse, 5’-CCCTATCCCCAGGATCTCTC-3’ (corresponding to nt positions 1225-1244 and 1453-1472 of the reference Genbank sequence accession no. NM\_021332.1, respectively) and purified (QIAGEN kit). Sequencing was performed in the PBRC Genomic Core Facility on an AB 3130 using the BigDye Terminator Cycle Sequencing kit version 1.1 (Perkin-Elmer Biosystems), with the reverse primer. The B6 and CAST SNP peak heights were compared using the AB Sequence Scanner v1.0 software program. A deviation from the expected ratio of one (equal amounts of each allele) suggests regulatory controls on RNA transcription or stability (35, 57).

*Indirect calorimetry.* The rate of carbon dioxide production (VCO\(_2\)) was measured by the Oxymax indirect calorimetry (Columbus Instruments) with airflow of 0.75 L/min.
Gastric emptying using the $^{13}$C-octanoate breath test. $^{13}$C-octanoate (Cambridge Isotopes Laboratories), a non-radioactive, stable isotope, is administered in a test meal (30). As the meal empties into the duodenum, the $^{13}$C-labelled octanoate is rapidly absorbed and transported via the portal system to the liver (25) where it is oxidized to CO$_2$ and excreted in the breath. The rate of $^{13}$CO$_2$ appearance (excretion curve) approximates the rate of gastric emptying (43, 48-50).

In the present study, singly-housed mice were trained to eat a semi-solid test meal containing 47% carbohydrate, 34.5% fat, 0.7% protein, 3.8% fiber, and 14% moisture by weight and 4.4 kcal/g by energy (Nutri-Cal®, Evsco Pharmaceuticals). Mice were acclimated to metabolic chambers for 24 h; VCO$_2$ and VO$_2$ were measured at 15 min intervals. Breath tests were performed after 18 h food (not water) deprivation. Baseline calorimetry measurements were taken at 15 min intervals and mice were then fed a test meal containing the metabolic tracer. The meal was administered over 10 min through a pre-weighed, 1-ml syringe inserted into the metabolic chamber through a hole. The food syringe was then replaced with a rubber stopper through which 10 ml breath samples were extracted from the chamber at 5 min intervals for the first 30 min (to capture the rapid rise in $^{13}$CO$_2$ excretion) and at 10 min intervals thereafter. Breath samples were immediately injected into evacuated 10-ml Exetainer tubes (Labco Limited, High Wycombe, England) for later analysis.

Analysis of gastric emptying data. Samples were analyzed using a trapping box or gas bench connected to a Finnigan Mat 252 isotope ratio mass spectrometer (Bremen, Germany). The percent dose excreted per hour was calculated using the measured VCO$_2$ (42). The area under the curve (AUC) for the cumulative $^{13}$CO$_2$ excretion from 0-6 h was determined by the trapezium method and used to calculate the gastric half-emptying time ($t$ $\frac{1}{2}$) (43). The rate of
CO₂ production was based on a mean of the VCO₂ values collected for each individual mouse in the experiment. The response variable (% dose administered per h) was analyzed two ways: 1) the AUC was calculated for each subject with respect to 10-min time intervals, and 2) the t½ AUC was established and then using linear interpolation, the t½ of the % dose administered was calculated. In both analyses, the response variable was analyzed with respect to cohort and strain. Neither a cohort-by-strain interaction, nor a cohort effect was observed, thus the simpler model was chosen and only the strain effect was tested.

_Gastric emptying by laparotomy._ Mice were housed separately and food (not water) deprived for 16-18 h. At time = 0 h, animals were fed preweighed chow for 1 h. At time = 3 h, mice were euthanized by isoflurane inhalation. The stomach was exposed by laparotomy, immediately excised at the pylorus and cardia, and its wet contents removed, frozen, lyophilized overnight and weighed. Gastric emptying (%) was calculated as (1-(dry weight of food recovered from the stomach/total food intake)) x 100 (51, 58).

_Effects of exendin-(9-39) on gastric emptying._ Exendin-(9-39) is a COOH-terminal fragment of exendin-4, a bioactive peptide which shares 50% structural homology with GLP-1 (19, 53) and antagonizes the _in vivo_ actions of GLP-1 (51). Administration of exendin-(9-39) to rats or human subjects increases gastric emptying (40) when given by peripheral but not central administration (21). In the present study, overnight fasted mice were injected i.p. with 100 µl of either phosphate buffered saline (PBS) or exendin-(9-39) (Bachem) (0.25 mg/kg) immediately before re-feeding. Mice were fed pre-weighed chow for 1 h, food deprived for another 1.5 h, and then euthanized. Gastric emptying rate was calculated as described above.
General statistical analyses. ANOVA was used to determine the effects of strain and treatment, i.e., exendin-(9-39) vs. PBS, on gastric emptying. The significance level was set at \( P < 0.05 \).

RESULTS

Identification of QTL for total food volume. We hypothesized that food volume may constitute a quantitative trait because in our paradigm the two diets from which the F2 mice composed their total intake contained different energy densities, i.e., carbohydrate/protein (3.61 kcal/g) and fat/protein (5.96 kcal/g). Thus we conducted a genome scan for this phenotype in a mapping population used previously (46). Genome scans for total food volume (g) over the 10-d phenotyping period, with and without baseline body weight as a covariate, are presented in Fig. 1. These analyses revealed a highly significant QTL (LOD=7.6, peak 14 cM, \( D17Mit198 \); 95% confidence interval, 10-26 cM) controlling total food volume adjusted for body weight (\( Tfv1 \)). The location of \( Tfv1 \) coincides with previously identified QTLs influencing carbohydrate (\( Mnic1 \)) and total kilocalorie intake (\( Kcal2 \)) (46), suggesting a common mechanism (Fig. 2). Analyses of total food volume also detected three suggestive QTL (LOD threshold = 2.5) on Chrs 2 (peak 76 cM), 6 (peak 46 cM), and 15 (peak 14 cM). The suggestive QTL for food volume on Chrs 2, 6, and 15 appear to depend on body weight, i.e., they disappeared when adjusted for body weight by regression (Fig. 1B). By contrast, the food volume QTL on Chr 17 strengthened considerably (LOD increased from 4.6 to 7.6) (Fig. 1B), suggesting that this locus has effects on both food intake and body weight but with effects in opposite direction (29). No other significant QTLs for food volume were detected, suggesting a unique contribution of the Chr 17 locus.
Total food volume in parental and congenic mice. The CAST strain consumed significantly greater food volume (expressed as total g per 20 g body weight) (40.1 ± 1.0 vs. 24.5 ± 3, 1.6 fold, \( P < 0.0001 \)), when compared with B6 mice, over the 10 d phenotyping period (Fig. 3A). In addition, the B6.CAST-17 congenic mice consumed greater food volume per body weight compared to B6 homozygote controls B6 (27.8 ± 0.9 vs. 21.7 ± 0.7 g, 1.3 fold; \( P < 0.0001 \)) (Fig. 3B), thus verifying the genetic linkage for increased total food volume on proximal Chr 17.

Gene expression candidates in stomach. Whole-genome microarray analysis using RNA prepared from stomach was performed to identify genes with expression differences due to the QTL. Within the QTL, 31 out of 523 genes (6%) were differentially expressed between B6.CAST-17 congenic and B6 homozygote controls (Table 1). Expression was increased in 18 genes and decreased in 13 genes. Out of 20,217 expressed genes outside of the QTL region, only 252 (~1%) were differentially expressed; 154 genes showed elevated expression and 98 were decreased in gastric RNA from B6.CAST-17 mice. An examination of known genes outside the QTL that could potentially affect gastric emptying and/or satiety (39) through trans-acting effects revealed none with differential expression, e.g., GLP-1 derived from post-translational processing of pro-glucagon (\( \text{Gcg} \)), ghrelin (\( \text{Ghrl} \)), cholecystokinin (\( \text{Cck} \)) and its two receptors (\( \text{Cckar}, \text{Cckbr} \)), gastrin (\( \text{Gast} \)), peptide YY (\( \text{Pyy} \)), and amylin (\( \text{Iapp} \)). Notably, expression of the \( \text{Glp1r} \) (glucagon-like peptide 1 receptor), located near the QTL peak, was significantly decreased \( (P = 0.01) \). qRT-PCR analysis of individual gastric RNA samples validated the significant decrease in \( \text{Glp1r} \) expression (by a factor of 2) in B6.CAST-17 mice.

Functional analysis of a SNP in the coding region of \( \text{Glp1r} \). Previously we reported a nonsynonymous single nucleotide polymorphism (SNP) that predicts an amino acid change
(C416Y) in the C-terminal intracellular domain of the GLP-1R (26). To assess whether this SNP results in generation of a GLP-1 receptor with altered signaling in response to GLP-1, an *in-vitro* assay was performed. Two independent populations of \( \text{Glp1r}^{\text{CAST}} \) and \( \text{Glp1r}^{B6} \) transfected CHO cells, which stably express equivalent levels of the respective transgenes, were generated and assayed for cAMP production in response to GLP-1. Administration of GLP-1 increased cAMP in both cell lines in a concentration-dependent manner. However, we did not observe an allele-specific difference in levels of cAMP accumulation, indicating that this nonsynonymous variant does not affect receptor signaling (Table 2). One caveat to be considered is that the CHO cells used in this experiment were hypodiploid which could result in abnormal chromosome counts and therefore mask subtle differences between the alleles being tested.

**Allelic imbalance (AI) test for Glp1r.** We next sought evidence for a regulatory polymorphism that could be responsible for modifying \( \text{Glp1r} \) expression between strains, in a tissue-specific manner. Quantification of allelic-specific \( \text{Glp1r} \) expression in F\( _1 \) heterozygous mice revealed a significant deviation from the theoretical 50:50 allelic ratio in stomach (Fig 4). Specifically, the mRNA level showed an altered ratio of 31% T and 69% C (CAST and B6 respectively), or a ratio of 2.22. By contrast, an altered ratio in cDNA was not found for either the pancreas or hypothalamus. These results suggest that a stomach specific, \textit{cis}-acting regulatory element (controlling RNA transcription or stability) underlies the strain difference in gastric \( \text{Glp1r} \) expression, rather than a trans-acting factor (e.g., transcription factors).

**Gastric emptying in the parental strains.** The rate of gastric emptying was determined in the parental strains after an overnight fast and administration of a test meal. In the \( ^{13}\text{C}\)-octanoate breath test, gastric emptying rate was significantly faster in CAST (by 20%) compared with B6 \[ t_{1/2}-\text{values} \pm \text{SE of the mean: } 113 \pm 4 \text{ vs. } 140 \pm 4 \text{ min respectively; } F(1,33.9) = 21.83, P < \]
0.0001] (Fig. 5). Fig. 5 illustrates the strain difference in the $^{13}$C excretion curve in response to a standardized meal. Following the lag phase, CAST mice (with reduced gastric Glp1r expression) showed an increased gastric emptying rate, clearly indicated by a curve shift to the left in the linear phase. The curve amplitude was similar between the two strains, indicating that hepatic oxidation of $^{13}$C octanoate was equivalent. Gastric emptying determined in this manner was then compared with the traditional method of analyzing gastric contents after meal ingestion. In a separate experiment, we measured the amount of food remaining in the stomach 2 h after re-feeding, and again observed a significantly higher gastric emptying rate in CAST mice compared with B6 (Fig. 6A).

Gastric emptying can be affected by intragastric volume (20), which varies in mouse strains of different size. The smaller CAST mice consumed 32% less of the test meal than B6 mice did, but also had 32% lower stomach mass. There was no difference in test meal size when expressed per stomach mass (CAST $4.0 \pm 0.2 \text{ g/g}$ vs. B6 $3.7 \pm 0.2 \text{ g/g}$, $P = 0.25$, data not shown), thus it is unlikely that meal volume contributed to the observed strain difference in gastric emptying. With respect to the B6.CAST-17 congenic and B6 mice, there was no difference in either body weight or test meal size.

*Gastric emptying in the congenic strain.* Gastric emptying rate was assessed by measuring the amount of lyophilized gastric contents that remained 2 h after re-feeding. Consistent with our observations in the parental strain CAST, the rate of gastric emptying was significantly higher in B6.CAST-17 congenic mice compared with homozygous B6 controls ($95.1 \pm 1.4\%$ vs $87.3 \pm 2.2\%$, $P < 0.05$) (Fig. 6B).

*Test of GLP-1R function using Exendin-(9-39).* The physiological effects of GLP-1 have been examined using GLP-1 receptor antagonists to block endogenous GLP-1 action, e.g.,
administration of exendin-(9-39) to rats or human subjects increases gastric emptying (40). Because Glp1r mRNA and protein expression in stomach is lower in the CAST parental and B6.CAST-17 congenic strains (26), we tested the hypothesis that decreased GLP-1 receptor function is responsible for the strain difference in gastric emptying. Consistent with previous experiments, saline-treated B6.CAST-17 congenic mice showed a small but significant increase in the rate of gastric emptying (11% compared with homozygous B6, \( P < 0.05 \)) (Fig. 7). Administration of exendin-(9-39) significantly increased gastric emptying in homozygous B6 mice by 10% (\( P < 0.05 \)) but not in B6.CAST-17 congenic mice (\( P = 0.78 \)) (Fig. 7). Thus B6.CAST-17 congenic mice exhibited significantly less GLP-1R-mediated inhibitory function relative to the homozygous B6 control line, which may contribute to their higher gastric emptying rate.

*Phenotypic analyses in Glp1r-/- mice.* We examined whether the presence or absence of the GLP-1R in vivo would alter a) the composition of self-selected macronutrient diet intake, or b) the rate of gastric emptying. Glp1r-/- mice consumed 2-fold more carbohydrate and less fat (by energy) compared with inbred B6 mice (51 ± 2%, \( n=17 \), vs. 30 ± 3%, \( n=1 \), \( P < 0.0001 \)) (Fig. 8). There was no strain difference in total food volume over 10 d (Glp1r-/- 22 ± 1 g vs. B6 20 ± 1 g, \( P = 0.22 \); Fig. 3C), although the GLP-1R deficient mice tended to have a lower energy intake per body weight (7%; \( P = 0.05 \); data not shown). We next examined whether GLP-1R deficiency would enhance gastric emptying. Unexpectedly, Glp1r-/- mice showed a slower rate of gastric emptying compared with B6 (85 ± 3%, \( n=17 \), vs. 93 ± 1%, \( n=14 \), for Glp1r-/- vs. B6, respectively, \( P < 0.05 \); data not shown).
DISCUSSION

In this study, we have identified a new QTL for total food volume ($Tfv1$) on mouse chromosome 17 and have verified linkage for this trait in a B6.CAST-17 congenic strain. This QTL region encompasses $Glp1r$ (glucagon-like peptide 1 receptor), encoding an important modulator of gastric emptying. We have shown that genetic variation in $Glp1r$ is associated with altered gastric emptying in mice. Evidence of genetic variation is that $Glp1r$ shows allelic imbalance in stomach but not pancreas or hypothalamus, suggesting the existence of a mutation(s) or sequence variant(s) in either tissue-specific regulatory elements or other non-coding regions. These data extend our previous observations of complex, tissue-specific expression differences in $Glp1r$ between the CAST and B6 strains (26). Moreover, we found that allele-specific $Glp1r$ expression in stomach is associated with an acceleration in gastric emptying rate, as well as an impaired response to GLP-1 receptor blockade, which suggests a functional polymorphism. These results provide evidence for a genetic component in the regulation of gastric emptying. Furthermore, these findings suggest the involvement of stomach GLP-1 receptors in the inhibitory action of GLP-1 on gastric emptying.

To more precisely understand the genetic loci contributing to the control of food intake and gastric emptying, we used microarray analysis to identify potential candidate genes located within the Chr 17 congenic donor region that were differentially expressed in the stomach of a congenic strain that consumes more energy and food volume per body weight. Thus we compared gene expression between the B6.CAST-17 congenic and the recipient strain. Microarray analysis revealed that within the QTL, 31 or ~6% of expressed genes were differential in stomach. Outside of the QTL, only 252 or ~1% of 20,217 expressed genes showed differential expression between B6.CAST-17 congenic and B6 mice. An examination of
all known genes, differentially expressed within or outside the QTL, found none other than Glp1r that could potentially affect gastric emptying and/or satiety (39) through cis- or trans-acting effects. Thus the results of this experiment confirmed Glp1r as a primary candidate with regard to differential gene expression.

The present study has revealed allele-specific expression in stomach, but not in hypothalamus or pancreas, due to lower expression of the CAST allele. This finding suggests the existence of a polymorphism that may contribute to the physiological effects of these QTLs. For example, reduced GLP-1 receptor expression in the stomach of CAST mice could lead to more rapid gastric emptying, thus permitting the consumption of larger food volumes and more calories, in the form of carbohydrate. To identify specific regulatory elements, we sequenced ~2.5 kb of the region upstream of the translation start site for Glp1r. Several polymorphisms were identified, which is not surprising given the high polymorphism rate between these strains. Additional studies are needed to identify the putative cis-acting regulatory variant(s) responsible for this tissue- and allele-specific expression.

The present study revealed an accelerated gastric emptying rate in parental CAST and B6.CAST-17 congenic mice which correlates with their lower Glp1r expression (26). Our evidence for inbred strain differences in gastric emptying rate extends the observations of Kirby et al. (24) and provides new information on the identity of candidate genes contributing to this phenotype. Our results are similar in magnitude to those observed between lean and obese humans (13%) (22), and consistent with the effects of known GLP-1 receptor agonists on food intake and gastric emptying (51). The modest effect size on gastric emptying rate in our study can be attributed to the typical size of individual genetic contributions to polygenic traits. As reviewed by Flint et al. (18), most QTLs detected in an inbred strain cross have small effects.
Therefore the molecular mechanisms of genes that underlie them produce small genetic effects, e.g., it has been estimated that the average effect size could go as low as 5%.

The present results support Glp1r as a functional candidate for this Chr 17 QTL region by showing that administration of the GLP-1R antagonist exendin-(9-39) led to an acceleration of gastric emptying rate in B6 mice, but had no effect in the B6.CAST-17 congenic mice. The demonstration of an impaired response to GLP-1 receptor blockade in the B6.CAST-17 congenic strain suggests the presence of a functional polymorphism. The mechanism by which basal expression and signaling of the endogenous GLP-1R could influence stomach emptying (and ultimately food intake) is not yet known but could result from genetic variation in the regulatory region of the gene, leading to reduced Glp1r expression (26).

Our findings suggest a peripheral effect of GLP-1R in stomach. GLP-1 receptors are expressed on gastric parietal cells (7, 41, 55) which suggests that GLP-1R might inhibit gastric emptying and/or gastric acid secretion through a direct mechanism. However little is known about the biological actions of GLP-1 in this tissue. Although the inhibitory effect of GLP-1 on gastric emptying likely involves neural transmission, GLP-1 retained its inhibitory effect on antral motility in pigs after vagal deafferentation (32). Moreover, smooth muscle cells from the human colon contract in response to GLP-1, and this effect was inhibited by exendin-(9-39) (2). Hence, it remains possible that GLP-1 modulates gastric emptying in part through direct interaction with the gastric GLP-1 receptor.

It is important to note that the regulation of gastric emptying depends not only on antral motility but also on the gastric body or corpus, the motility of which is regulated by a number of gastrointestinal peptides via a vagal afferent pathway. For example, CCK stimulates vagal afferent neurons to relax the body of the stomach and thereby to decrease the pressure gradient
between stomach and duodenum (36-37), resulting in an inhibitory effect on gastric emptying.

With this in mind, we examined all known genes encoding gastrointestinal peptides and/or their receptors, both within and outside the congenic interval, and found none that were differentially expressed in the stomach of B6.CAST-17 congenic vs. wildtype mice, other than Glp1r.

Genetic variation in the response of mechanical receptors to gastric distension could also influence rates of gastric emptying, satiety, and subsequent food intake through a neural reflex arc. In this regard, CCK-1 receptor-deficient OLETF rats have been shown to exhibit decreased behavioral and vagal responsiveness to gastric distension, although they did not show altered gastric emptying rates (12). To our knowledge, strain differences in response to distension, e.g., in vagal nerve activity, have not been reported. However the possibility that a putative deficit in vagal responsiveness to gastric distension in B6.CAST-17 congenic mice accounts for their increased gastric emptying rate, thus allowing them to consume higher food volumes, cannot be discounted.

We did not observe a functional effect of the C416Y Glp1r variant (26) in CHO cells. Compared to the parental B6 strain receptor gene, the variant CAST Glp1r showed similar efficacy in transducing cAMP production by GLP-1. Thus it appears that the mechanism by which the variant GLP-1R protein could influence stomach emptying (and ultimately food intake) is not due to this coding polymorphism.

We examined the physiological effects of endogenous GLP-1 on nutrient intake in Glp1r-/- mice and found that absence of the GLP-1R altered macronutrient diet selection, resulting in the preferential consumption and higher intake of carbohydrate. By contrast, there were no differences in total calories or food volume between the Glp1r-/- and inbred C57BL/6J mice. These results are consistent with a previous investigation of Glp1r-/- mice on the outbred CD1
genetic background which found no effects of the targeted GLP-1R mutation on food intake after a 20 h fast (44). Nevertheless, the preferential carbohydrate intake displayed by the *Glp1r/-* mice was somewhat surprising. Due to impaired glucose-stimulated insulin secretion, *Glp1r/-* mice exhibit mild fasting hyperglycemia and glucose intolerance after oral or intraperitoneal loading (44). Thus we hypothesized that *Glp1r/-* mice would select proportionately more calories from fat. This proposition was based on substantial evidence that mildly diabetic rats select higher amounts of fat from macronutrient sources than nondiabetic rats, due to the conditioning effects of substrate utilization on feeding behavior (3, 23, 38). The discrepancies observed in the phenotypic comparisons between *Glp1r/-* mice and B6.CAST-17 congenic mice could be due to environmental (satiety model) or genetic factors (27), e.g., the genetic background was composed of 129/J, CD1, and C57BL/6J genomic DNA, but contained none from the CAST/Ei. Furthermore, the B6,CD1.129Glp1rtm_Drucker mice studied here were generated through only 6 backcross generations and may still contain regions of contaminating CD1 DNA throughout the genome. Therefore although the *Glp1r/-* mouse is a good model for examining GLP-1R function, it is not suitable for evaluating our *Glp1r* variant, due to the unique genetic and genomic features attributed to the CAST donor segment.

We have identified a highly significant locus mapping to proximal mouse chromosome 17 that is genetically linked to the increased consumption of carbohydrate, total calories, and food volume. Using an interval-specific microarray of stomach, we identified *Glp1r* as the only expression candidate known to potentially affect gastric emptying and/or satiety. Allele-specific expression analyses indicated the differential expression in stomach was due to cis effects of a polymorphism within the congenic donor region. Physiologic studies employing pharmacological antagonism of the GLP-1 receptor provided evidence for a functional effect of
this genetic variant. We conclude that Glp1r is a compelling functional candidate gene for this complex quantitative trait locus.

**Perspectives and Significance**

To our knowledge, there have been no previous studies directed at uncovering genetic variants in Glp1r that are associated with gastric emptying phenotypes. Other studies aimed at identifying polymorphisms in the human GLP-1R gene have focused on possible linkage with diabetes or obesity and thus far none has been reported. Genetic polymorphisms may be responsible for the wide variation in individual drug responses. An emerging pharmaco-genetics field is aimed at the detection of genetic variation to help predict drug metabolism and patient response. With regard to GLP-1, its ability to stimulate glucose-dependent insulin secretion has led to the development of therapeutic agents to treat type 2 diabetes. For example, pharmacological activation of the GLP-1R with Exenatide reduces body weight and improves glycemic control in diabetes, yet there is a gradation in the clinical response (4). Similarly, some patients treated with GLP-1R agonists experience nausea; however the relation between the presence of nausea and the degree of inhibition of gastric emptying following these drugs remains poorly understood. Our findings illustrate that genetic differences may contribute to the differential expression and action of the GLP-1R in mouse stomach. Future analyses of genetic and functional differences in Glp1r genotypes may enhance our understanding of the control of gastric emptying and/or individual differences in response to incretin-based therapies.
ACKNOWLEDGMENTS

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Table 1. Differentially expressed genes within the donor region of B6.CAST-17 as identified by microarray (positive value in fold difference indicates increased expression, and negative value indicates decreased expression, in congenic mice). Mm, mus musculus.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>P-value</th>
<th>Fold difference</th>
<th>UniGene</th>
<th>Gene symbol</th>
<th>Gene name</th>
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</thead>
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<tr>
<td>904816</td>
<td>0.00068</td>
<td>-5.34</td>
<td>Mm.22506</td>
<td>D17H6S56E-5</td>
<td>DNA segment, Chr 17, human D6S56E 5</td>
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<tr>
<td>761588</td>
<td>0.021</td>
<td>-5.30</td>
<td>Mm.267998</td>
<td>Taf11</td>
<td>TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor</td>
</tr>
<tr>
<td>611571</td>
<td>0.040</td>
<td>-4.15</td>
<td>Mm.3135</td>
<td>Igfals</td>
<td>Insulin-like growth factor binding protein, acid labile subunit</td>
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<td>716213</td>
<td>0.030</td>
<td>-3.88</td>
<td>Mm.292869</td>
<td>Decr2</td>
<td>2-4-dienoyl-Coenzyme A reductase 2, peroxisomal</td>
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<td>906572</td>
<td>0.043</td>
<td>-3.12</td>
<td>Mm.57043</td>
<td>Nfkbie</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon</td>
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<tr>
<td>300048</td>
<td>0.022</td>
<td>-2.92</td>
<td>Mm.9277</td>
<td>Pla2g7</td>
<td>Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)</td>
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<tr>
<td>638437</td>
<td>0.033</td>
<td>-2.70</td>
<td>Mm.87776M m.195061 Mm.195061</td>
<td>H2-T17H2-T10H2-T9H2-T22</td>
<td>Histocompatibility 2, T region locus 17</td>
</tr>
<tr>
<td>370857</td>
<td>0.001</td>
<td>-2.65</td>
<td>Mm.30145</td>
<td>Enpp5</td>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase 5</td>
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<tr>
<td>431279</td>
<td>0.005</td>
<td>-2.52</td>
<td>Mm.235338</td>
<td>H2-EalH2-Aa</td>
<td>Histocompatibility 2, class II antigen E alphahistocompatibility 2, class II antigen A, alpha</td>
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<td>762178</td>
<td>0.021</td>
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<td>Mm.215162</td>
<td>Prdm9</td>
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<td>465717</td>
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<td>-2.35</td>
<td>Mm.285322</td>
<td>Mtc1</td>
<td>Mitochondrial carrier homolog 1 (C. elegans)</td>
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<tr>
<td>653170</td>
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<td>-2.30</td>
<td>Mm.28420</td>
<td>Vars2</td>
<td>Valyl-tRNA synthetase 2</td>
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<td>706669</td>
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<td>1110038B12Rik</td>
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<td>558496</td>
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<td>Mm.35016</td>
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<td>919204</td>
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<td>-1.57</td>
<td>Mm.30496</td>
<td>1200007D18Rik</td>
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<td>591309</td>
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<td>-1.50</td>
<td>Mm.140772</td>
<td>Glp1r</td>
<td>Glucagon-like peptide 1 receptor</td>
</tr>
<tr>
<td>Gene ID</td>
<td>FDR</td>
<td>Fold Change</td>
<td>Accession Number</td>
<td>Description</td>
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</tr>
<tr>
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<td>-----------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>346004</td>
<td>0.019</td>
<td>1.55</td>
<td>null</td>
<td>Gm935 Gene model 935, (NCBI)</td>
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<tr>
<td>673097</td>
<td>0.005</td>
<td>1.65</td>
<td>Mm.15252</td>
<td>Srpk1 Serine/arginine-rich protein specific kinase 1</td>
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<tr>
<td>915442</td>
<td>0.031</td>
<td>1.65</td>
<td>Mm.379295</td>
<td>Glo1 Glyoxalase 1</td>
<td></td>
</tr>
<tr>
<td>915872</td>
<td>0.014</td>
<td>2.17</td>
<td>Mm.26688</td>
<td>Thbs2 Thrombospondin 2</td>
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</tr>
<tr>
<td>920440</td>
<td>0.029</td>
<td>2.19</td>
<td>Mm.328931</td>
<td>Pim1 Proviral integration site 1</td>
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<tr>
<td>869338</td>
<td>0.036</td>
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<td>Mm.79993</td>
<td>Dact2 Dapper homolog 2, antagonist of beta-catenin (xenopus)</td>
<td></td>
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<td>467910</td>
<td>0.009</td>
<td>2.26</td>
<td>null</td>
<td>Btnl1 Butyrophilin-like 1</td>
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<tr>
<td>849986</td>
<td>0.017</td>
<td>2.64</td>
<td>Mm.214638</td>
<td>Tmprss3 Transmembrane protease, serine 3</td>
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<tr>
<td>480967</td>
<td>0.003</td>
<td>3.09</td>
<td>Mm.2443</td>
<td>0610031G08Rik RIKEN cDNA 0610031G08 gene</td>
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<tr>
<td>682540</td>
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<td>3.40</td>
<td>Mm.24192</td>
<td>Msh5 MutS homolog 5 (E. coli)</td>
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<td>837168</td>
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<td>6.63</td>
<td>Mm.381072</td>
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<td>898097</td>
<td>0.036</td>
<td>6.66</td>
<td>Mm.275521</td>
<td>Wtap Wilms' tumour 1-associating protein</td>
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<td>560648</td>
<td>0.004</td>
<td>51.35</td>
<td>Mm.358604</td>
<td>H2-Ob Histocompatibility 2, O region beta locus</td>
<td></td>
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</tbody>
</table>

Genes were selected based on a significant ($P < 0.05$) and differential fold change in expression $> 1.5$. Fold change refers to the normalized signal intensity of a given gene in B6.CAST-17 homozygous relative to B6.CAST-17 wild-type mice. *The curated gene information is not available from Applied Biosystems at this time.
Table 2. cAMP production after stimulation with GLP-1(7-36)amide. CHO cells expressing B6 GLP1R and CAST GLP1R were stimulated with two concentrations of GLP-1(7-36) amide for 8 min and the production of cAMP (fmol/2.3 x 10^5 cells) was determined by enzyme immunoassay, performed in triplicate. The data are presented as means ± S.E.M.

<table>
<thead>
<tr>
<th>Concentrations of GLP-1(7-36)amide</th>
<th>0 nm</th>
<th>100 nm</th>
<th>500 nm</th>
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<tr>
<td>pC-DNA3.1</td>
<td>5.14 ± 0.64</td>
<td>3.10 ± 0.40</td>
<td>4.48 ± 0.98</td>
</tr>
<tr>
<td>B6 GLP-1R</td>
<td>7.90 ± 2.25</td>
<td>35.19 ± 7.50</td>
<td>102.73 ± 7.09</td>
</tr>
<tr>
<td>CAST GLP-1R</td>
<td>8.52 ± 1.62</td>
<td>45.50 ± 8.07</td>
<td>93.94 ± 5.39</td>
</tr>
</tbody>
</table>
REFERENCES


26. **Kumar KG, Poole AC, York B, Volaufova J, Zuberi A, and Smith Richards BK.** Quantitative trait loci for carbohydrate and total energy intake on mouse Chromosome 17:


FIGURE LEGENDS

Fig. 1. Results of genome-wide QTL analysis for loci linked to total food volume (Tfv1) in an F2 population derived from the CAST and B6 inbred strains. Chromosome (Chr) numbers are represented on the X-axis. The Y-axis represents the LOD score. The experiment-wide significant (LOD > 3.5; \( P < 0.05 \)) and suggestive (LOD > 2.6; \( P < 0.10 \)) levels of genetic linkage were determined by permutation analyses (10). A: Absolute intake. B: Intake adjusted for baseline body weight by regression.

Fig. 2. Interval map for the Tfv1 QTL located on Chr 17, including coincident QTL for macronutrient intake-carbohydrate 1 (Mnic1) and total kilocalories (Kcal2). The abcissa represents the logarithm of the odds ratio (LOD) and the horizontal line indicates the LOD threshold for significant linkage (3.5). The ordinate contains symbols for MIT markers.

Fig. 3. Phenotypic strain differences in total food volume (g) per body weight, consumed over 10 d from a choice between the fat/protein and carbohydrate/protein diets. A: Inbred strain CAST (n=38) vs. B6 (n=35) were phenotyped as part of the QTL study, which included inbred strain controls in every F2 breeding cohort (1). B: Congenic B6.CAST-17 (B6.C-17) (n=20) vs. homozygous B6 (n=25). C: GLP-1R(-/-) (n=17) vs. inbred B6 (n=14) mice. \(*P < 0.0001\).

Fig. 4. Detection of allelic-specific gene expression using a sequencing-based assay; differences in the relative peak heights (in relative fluorescent units) were used to detect allelic imbalance. Sequence traces were obtained by amplifying the cDNA from F1 (B6 x B6.CAST-17 congeneric) and genomic DNA (gDNA) from C57BL/6J and CAST/Ei, for a region encompassing the single
nucleotide polymorphism (SNP) at 1247 bp of mRNA. Sequence traces from gDNA (top row) vs. cDNA (bottom row) of the two alleles are illustrated. The ratio in genomic DNA shows the theoretical 50:50 ratio as indicated by the arrow. In contrast, the cDNA shows an altered ratio of 31% T (red color) and 69% C (blue color) representing CAST and B6 respectively, or a ratio of 2.22, in stomach but not in hypothalamus or pancreas. Because the genomic DNA is at the theoretical ratio, no correction is required.

**Fig. 5.** (A) Gastric emptying in CAST ($n=28$) (●) and B6 ($n=30$) (○) mouse inbred strains as measured by the $^{13}$C-octanoate breath test. Time 0 designates the end of the test meal. The amount of $^{13}$CO$_2$ recovery in the breath samples, determined by isotope ratio mass spectrometry, is expressed as percentage of dose/hour curve after ingestion of a semi-solid test meal. The $t_{50}$ after the test meal for each of the two strains is represented by the dashed line.

**Fig. 6.** Percent gastric emptying in A: CAST ($n=11$) and B6 ($n=12$) mouse inbred strains and B: B6.CAST-17 (B6.C-17) homozygous congenic ($n=6$) and homozygous B6 ($n=7$) mice, as measured by the amount of lyophilized gastric contents that remained 2 h after re-feeding. *$P < 0.05$. #$P = 0.05$.

**Fig. 7.** Effects of strain and the GLP-1R antagonist exendin-(9-39) on gastric emptying in B6.CAST-17 (B6.CAST) homozygous congenic and homozygous B6 mice. *$P < 0.05$ vs. PBS. $n=6$ mice per group.
Fig. 8. Percent (%) of total kcal from fat and carbohydrate in C57BL/6J (n=14) and Glp1r<sup>−/−</sup> (n=17) mice selecting from a choice between carbohydrate/protein and fat/protein diets for 10 d. 

$P < 0.0001$. 
Fig. 1
Fig. 3
Fig. 4
CAST $t_{1/2} = 113 \pm 4$

B6 $t_{1/2} = 140 \pm 4$
Fig. 6
Macronutrient preference (%)

- B6 (14)
- Glp1r<sup>−/−</sup> (17)

(Protein intake was held constant at 22% of energy)