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

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<sup>1</sup>Divisions of Experimental Obesity, and <sup>2</sup>Nutrition and Chronic Diseases, <sup>3</sup>Stable Isotope Laboratory, Pennington Biomedical Research Center, Louisiana State University, Baton Rouge; <sup>4</sup>Biostatistics Program, Louisiana State University Health Sciences Center, New Orleans, Louisiana; <sup>5</sup>The Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, University of Toronto, Toronto, Ontario, Canada; and <sup>6</sup>The Jackson Laboratory, Bar Harbor, Maine

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**Kumar KG, Byerley LO, Volaufova J, Drucker DJ, Churchill GA, Li R, York B, Zuberi A, Richards BK.** Genetic variation in *Glp1r* expression influences the rate of gastric emptying in mice. *Am J Physiol Regul Integr Comp Physiol* 294: R362–R371, 2008. First published December 12, 2007; doi:10.1152/ajpregu.00640.2007.—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 F<sub>2</sub> 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*) (log of the odds ratio = 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.

quantitative trait locus; glucagon-like peptide 1 receptor

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 (GLP-1R) antagonist exendin-(9-39) stimulates food intake (14, 54).

The 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 (Ref. 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, in gastrointestinal tract, in lung, in kidney, in heart, and throughout the brain (7, 8, 16). The satiety-promoting effects of GLP-1 implicate *Glp1r* as a physiological 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) × 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 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 physiological effects of GLP-1. Thus we hypothesized that variation in *Glp1r* could influence nutrient intake through changes in either the

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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 F<sub>1</sub> 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-1R 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-day 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 an ~38-cM CAST donor segment on the B6 background. This CAST segment extends at least from proximal marker *D17Mit19* at 4.7 Mb to distal marker *D17Mit50* at 44.8 Mb, 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 corn cob 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 Pennington Biomedical Research Center 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 days. 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 Ref. 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>. Significance was assessed by permutation analysis (10); the log of the odds ratio (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°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 ~1,000 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 transcribed to DIG-labeled cRNA using AB chemiluminescent RT-IVT kit version 2.0. 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 version 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 (Ref. 5; <http://www.bioconductor.org>). Features with a signal/noise value of ≥3.0 and quality flag value of <5,000 were considered “detected” and subjected to analysis. The transformed data were analyzed by ANOVA using Spotfire DecisionSite Software version 16.0 (Spotfire, Somerville, MA). Differential expression was defined as a fold change ≥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; Ref. 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 DMEM containing 10% fetal bovine serum. Zeo<sup>R</sup> CHO cells expressing the empty vector pcDNA3.1 were used as a negative control. Two independent populations of *Glp1r*<sup>CAST</sup> and *Glp1r*<sup>B6</sup> 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 and 500 nM) in the presence of 3-isobutyl-1-methylxanthine (IBMX; 1 µM) for 8 min. 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 × B6.CAST-17) F<sub>1</sub> mice (11, 28). A 247-bp amplicon spanning the previously identified SNP at position 1247 (26) was generated using the oligonucleotide primers: forward, 5′-GTC-CAGATGGAGTTTCGGAA-3′ and reverse, 5′-CCCTATCCCCAG-GATCTCTC-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 version 1.0 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 ( $\dot{V}\text{CO}_2$ ) was measured by the Oxymax indirect calorimeter (Columbus Instruments) with airflow of 0.75 l/min.

**Gastric emptying using the  $^{13}\text{C}$ -octanoate breath test.**  $^{13}\text{C}$ -octanoate (Cambridge Isotopes Laboratories), a non-radioactive stable isotope, is administered in a test meal (30). As the meal empties into the duodenum, the  $^{13}\text{C}$ -labeled octanoate is rapidly absorbed and transported via the portal system to the liver (25) where it is oxidized to  $\text{CO}_2$  and excreted in the breath. The rate of  $^{13}\text{CO}_2$  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;  $\dot{V}\text{CO}_2$  and oxygen uptake were measured at 15-min intervals. Breath tests were performed after 18 h of 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 preweighed, 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  $^{13}\text{CO}_2$  excretion) and at 10-min intervals thereafter. Breath samples were immediately injected into evacuated 10-ml Exetainer tubes (Labco, High Wycombe, UK) 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  $\dot{V}\text{CO}_2$  (42). The area under the curve (AUC) for the cumulative  $^{13}\text{CO}_2$  excretion from 0 to 6 h was determined by the trapezoid method and used to calculate the gastric half-emptying time ( $t_{1/2}$ ) (43). The rate of  $\text{CO}_2$  production was based on a mean of the  $\dot{V}\text{CO}_2$  values collected for each individual mouse in the experiment. The response variable (% dose administered per hour) was analyzed in two ways: 1) the AUC was calculated for each subject with respect to 10-min time intervals, and 2) the  $t_{1/2}$  AUC was established and then, using linear interpolation, the  $t_{1/2}$  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 and immediately excised at the pylorus and cardia, and its wet contents were removed, frozen, lyophilized overnight, and weighed. Gastric emptying (%) was calculated as  $[1 - (\text{dry weight of food recovered from the stomach}/\text{total food intake})] \times 100$  (51, 58).

**Effects of exendin-(9-39) on gastric emptying.** Exendin-(9-39) is a COOH-terminal fragment of exendin-4, a bioactive peptide that 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 intraperitoneally with 100  $\mu\text{l}$  of either PBS or exendin-(9-39) (Bachem) (0.25 mg/kg) immediately before re-feeding. Mice were fed preweighed 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  $F_2$  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-day 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 QTLs 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/20 g body wt) ( $40.1 \pm 1.0$  vs.  $24.5 \pm 3$ , 1.6-fold,  $P < 0.0001$ ) when compared with B6 mice over the 10-day phenotyping period (Fig. 3A). In addition, the B6.CAST-17 congenic mice consumed greater food volume per body weight compared with B6 homozygote controls B6 ( $27.8 \pm 0.9$  vs.  $21.7 \pm 0.7$  g, 1.3-fold;  $P < 0.0001$ ) (Fig. 3B), thus verifying the genetic linkage for increased total food volume on proximal Chr 17.

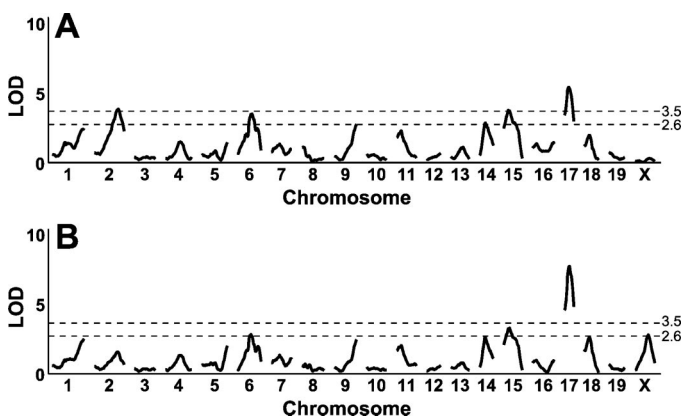


Fig. 1. Results of genome-wide quantitative trait locus (QTL) analysis for loci linked to total food volume (*Tfv1*) in an  $F_2$  population derived from the CAST and B6 inbred strains. Chromosome (Chr) numbers are represented on the x-axis. The y-axis represents the LOD score. The experiment-wide significant [ $\log$  of the odds ratio (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.

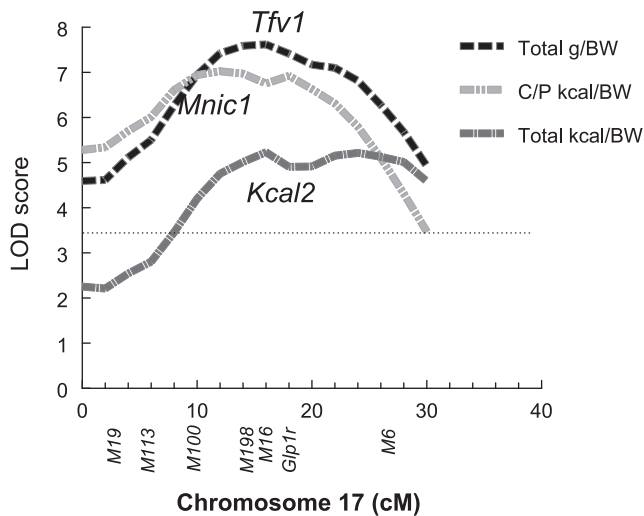


Fig. 2. Interval map for the *Tfv1* QTL located on Chr 17, including coincident QTL for macronutrient intake-carbohydrate (*Mnic1*) and total kilocalories (*Kcal2*). The abscissa represents the LOD, and the horizontal line indicates the LOD threshold for significant linkage (3.5). The ordinate contains symbols for MIT markers. BW, body weight; C/P, carbohydrate/protein.

**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 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 posttranslational processing of pro-glucagon (*Gcg*), ghrelin (*Ghrl*), cholecystokinin (*Cck*) and its two receptors (*Cckar*, *Cckbr*), gastrin (*Gast*), peptide YY (*Pyy*), and amylin (*Iapp*). Notably, expression of

*Glp1r* located near the QTL peak, was significantly decreased ( $P = 0.01$ ). qRT-PCR analysis of individual gastric RNA samples validated the significant decrease in *Glp1r* expression (by a factor of 2) in B6.CAST-17 mice.

**Functional analysis of a SNP in the coding region of *Glp1r*.** Previously, we reported a nonsynonymous single nucleotide polymorphism (SNP) that predicts an amino acid change (C416Y) in the COOH-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 *Glp1r*<sup>CAST</sup> and *Glp1r*<sup>B6</sup> transfected CHO cells, which stably express equivalent levels of the respective trans-genes, 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 test for *Glp1r*.** We next sought evidence for a regulatory polymorphism that could be responsible for modifying *Glp1r* expression between strains, in a tissue-specific manner. Quantification of allelic-specific *Glp1r* expression in F<sub>1</sub> 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, *cis*-acting regulatory element (controlling RNA transcription or stability) underlies the strain difference in gastric *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

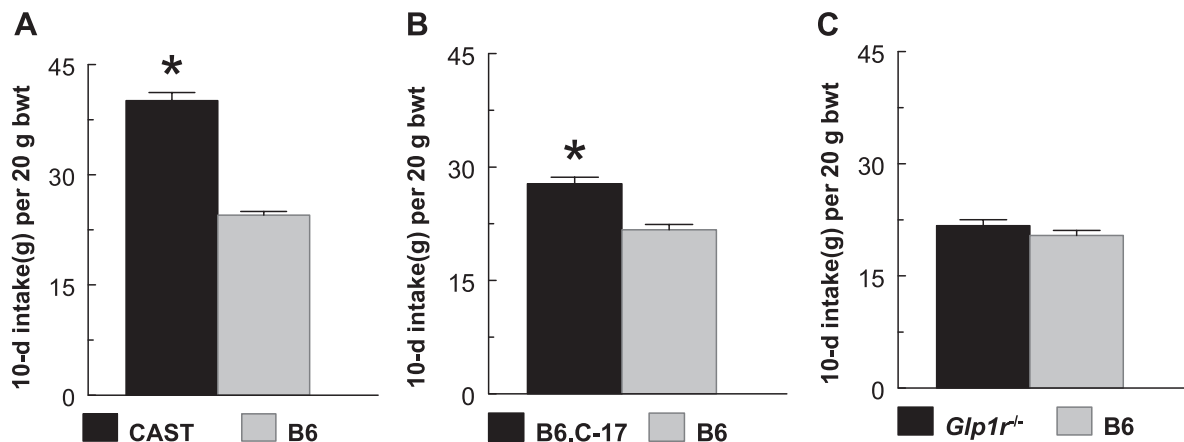


Fig. 3. Phenotypic strain differences in total food volume (g) per body weight consumed over 10 days from a choice between the fat/protein and carbohydrate/protein diets. A: inbred strain CAST ( $n = 38$ ) vs. B6 ( $n = 35$ ) were phenotyped as part of the QTL study, which included inbred strain controls in every F<sub>2</sub> 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. \* $P < 0.0001$ .

Table 1. Differentially expressed genes in stomach (positive value in fold difference indicates increased expression, and negative value indicates decreased expression, in congenic mice)

Probe ID	P Value	Fold Difference	UniGene	Gene Symbol	Gene Name
904816	0.00068	-5.34	Mm.22506	D17H6S56E-5	DNA segment, Chr 17, human D6S56E 5
761588	0.021	-5.30	Mm.267998	Taf11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor
611571	0.040	-4.15	Mm.3135	Igfals	Insulin-like growth factor binding protein, acid labile subunit
716213	0.030	-3.88	Mm.292869	Decr2	2-4-dienoyl-Coenzyme A reductase 2, peroxisomal
906572	0.043	-3.12	Mm.57043	Nfkbie	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
300048	0.022	-2.92	Mm.9277	Pla2g7	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
638437	0.033	-2.70	Mm.195061	H2-T22	Histocompatibility 2, T region locus 22
370857	0.001	-2.65	Mm.30145	Enpp5	Ectonucleotide pyrophosphatase/phosphodiesterase 5
431279	0.005	-2.52	Mm.235338	H2-Aa	Histocompatibility 2, class II antigen A, alpha
762178	0.021	-2.43	Mm.215162	Prdm9	VPR domain containing 9
465717	0.003	-2.35	Mm.285322	Mtch1	Mitochondrial carrier homolog 1 ( <i>C. elegans</i> )
653170	0.012	-2.30	Mm.32002	Vars2	Valyl-tRNA synthetase 2
669667	0.023	-2.05	Mm.276405	Fkbp5	FK506 binding protein 5
706669	0.002	-1.95	Mm.28349	1500032D16Rik	RIKEN cDNA 1500032D16 gene
537031	0.040	-1.73	Mm.28895	1110038B12Rik	RIKEN cDNA 1110038B12 gene
558496	0.004	-1.62	Mm.439648	H2-T23	Histocompatibility 2, T region locus 23
919204	0.028	-1.57	Mm.30496	Ergic1	Endoplasmic reticulum-golgi intermediate compartment (ERGIC) 1
591309	0.020	-1.50	Mm.140772	Glp1r	Glucagon-like peptide 1 receptor
346004	0.019	1.55	Mm.291772	Npw	Neuropeptide W
673097	0.005	1.65	Mm.15252	Srpkl	Serine/arginine-rich protein specific kinase 1
915442	0.031	1.65	Mm.261984	Glo1	Glyoxalase 1
915872	0.014	2.17	Mm.26688	Thbs2	Thrombospondin 2
920440	0.029	2.19	Mm.328931	Pim1	Proviral integration site 1
869338	0.036	2.24	Mm.79993	Dact2	Dapper homolog 2, antagonist of beta-catenin ( <i>xenopus</i> )
467910	0.0009	2.26	Mm.440426	Btn1l	Butyrophilin-like 1
849986	0.017	2.64	Mm.214638	Tmprss3	Transmembrane protease, serine 3
480967	0.003	3.09	Mm.2443	Bak1	BCL2-antagonist/killer 1
682540	0.017	3.40	Mm.24192	Msh5	MutS homolog 5 ( <i>E. coli</i> )
837168	0.025	6.63	Mm.445985	BC026600	cDNA sequence BC026600
898097	0.036	6.66	Mm.275521	Wtap	Wilms' tumor 1-associating protein
560648	0.004	51.35	Mm.327358	H2-Ob	Histocompatibility 2, O region beta locus

Genes were selected based on a significant ( $P < 0.05$ ) and differential fold change in expression of  $\geq 1.5$ . 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. Mm, mus musculus. \*The curated gene information is not available from Applied Biosystems at this time.

[ $^{13}\text{C}$ ]octanoate breath test, gastric emptying rate was significantly faster in CAST (by 20%) compared with B6 [ $t_{1/2}$  values  $\pm$  SE of the mean:  $113 \pm 4$  vs.  $140 \pm 4$  min, respectively;  $F(1,33.9) = 21.83$ ,  $P < 0.0001$ ] (Fig. 5). Figure 5 illustrates the strain difference in the  $^{13}\text{C}$  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

Table 2. cAMP production after stimulation with GLP-1(7-36)amide

	Concentrations of GLP-1(7-36)amide		
	0 nm	100 nm	500 nm
pC-DNA3.1	$5.14 \pm 0.64$	$3.10 \pm 0.40$	$4.48 \pm 0.98$
B6 GLP-1R	$7.90 \pm 2.25$	$35.19 \pm 7.50$	$102.73 \pm 7.09$
CAST GLP-1R	$8.52 \pm 1.62$	$45.50 \pm 8.07$	$93.94 \pm 5.39$

Data are means  $\pm$  SE. CHO (Chinese hamster ovary) cells expressing B6 glucagon-like peptide 1 receptor (GLP1R) and CAST GLP1R were stimulated with two concentrations of GLP-1(7-36) amide for 8 min, and the production of cAMP (fmol/ $2.3 \times 10^5$  cells) was determined by enzyme immunoassay, performed in triplicate.

similar between the two strains, indicating that hepatic oxidation of  $^{13}\text{C}$  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 but also had 32% lower stomach mass. There was no difference in test meal size when expressed per stomach mass (CAST  $4.0 \pm 0.2$  g/g vs. B6  $3.7 \pm 0.2$  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

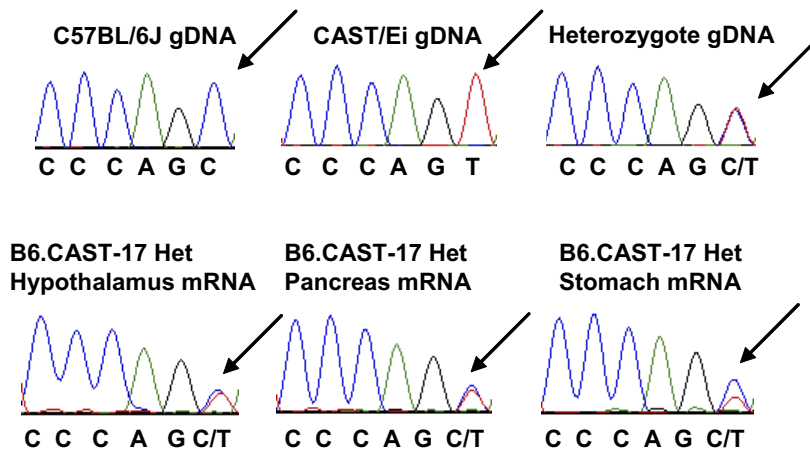


Fig. 4. Detection of allelic-specific gene expression using a sequencing-based assay; differences in the relative peak heights (in relative fluorescent units) were used to detect allelic imbalance. Sequence traces were obtained by amplifying the cDNA from F<sub>1</sub> mice (B6 × B6.CAST-17 congenic) and genomic DNA (gDNA) from C57BL/6J and CAST/Ei for a region encompassing the single nucleotide polymorphism (SNP) at 1,247 bp of mRNA. Sequence traces from gDNA (top) vs. cDNA (bottom) 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) and 69% C (blue), 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.

gastric emptying was significantly higher in B6.CAST-17 congenic mice compared with homozygous B6 controls (95 ± 1% vs. 87 ± 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.

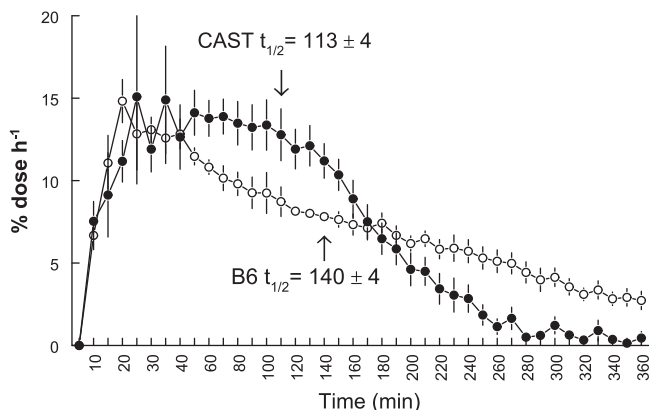


Fig. 5. Gastric emptying in CAST (*n* = 28) (●) and B6 (*n* = 30) (○) mouse inbred strains as measured by the [<sup>13</sup>C]octanoate breath test. Time 0 designates the end of the test meal. The amount of <sup>13</sup>CO<sub>2</sub> recovery in the breath samples, determined by isotope ratio mass spectrometry, is expressed as percentage of dose/hour curve after ingestion of a semi-solid test meal. The *t*<sub>50</sub> after the test meal for each of the two strains is represented by an arrow.

**Phenotypic analyses in *Glp1r*<sup>-/-</sup> mice.** We examined whether the presence or absence of the GLP-1R in vivo would alter 1) the composition of self-selected macronutrient diet intake or 2) the rate of gastric emptying. *Glp1r*<sup>-/-</sup> mice consumed twofold more carbohydrate and less fat (by energy) compared with inbred B6 mice (51 ± 2%, *n* = 17, vs. 30 ± 3%, *n* = 14, *P* < 0.0001) (Fig. 8). There was no strain difference in total food volume over 10 days (*Glp1r*<sup>-/-</sup> 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*<sup>-/-</sup> mice showed a slower rate of gastric emptying compared with B6 (85 ± 3%, *n* = 17, vs. 93 ± 1%, *n* = 14, for *Glp1r*<sup>-/-</sup> 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*, encoding an important mod-

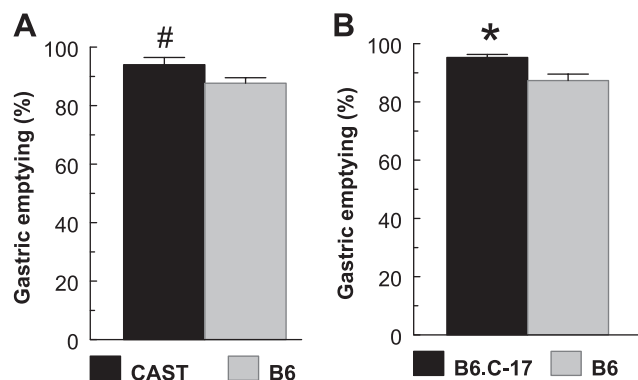


Fig. 6. Percent gastric emptying in CAST (*n* = 11) and B6 (*n* = 12) mouse inbred strains (A) and B6.CAST-17 (B6.C-17) homozygous congenic (*n* = 6) and homozygous B6 (*n* = 7) mice (B), as measured by the amount of lyophilized gastric contents that remained 2 h after re-feeding. \**P* < 0.05. #*P*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

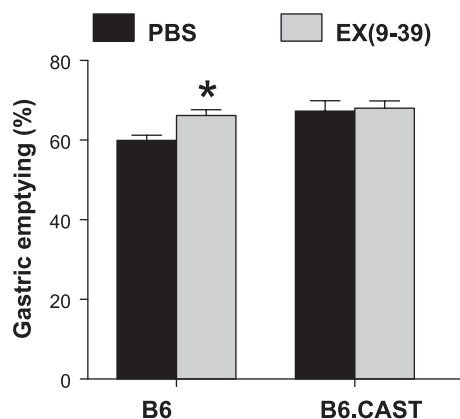


Fig. 7. Effects of strain and the GLP-1R antagonist exendin-(9-39) on gastric emptying in B6.CAST-17 (B6.CAST) homozygous congenic and homozygous B6 mice. \* $P < 0.05$  vs. PBS.  $n = 6$  mice per group.

ulator 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 noncoding 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 that 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 are 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 is consistent with 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

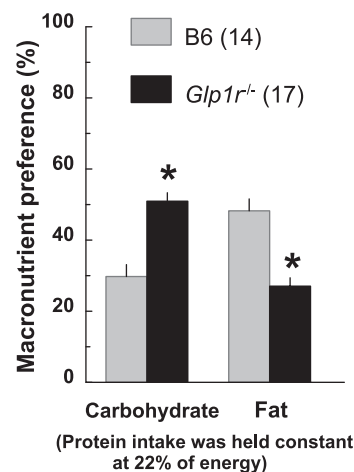


Fig. 8. Percent (%) of total kcal from fat and carbohydrate in C57BL/6J ( $n = 14$ ) and *Glp1r*<sup>-/-</sup> ( $n = 17$ ) mice selecting from a choice between carbohydrate/protein and fat/protein diets for 10 days. \* $P < 0.0001$ .

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 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. wild-type 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 with 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*<sup>-/-</sup> 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*<sup>-/-</sup> and inbred C57BL/6J mice. These results are consistent with a previous investigation of *Glp1r*<sup>-/-</sup> 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*<sup>-/-</sup> mice was somewhat surprising. Due to impaired glucose-stimulated insulin secretion, *Glp1r*<sup>-/-</sup> mice exhibit mild fasting hyperglycemia and glucose intolerance after oral or intraperitoneal loading (44). Thus we hypothesized that *Glp1r*<sup>-/-</sup> 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*<sup>-/-</sup> 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,CD1,129-*Glp1r*<sup>tm1Ddr</sup> mice studied here were generated through only six backcross generations and may still contain regions of contaminating CD1 DNA throughout the genome. Therefore, although the *Glp1r*<sup>-/-</sup> 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 Chr 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. Physiological 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.

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