Human Galanin (GAL) and Galanin 1 Receptor (GALR1) Variations Are Not Involved in Fat Intake and Early Onset Obesity[1,2]

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ABSTRACT The neuropeptide galanin (GAL) is involved in food intake and in fat ingestion. Presumably, these effects are conveyed via the galanin 1 receptor (GALR1). We screened the coding region of *GAL* (including 444 bp of its promoter region) and *GALR1* for mutations using single-strand conformation polymorphism analysis and denaturing HPLC in up to 191 obese children and adolescents and 106 healthy underweight young adults (students). In *GAL*, we identified 3 novel single nucleotide polymorphisms (SNPs; silent: g.−419T→C, g.−244G→A; missense: g.47C→T: Ala16Val) and one infrequent missense variation (c.253A→G: Asn85Asp), and in *GALR1* 2 novel SNPs (silent: c.150C→T, missense: c.793A→T: Ile265Phe). To test for an association with obesity, we genotyped 7 SNPs (*GAL*: g.−244G→A, g.47C→T, rs7101947, rs1042577, rs3136540; *GALR1:* c.150C→T, c.793A→T) in up to 322 obese children and adolescents compared with up to 277 healthy underweight and normal weight young adults. Furthermore, we analyzed these SNPs with respect to potential effects on the percentage of energy consumed as fat in obese children and adolescents. Allele and genotype frequencies did not differ among the groups tested. In addition, we performed a pedigree transmission disequilibrium test (PDT) for one SNP (*GAL:* g.−244G→A) in 610 (518 independent) obesity-trios (obese child or adolescent and both of its parents). However, the PDT for SNP *GAL* g.−244G→A revealed no transmission disequilibrium. We conclude that the analyzed SNPs in *GAL* and *GALR1* do not play a major role in early onset obesity or dietary fat intake in the obese children and adolescents of our study groups. J. Nutr. 135: 1387–1392, 2005.

KEY WORDS: • *percentage fat intake* • *body weight* • *adolescents* • *association*

There is a large body of evidence indicating that galanin (GAL)[4] has a role in appetite and body weight regulation. Examples for involvement in centrally regulated mechanisms include the following: *1*) GAL causes an increase in dietary fat intake (1); *2*) there is an interplay between GAL and leptin in the hypothalamic control of feeding (2); and *3*) GAL neurons project from the anterior parvocellular region of the paraventricular nucleus to the median eminence (3). The paraventricular nucleus, which is important in the control of food intake, is positively related to the natural preference for high-fat diets in rats. Thus it is conceivable that hypothalamic GAL signaling can mediate the development of obesity induced by overconsumption of fat. Peripherally, GAL reduces energy expenditure and sympathetic activation of brown adipose tissue (4), modulates intestinal motility (5), and affects food consumption (6).

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[4] Abbreviations used: ARMS, amplification refractory mutation system; dHPLC, denaturing HPLC; GAL, galanin; GALR 1, 2 or 3, galanin receptors 1, 2 or 3; HF, high-fat; IBD, identity by descent; LD, linkage disequilibrium; LF, low-fat; PDT, pedigree transmission disequilibrium test; RFLP, restriction fragment length polymorphism analyses; SNP, single nucleotide polymorphism; SSCP, single-strand conformation polymorphism.

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Transgenic mice overexpressing GAL are obese and have increased abdominal fat pads and cholesterol levels compared with wild-type mice (7), or have feeding patterns and body weights indistinguishable from wild-type controls (8). However, *Npy*$^{-/-}$ and *Gal*$^{-/-}$ mice (double knockout) develop obesity and endocrine dysfunction, whereas both phenotypes are not present in single knockouts (9).

The mature human galanin peptide consists of 30 amino acids and is proteolytically processed from a precursor (prepro-galanin) (10). The effect of GAL is mediated through G-protein–coupled receptors (*GALR1*, *GALR2*, *GALR3*) (11); however, the specific role of each receptor subtype in mediating the physiologic or pathologic effects of GAL remains to be elucidated (7). Nonetheless, the widespread expression of *GALR1* in brain, spinal cord, gut, and pancreas suggests that GALR1 might be broadly implicated in neurotransmitter release with potential effects on feeding, emotion, memory, nociception, glucose homeostasis, and gut secretion/motility (12). In *Galr1*$^{-/-}$ mice, no effect on body weight was found; however, a role of Galr1 in feeding behavior and body weight regulation has not been excluded (13).

In humans, intake of high-fat diets can result in obesity (14,15). Heritability of fat mass, body weight (16), as well as food preferences (17) was demonstrated in twin studies, e.g., a higher correlation for the percentage of energy consumed as fat was observed in monozygotic compared with dizygotic twins (18). It was also suggested that high-fat (HF) and low-fat (LF) phenotypes can be used as a conceptual tool to investigate the relation between genetic and environmental (diet) factors in the control of body weight (19).

A genome-wide linkage scan (genome scan) is used to identify chromosomal regions that are linked to the phenotype under study. Combined with functional or fine mapping evidence, the information is integrated to determine candidate genes. We recently performed a genome scan for obesity, based on 89 families comprising ≥2 obese siblings and both of their biological parents ("obesity families"; $n = 369$; criteria: one offspring BMI ≥ 95th and at least one sibling BMI ≥ 90th percentile), and a confirmatory sample of 76 "obesity families" with ≥2 obese siblings ($n = 315$; same criteria as above). In both samples, we observed a peak signal for the chromosomal region 11q11 in the vicinity of the *GAL* locus (20), which was mapped to human chromosome 11q13.3–13.5 (10).

The rationale of this study was to assess the following: *1*) whether variations in *GAL* and *GALR1* are involved in body weight regulation and the percentage of energy consumed as fat; and *2*) whether *GAL* is the positional candidate gene underlying the observed peak on chromosome 11q11.

SUBJECTS AND METHODS

Study groups. The ascertainment strategy for our study groups was described previously (21). In total, 4 study groups were used for analysis (**Table 1**). All obese subjects had an age- and gender-specific BMI ≥ 90th percentile, all healthy underweight young adults (students) had a BMI < 15th percentile, and all normal weight young adults (students) had a BMI between the 40th and 60th percentiles. Written informed consent was given by all participants and in case of minors, their parents. The study was approved by the Ethics Committee of the University of Marburg. Detailed methods and material including primer sequences are provided as online supplemental material.

Mutation screen. For *GAL*, 6 PCR fragments encompassing all exons and the predicted promoter, and for *GALR1*, 3 amplicons encompassing exon 1, 2, and 3 were used for PCR amplification. The PCR fragments encompassing *GAL* promoter, exon 1 and 2, as well as the *GALR1* amplicons for exon 1 and 3, were digested before single-strand conformation polymorphism (SSCP) analysis. SSCP was performed as described previously (22). SSCP gels (15% acrylamide gels 37.5:1, Q-BIOgene) for *GAL* promoter, exon 1 and 2 amplicons were run at both room temperature and 4°C; all *GALR1* amplicons were run at 4°C. All gels were silver-stained. PCR amplicons of *GAL*, and *GALR1* with aberrant SSCP patterns were resequenced. Mutation screening for the remaining *GAL* exons (4,5,6) was performed with denaturing HPLC (dHPLC; Transgenomic WAVE® system). The temperatures for optimal separation of homo- and heteroduplexes were deduced from the WAVEMAKER software (Transgenomic) as 62, 63, and 64°C for exon 4, 57°C for exon 5, and 63 and 65°C for exon 6. All chromatograms were compared with chromatograms of resequenced wild-type samples. The mutation screen for *GAL* was carried out in a subgroup of study group 1 (142 obese children and adolescents, 94 underweight controls) and for *GALR1* in study group 4, which partly overlapped study group 1 (Table 1).

Genotyping. Five *GAL* single nucleotide polymorphism (SNPs; g.−244G→A, g.47C→T, rs7101947, rs3136540, rs1042577) were genotyped by PCR-based restriction fragment length polymorphism

TABLE 1

Anthropometric data of obese children and adolescents (cases), underweight and normal weight young adults (controls) of 4 study groups investigated for GAL (1–3) and GALR1 (3 and 4)[1]

Study group[2]	*n*	Sex female/male	BMI	Age	Mean fat intake[3]
		n/n	*kg/m²*	*y*	*%*
1) Obese children and adolescents	322	170/152	32.7 ± 6.5	14.0 ± 2.5	ND[4]
Normal weight controls	95	49/46	21.9 ± 1.1	24.7 ± 2.6	ND
Healthy underweight controls	182	79/103	18.4 ± 1.1	25.6 ± 3.9	ND
2) Obese children and adolescents	518	286/232	32.0 ± 6.0	13.6 ± 2.9	ND
Obese siblings	92	47/45	28.1 ± 4.9	15.2 ± 4.6	ND
Parents	1036	518/518	30.1 ± 6.1	42.7 ± 6.0	ND
3) Obese children and adolescents, LF consumers	45	29/16	31.1 ± 5.4	13.6 ± 2.2	32.1 ± 4.2
Obese children and adolescents, HF consumers	50	22/28	31.7 ± 5.9	13.4 ± 2.0	45.2 ± 2.1
4) Obese children and adolescents	191	96/95	35.1 ± 6.2	14.4 ± 3.3	ND
Healthy underweight controls	106	40/66	18.4 ± 1.2	25.5 ± 3.8	ND

[1] Values are means ± SD or number.
[2] Study group 3 is part of study group 1; study group 4 partially overlaps study group 1.
[3] Measured by Leeds FFQ.
[4] ND, not determined.

analyses (PCR-RFLP). For *GALR1*, 2 SNPs (c.150C→T, c.793A→T) were genotyped by tetra-amplification refractory mutation system (ARMS)-PCR or ARMS-PCR. For validity of the genotypes, alleles were determined independently by at least 2 experienced individuals. Discrepancies were resolved unambiguously either by reaching consensus or by retyping.

Genotype analysis was performed in study group 4 for *GALR1* and in study group 1 for *GAL*. Of study group 1 (*GAL*), 49 (18 men) index patients of the obese individuals (mean age 13.7 ± 2.9 y, mean BMI 32.0 ± 5.6 kg/m^2) were included in our original linkage study (20). These individuals had the highest "Identity By Descent" (IBD) sharing at the linkage region on chromosome 11q11 (marker D11S1313, Maximum Likelihood Binomial LOD Score = 1.65) (20). Because the linkage signal emerged largely from these individuals, we hypothesized that they would be enriched with relevant mutations in a candidate gene for obesity at the chromosome 11q11 locus.

SNP g.−244G→A was resequenced for pedigree transmission disequilibrium test (PDT) analysis in 610 families (518 independent), presented as study group 2 (independent of study group 1). For resequencing, a genomic fragment of 2139 bp (encompassing g.−244G→A) was generated and used as template for a 2nd PCR. Sequencing reactions were electrophoresed on ABI 3700 automated sequencers. Trace files were inspected visually in gap.4 (23). Resequencing by pyrosequencing was performed with the PSQ 96 SNP Reagent Kit (Pyrosequencing) according to the manufacturer on a PSQ96MA machine.

In study group 3, a subgroup of the obese individuals in study group 1, we genotyped both *GAL* and *GALR1* SNPs in obese adolescents within LF (i.e., consuming 21–36% of total energy as fat) or HF (i.e., consuming 43–50% of total energy as fat) quartiles for the percentage of energy.

FFQ. The percentage of energy consumed as fat was assessed using the "Leeds Food Frequency Questionnaire" [Leeds FFQ, Leeds Food and Nutrition Survey (24)], which was adapted for German nutritional habits. Consistency of the adapted FFQ was evaluated using a short FFQ validated in Germany (25). Evidence for a substantial construct overlap of the questionnaires was indicated by Pearson's correlation coefficient for the scale "fat intake" ($r = 0.693$, $P < 0.001$, 2-sided). Adolescents were asked to indicate intake frequency for every food item and to record whether the FFQ was filled in by themselves, with the help of their parents, or entirely by the parents. Eleven of 169 returned FFQs were disregarded because more than 5 food items were missing.

Statistics. Between-group differences in genotype frequencies were investigated by 2-sided asymptotic or, in the case of reduced cell counts, exact Cochran-Armitage trend tests. Two-sided asymptotic Pearson's χ^2 or Fisher's Exact Test was used to analyze between-group differences in allele frequencies. Note that the number of patients in different quartiles for the percentage of energy consumed as fat varied because quartile cutoff values were set to percentage values that were available in integers only.

The transmission disequilibrium test uses family trios composed of affected persons and both of their parents, comparing the total number of transmissions and nontransmissions of a specific allele over all heterozygous parents (26). If the allele in question is neither linked nor associated with disease risk (here, obesity risk), the expected ratio of allele transmissions compared with nontransmissions is 1. In addition, the PDT used in this study (for SNP g.−244G→A in *GAL*) allows correction for dependencies due to multiple children within nuclear families (27).

Because homozygous 793-T carriers were not observed for the missense SNP (c.793A→T) in *GALR1* in the 2 groups with different fat intake, Fisher's Exact Test was performed to investigate differences in allele and genotype frequencies. Linkage disequilibrium between these 2 *GALR1* SNPs was investigated with the program EH, version 1.11 (28).

RESULTS

Galanin. We screened the coding region of *GAL* plus the putative promoter region of 444 bp (position 100228–100671 of GenBank entry AP003096) for mutations. By the resequencing of PCR products showing aberrant SSCP or dHPLC patterns, we identified 4 novel variations: *1*) g.−419T→C in the putative promoter, 2) g.−244G→A in the untranslated first exon, located in a GC-box [CCC**G**CC] (29), *3*) g.47C→T in exon 2 leading to a conservative amino acid exchange (Ala16Val) and *4*) c.253A→G in exon 5 leading to a nonconservative amino acid exchange (Asn85Asp). Three known SNPs were confirmed: *1*) rs7101947 (exon 1, 5′UTR), *2*) rs1042577 (intron 5), and *3*) rs3136540 (exon 6, 3′UTR). By resequencing 518 independent obesity trios (study group 2), 4 infrequent sequence variations were identified: g.−460C→A, g.−363C→T, g.−344C→T, g.−22C→T, which were not analyzed further.

Two novel (silent: g.−244G→A; missense: g.47C→T: Ala16Val) and 3 known SNPs (rs7101947, rs1042577, rs3136540) of *GAL* were genotyped by PCR-RFLP in up to 322 obese children and adolescents and up to 182 healthy underweight and 95 normal weight controls (study group 1). Genotype and allele frequencies of obese children and adolescents and controls did not differ for any of these SNPs (all $P > 0.05$; **Table 2**). Furthermore, genotype and allele frequencies did not differ for the analyzed SNPs in obese patients of the low and high quartile of the percentage of energy consumed as fat (study group 3; Table 2). Nonconservative missense variation Asn85Asp (c.253A→G) was infrequent and therefore excluded from further analyses.

For SNP g.−244G→A, which is in complete linkage disequilibrium (LD, $D' = 1$) with Ala16Val (g.47C→T), a PDT in 610 trios (518 independent; study group 2) was performed because we observed a slightly increased frequency of the g.−244 A-allele in the obese sample (3.4%) compared with normal weight and underweight individuals (1.1%, nominal $P = 0.10$, Table 2). This trend was not substantiated in the family-based association study (PDT); the A-allele was not preferentially transmitted to obese index patients (nominal $P = 0.7$).

In those individuals who substantially contributed to the peak on chromosome 11q11 (study group 1) in our genome scan (20), we did not detect increased frequencies of any of the analyzed alleles, nor was there an increased rate of homozygotes (data not shown). Hence, we found no evidence that *GAL* might be the positional candidate gene underlying the peak observed in the genome scan.

Galanin receptor 1. Two novel SNPs, which were in linkage disequilibrium (nominal $P < 0.05$), were identified by SSCP in the coding region of *GALR1*: *1*) silent c.150C→T; Gly50 in exon 1 and *2*) missense g.793A→T; Ile265Phe in exon 3. The genotype and allele frequencies of the 2 SNPs did not differ in obese children and adolescents and underweight controls (study group 4; **Table 3**). Missense SNP Ile265Phe (g.793A→T) was found in 3 of 45 individuals (6.67%) of the low quartile and 8 of 50 individuals (16.0%) of the high quartile for the percentage of energy consumed as fat (study group 3; Table 3); however, association tests (Fisher's Exact Test) were not significant ($P > 0.05$).

DISCUSSION

Galanin. A mutation screen of the entire coding and putative promoter region of *GAL* revealed 3 novel SNPs and 5 novel infrequent variations. To our knowledge, this study represents the first systematic mutation screen of *GAL*. We initially investigated the association of 2 novel (g.−244G→A, g.47C→T: Ala16Val) and 3 known SNPs (rs7101947,

TABLE 2

Genotype frequencies of 5 GAL SNPs in obese children and adolescents (cases) compared with underweight and normal weight young adults (controls), and in obese children and adolescents of different quartiles for percentage of energy consumed as fat (LF vs. HF consumers)[1]

Genotype	Cases[2]		Controls[2]		LF consumers[3]		HF consumers[3]	
	n	%	*n*	%	*n*	%	*n*	%
rs7101947								
TT	140	43.5	138	50.0	21	46.7	22	45.8
TC	154	47.8	108	39.1	21	46.7	20	41.7
CC	28	8.7	30	10.9	3	6.7	6	12.5
g.-244G→A								
GG	311	96.6	274	98.9	43	95.6	48	100.0
GA	11	3.4	3	1.1	2	4.4	0	0
AA	0	0	0	0	0	0	0	0
g.47C→T: Ala16Val								
CC	311	96.6	274	98.9	43	95.6	48	100.0
CT	11	3.4	3	1.1	2	4.4	0	0
TT	0	0	0	0	0	0	0	0
rs3136540								
CC	159	49.4	146	52.7	23	51.1	23	47.9
CT	143	44.4	111	40.1	21	46.7	22	45.8
TT	20	6.2	20	7.2	1	2.2	3	6.3
rs1042577								
CC	130	40.4	122	44.7	18	40.0	19	39.6
CT	152	47.2	118	43.2	22	48.9	25	52.1
TT	40	12.4	33	12.1	5	11.1	4	8.3

[1] All genotypes are in Hardy-Weinberg equilibrium; $P > 0.05$ for all SNPs of *GAL* for the comparisons: cases vs. controls and LF consumers vs. HF consumers.
[2] Study group 1 from Table 1.
[3] Study group 3 from Table 1.

rs1042577, rs3136540) of *GAL* with obesity. Subsequently, we analyzed these SNPs for association with the percentage of energy consumed as fat in obese individuals because certain patterns of eating behavior, such as fat preference, contribute to the development of obesity (14,15). None of the tests revealed a positive finding. Additionally, there is no evidence that *GAL* might be the positional candidate gene explaining the peak on chromosome 11q11 observed in our genome scan (20).

Furthermore, a PDT for g.−244G→A in 610 obesity trios revealed no transmission disequilibrium. We chose this SNP for PDT analysis for the following reasons: *1*) it might have a functional effect itself; *2*) it is in complete LD with Ala16Val (g.47C→T), which might also be functionally relevant; and *3*) we observed a slightly increased frequency of the g.−244 A-allele in the obese sample compared with controls. In detail, *1*) g.−244G→A is located in a GC-Box in the untranslated first exon where the g.−244 A-allele might result in the loss of

TABLE 3

Genotype frequencies of 2 GALR1 SNPs in obese children and adolescents (cases) compared with underweight and normal weight young adults (controls), and in obese children and adolescents of different quartiles for the percentage of energy consumed as fat (LF vs. HF consumers)[1]

Genotype	Cases[2]		Controls[2]		LF consumers[3]		HF consumers[3]	
	n	%	*n*	%	*n*	%	*n*	%
c.150C→T								
CC	64	35.0	40	38.5	19	44.2	19	38.0
CT	86	47.0	45	53.3	21	48.8	23	46.0
TT	33	18.0	19	18.3	3	7.0	8	16.0
c.793A→T: Ile265Phe								
AA	167	89.3	97	91.5	42	93.3	42	84.0
AT	20	10.7	8	7.6	3	6.7	8	16.0
TT	0	0	1	0.9	0	0	0	0

[1] All genotypes are in Hardy-Weinberg equilibrium; $P > 0.05$ for all SNPs of *GALR1* for the comparisons: cases vs. controls and LF consumers vs. HF consumers.
[2] Study group 4 from Table 1.
[3] Study group 3 from Table 1.

a SP1 binding site (29). However, the regulatory relevance of this variation must be analyzed in functional studies. *2*) The conservative missense variation Ala16Val (g.47C→T) is located in the hydrophobic signal peptide of preprogalanin. However, at amino acid position 16, there is Ala in rats and cows, Val in mice and Ser in pigs (10). Hence, this position is not conserved, deeming an effect of the missense variation rather unlikely.

Because we did not find an association (in either the case-control or family-based approach) with obesity, it seems unlikely that novel SNPs g.−244G→A and Ala16Val (g.47C→T) are relevant for body weight regulation in the obese children and adolescents of our study groups.

Galanin 1 receptor. In the coding region of *GALR1* 2 novel SNPs were identified. Whereas g.150C→T in exon 1 is silent, c.793A→T in exon 3 causes the conservative missense variation Ile265Phe. Although it is presently unknown whether one of these SNPs might alter GALR1 function, we initially analyzed the SNPs for association in study groups of different body weight. The comparison of obese children and adolescents with underweight controls revealed no association for the 2 SNPs. Subsequently, we tested for association in obese patients whose reported fat intake was in the lowest and highest quartile for the percentage of energy consumed as fat. We found a slightly higher, although not significant occurrence ($P = 0.2$ for genotypes and $P = 0.22$ for alleles) of the less frequent allele of SNP Ile265Phe (c.793A→T) in those obese patients in the highest quartile for the percentage of energy consumed as fat. Assuming that the allele frequencies of this polymorphism are in fact in the reported magnitude in our overweight groups for the highest and lowest quartile of the percentage of energy consumed as fat, a 2-sided test at a significance level of 5% with the present sample size has a power of 28%. To investigate this further, a total of 150 individuals per group would be necessary to perform a one-sided test at a significance level of 5% with 80% power.

Sensitivity of the FFQ. Only very few studies have included dietary fat intake as a phenotype in a candidate gene study (30,31). Recently, a FFQ was used to measure the ratio of dietary polyunsaturated to saturated fatty acids (P:S ratio); an association of the P:S ratio with a PPARγ polymorphism was described (31). Hence, the FFQ might be considered a sensitive tool with which to measure dietary macronutrient composition. We have to emphasize that the definition of the upper and lower quartile for energy intake from fat is based solely on the results of the Leeds FFQ. In our study, the Leeds FFQs were completed by obese adolescents, partially with the help of or entirely by their parents. One has to keep in mind that estimating absolute values (kJ) for habitual fat consumption is difficult. Nevertheless, the Leeds FFQ can be reliably used to classify individuals on the basis of energy or nutrient intakes within a sample. We found a significant difference in BMI (2.4 kg/m^2, $P < 0.001$) between the lowest and highest quartiles of the percentage of energy intake from fat. A positive relation between relative fat intake and body weight has been described in various studies (32,33), thus indirectly confirming our measurement of the percentage of energy consumed as fat.

In a recent study, we identified negative association of an infrequent variation in the *MC4R* gene with obesity (34) proving that a SNP-based approach for analyzing the association with complex phenotypes was successful. At the *GAL* and *GALR1* loci, we identified a total of 10 novel sequence variations. However, we did not obtain evidence for an involvement of *GAL* or *GALR1* in body weight regulation or the percentage of energy consumed as fat in our study groups. Also, we did not find evidence that *GAL* is the positional candidate gene underlying the peak on chromosome 11q11. Therefore, we conclude that the analyzed SNPs in *GAL* and *GALR1* do not play a major role in early onset obesity or dietary fat intake.

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