

Sequence variants in the melatonin-related receptor gene (*GPR50*) associate with circulating triglyceride and HDL levels

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Abstract The gene encoding the melatonin-related receptor (*GPR50*) is highly expressed within hypothalamic nuclei concerned with the control of body weight and metabolism. We screened *GPR50* for mutations in an obese cohort and identified an insertion of four amino acid residues (TTGH) at position 501, two common coding polymorphisms (T528A and V602I), and one noncoding polymorphism (C-16X2GPR50T). Single-nucleotide polymorphisms were then typed in 500 English Caucasian subjects, and associations were sought to intermediate obesity phenotypes. Although no association was seen with body mass index, carriers of two copies of the mutant allele at C-16X2GPR50T, Ins501Del, and A1582G had significantly higher fasting circulating triglyceride levels ($P < 0.05$). In a separate set of 585 subjects, the associations were replicated, with statistically significant effects of similar magnitude and direction. The association of C-16X2GPR50T with fasting triglycerides was highly significant ($P < 0.001$). In addition, a significant association between C-16X2GPR50T and circulating HDL levels was observed in the combined population, with C-16X2GPR50T carriers having significantly lower circulating HDL-cholesterol levels (1.39 mM) than wild-type subjects (1.47 mM) ($P < 0.01$). These findings suggest a previously unexpected role for this orphan receptor in the regulation of lipid metabolism that warrants further investigation.—Bhattacharyya, S., J. Luan, B. Challis, J. Keogh, C. Montague, J. Brennand, J. Morten, S. Lowenbeim, S. Jenkins, I. S. Farooqi, N. J. Wareham, and S. O'Rahilly. **Sequence variants in the melatonin-related receptor gene (*GPR50*) associate with circulating triglyceride and HDL levels.** *J. Lipid Res.* 2006. 47: 761–766.

Supplementary key words bipolar • metabolic • neuronal

Mutations in at least six different genes expressed in, or acting on, the hypothalamus have been shown to result in severe early-onset human obesity (1). Indeed, there is as

yet no compelling instance of a monogenic form of obesity that does not involve a disturbance of hypothalamic function. In our own large cohort of patients with severe early-onset obesity, <10% of subjects have a mutation in any of these known genes. It is plausible, therefore, that dysfunction of other signaling molecules expressed in the hypothalamus might underlie some as yet uncharacterized cases of severe human obesity. The orphan G protein-coupled receptor *GPR50* (also known as the melatonin-related receptor) is expressed within the dorsal medial hypothalamus, lateral hypothalamus, and arcuate nucleus within the rodent brain (2). The human gene is located on the X chromosome. Other than the site of its expression and its structural similarity to the melatonin receptor (3), little is known of its biology (4). In this study, we have examined the coding region and immediate 5' and 3' flanking sequences of *GPR50* for mutations in a cohort of subjects with severe early-onset obesity (mean age of onset of obesity, 5 years; mean body mass index standard deviation score, 4.2). Having detected common single-nucleotide polymorphisms (SNPs) within this gene and determined their allele frequency in 100 alleles from control subjects, we examined their association with quantitative metabolic traits in a population-based study. Having found a surprising association of certain polymorphisms with plasma triglyceride levels, we undertook a replication study, which confirmed our original observations.

RESEARCH DESIGN AND METHODS

Study populations

All studies were approved by the Anglia and Oxford Multi-regional Ethics Committee, and subjects were recruited after appropriate informed consent.

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Ninety-five unrelated individuals were randomly selected from a cohort of patients with severe early-onset obesity recruited as part of the Genetics of Obesity Study. In all probands within this cohort, obesity arose before the age of 10 years and their body mass index (BMI) was >4 standard deviations above the population mean for their sex and age. Individuals in whom evidence for a recognized clinical syndrome, a known monogenic syndromic basis, or a structural cause for their obesity was known were excluded (1). Patients were screened for mutations in *GPR50* by a combination of dHPLC and direct nucleotide sequencing.

The Medical Research Council Ely Study is a prospective population-based cohort study of the pathogenesis of type 2 diabetes and related metabolic disorders (5). It involves an ethnically homogeneous Caucasian population and is of particular value because phenotypic data have been recorded on individuals both at the outset and again after 4.5 years. Subjects were all aged between 40 and 65 years at baseline. We screened the *GPR50* gene in 50 randomly selected subjects to determine the allele frequency of the SNPs identified in the patients with severe early-onset obesity using the same methods. Association studies were performed in 500 subjects initially (test set) and then in 585 subjects (replication set); these two sets were genotyped

using restriction fragment-length polymorphism and TaqMan-based assays, as described below.

PCR, sequencing, and genotyping

Primers were designed using a 1,939 bp fragment of *GPR50* electronically deposited under the accession identifier NM_004224 (4). Genes were amplified from genomic DNA by PCR. Genomic DNA was isolated from whole blood using a QIAamp blood kit (Qiagen, London, UK). PCR was performed using BioTaq (Bioline, London, UK) and carried out as recommended by the manufacturer. Thirty-five cycles (30 s at 96°C, 40 s at 60°C, and 40 s at 72°C) were performed using a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, MA). Primers were designed, fragments were separated using dHPLC (Transgenomics, Omaha, NE), and polymorphisms were detected within *GPR50* exon 2 using standard protocols. All primers used in the PCR, sequencing, and genotyping are listed in **Table 1**. Sequencing was carried out using BigDye terminator chemistry (Applied Biosystems, Foster City, CA) and electrophoresed on an ABI 377 automated DNA sequencer (Applied Biosystems).

C-16X2GPR50T was genotyped by PCR amplification of the fragment containing this polymorphism and then digestion with

TABLE 1. Primer list

Detection	Primer
Sequence scanning	
PCR1FGPR50X1	GCTGTCATTTGCTCTCTGAC
PCR1RGPR50X1	CAGGGATGTTCAATATGAGG
PCR2FGPR50X1	CTGTCATTTGCTCTCTGACTC
PCR2RGPR50X1	TATGAGGGCACTGTCCATAG
SEQ1FGPR50X1	AACAGGTGTTTGTCTGTCTG
SEQ1RGPR50X1	GGGAGCTCAAAAACCTAACG
SEQ2FGPR50X1	GTTTGCTGTCTGGACCTG
SEQ2RGPR50X1	TAGGGGTTGCAAGTCTCTG
dHPLC scanning	
1LWAVEGPR50X2	ACCACTTATTCTTCTCTGTCTTTC
1RWAVEGPR50X2	GGACAGCCAGGACCGTCA
2LWAVEGPR50X2	ACTGCTACATCTGCCACAGC
2RWAVEGPR50X2	AAGATCACAAACATGGTTAGAAAA
3LWAVEGPR50X2	CGTGAGGATCTGGACCAAG
3RWAVEGPR50X2	CTGATGAGGCCAGAGAAGAA
4LWAVEGPR50X2	CCTCAACGCTGTGATCTACG
4RWAVEGPR50X2	TGGTGGGTAGAGGCAGATTT
5LWAVEGPR50X2	GAATGTTCCATTACCTGGTG
5RWAVEGPR50X2	GACATGGTGGCCAGTGAT
6LWAVEGPR50X2	TCTGTCCATTTCAAGGGTGA
6RWAVEGPR50X2	GACTCAGGGAGGTCACCTGTC
7LWAVEGPR50X2	TAAGCCCACTGCTGCTGAC
7RWAVEGPR50X2	CAGTAAGGCATCTCACTTTGGA
Single-nucleotide polymorphism detection (by restriction fragment-length polymorphism)	
XHOIGPR50FA	CCTTCCCCTGCACCTTAAAT
XHOIGPR50RA	AGATGTTGCCTGAAAAACATCTC
XHOIGPR50FB	CCTTCCCCTGCACCTTAAAT
XHOIGPR50RB	TGTTGCCTGAAAAACATCTC
SACHIGPR50FA	AAACCCATCAAGCCAGCTAC
SACHIGPR50RA	GAGCTCAGGGTTGTCAGCAGCCG
SACHIGPR50FB	AAACCCATCAAGCCAGCTAC
SACHIGPR50RB	CTCAGGGTTGTCAGCAGCCG
Polymorphism detection (by 5' nuclease assay)	
Ins501Del Forward_FAMfluor	TGGCAGCCACTCCAAGTCTGC
Ins501Del Reverse	GTAGTGGCAGGCTTGGGATAG
G1804APCRF	CACTACCAGTACCAATGATTACCATGA
G1804APCRR	AGCCATTTTCATCAGGATCATCTTC
G1804A_A_allele_FAMfluor	TGTCGTGGTTATTGAT
G1804A_G_allele_VICfluor	CGTGGTTGTTGATGTT

TABLE 2. Variant sequences in the melatonin-related receptor found in 95 unrelated subjects with severe early-onset obesity

Gene	Polymorphism (Nucleotide)	Coding Change (Amino Acid)	Type of Change	Allele Frequency
<i>GPR50</i>	C-16X2GPR50T*	—	Silent	11%
	A1582G	Ins501Del	Nonconservative	44%
	G1804A	T528A	Nonconservative	44%
		V602I	Conservative	40%

-number, Signifies number of base pairs upstream of the first codon of *GPR50* exon 2.

* The nucleotide change that arises by the insertion is as follows: ccc-(aCC-ACT-GGC-CAC)-Atc-aag-cca P(501)-(T-T-G-H)-I-K-P(504).

Amino acid positions within *GPR50* of the proline residues are shown.

the restriction enzyme *XhoI* (New England Biolabs, London, UK), in accordance with the manufacturer's protocol.

A1582G (T528A) was also typed after generation of a forced restriction enzyme site. Digestion of the PCR product was performed with the restriction enzyme *SacI* (New England Biolabs).

Ins501Del and G1804A (V602I) were genotyped using a semi-automated fluorescent technology (Applied Biosystems). TaqMan 5' nuclease assays were designed and conducted according to standard procedures.

Statistical methodology

Linkage disequilibrium tests and haplotype analyses were performed using the expectation-maximization algorithm. Association analyses between genotype and phenotype were performed using the MIXED procedure in the Statistical Analysis System (SAS for Windows, version 8.2); this allows the incorporation of unbalanced repeated data, which improves the power of the study. To test for associations in males and females together, the variables were first tested for the existence of a significant gender difference and were then adjusted accordingly. Insulin, circulating triglyceride levels, LDL, and HDL all required correction to permit a global analysis. For BMI, tests were adjusted for age and sex.

RESULTS

We identified a number of variants in the coding region of *GPR50* in 95 subjects with severe early-onset obesity (Table 2): an insertion of four amino acid residues (TTGH) at position 501 of the receptor (Fig. 1) within its C-terminal tail, two other common coding changes [T528A (A1582G) (rs561077) and V602I (G1804A) (rs13440581)], and one noncoding polymorphism (C-16X2GPR50T) (rs1202874). The insertion occurs within its unusually long proline-rich and putatively functionally important cytoplasmic C-terminal tail and has been reported previously (6). The variant sequences at these three loci were found at similar allele frequencies in normal-weight Caucasians (data not shown); thus, major loss-of-function mutations in *GPR50* do not appear to be a common cause of severe human obesity.

To examine whether genetic variation in *GPR50* might contribute to the inherited component of variation in obesity and other intermediate metabolic traits, we genotyped 500 unrelated English Caucasian subjects from the Isle of Ely prospective study at the four loci using both

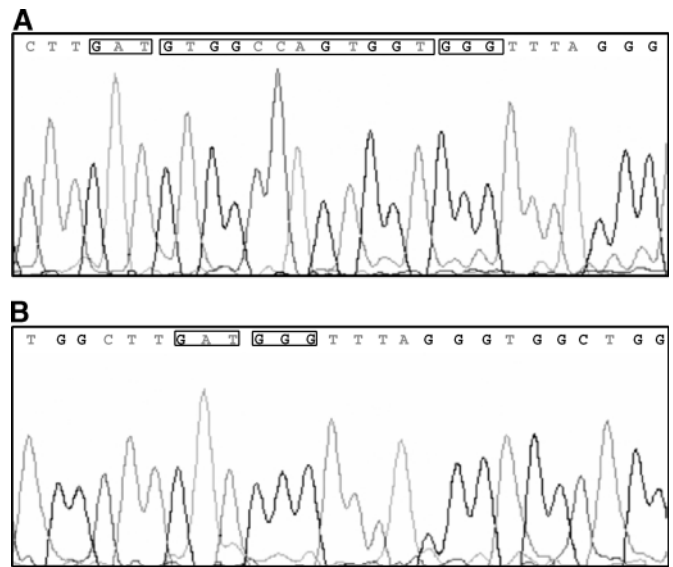


Fig. 1. *GPR50* genomic sequence electrophoregrams in male subjects carrying Ins501 (A) and 501Del (B). The 12 bp insertion and flanking three nucleotides on either side are highlighted.

restriction fragment-length polymorphism digestion and TaqMan technology. We recorded valid genotypes for 98% of the population for C-16X2GPR50T and 96% for Ins501Del, A1582G (T528A), and G1804A (V602I). In a population of 1,085 individuals consisting of 450 males and 635 females, one would expect 514 11(females), 114 12(females), 7 22(females), 405 hemizygous 1(males), and 45 hemizygous 2(males), for a polymorphism of allele frequency (f) of 0.1; values are 228, 305, 102, 270, and 180, respectively, at $f = 0.4$.

Allele frequencies of the mutant alleles at C-16X2GPR50T, Ins501Del, A1582G (T528A), and G1804A (V602I) were 0.11, 0.44, 0.44, and 0.40, respectively, with Ins501Del and A1582G (T528A) existing in complete linkage disequilibrium. Because *GPR50* is an X-chromosomal gene, haplotypes and hence linkage disequilibrium can be determined unambiguously in males. The quality of the generated genotypes was ensured by testing for violation of Hardy-Weinberg equilibrium in male and female subjects separately and by checking for the presence of only a single allele at each of the four polymorphic loci in males.

Table 3 provides pair-wise linkage disequilibrium estimates for the polymorphisms within *GPR50*. Complete link-

TABLE 3. Pairwise linkage disequilibrium values (D') between the four polymorphic loci [C-16X2GPR50T, Ins501Del, A1582G (T528A), and G1804A (V602I)] presented in 5' to 3' order across *GPR50*

Locus 1	Locus 2	D' (Males)	D' (Females)
C-16X2GPR50T	Ins501Del	0.689	0.725
C-16X2GPR50T	A1582G (T528A)	0.654	0.706
C-16X2GPR50T	G1804A (V602I)	0.743	0.746
Ins501Del	A1582G (T528A)	1	1
Ins501Del	G1804A (V602I)	0.524	0.537
A1582G (T528A)	G1804A (V602I)	0.559	0.538

TABLE 4. Results from an association study between the four polymorphisms within *GPR50* [C-16X2GPR50T, Ins501Del, A1582G (T528A), and G1804A (V602I)] and serum triglyceride levels

Polymorphism	Serum Triglyceride Levels		<i>P</i>
	Alleles 11 and 12	Allele 22	
	<i>mM</i>		
Test set (n = 500)			
C-16X2GPR50T	1.18 (1.14–1.22)	1.33 (1.25–1.41)	<0.01
Ins501Del	1.20 (1.13–1.27)	1.31 (1.22–1.41)	<0.05
A1582G (T528A)	1.20 (1.13–1.26)	1.31 (1.22–1.41)	<0.05
G1804A (V602I)	1.21 (1.13–1.30)	1.32 (1.22–1.44)	<0.05
Replication set (n = 585)			
C-16X2GPR50T	1.18 (1.13–1.23)	1.31 (1.25–1.37)	<0.01
Ins501Del	1.19 (1.13–1.25)	1.30 (1.22–1.39)	<0.05
A1582G (T528A)	1.19 (1.13–1.25)	1.30 (1.22–1.40)	<0.05
G1804A (V602I)	1.22 (1.14–1.30)	1.27 (1.18–1.36)	ns (<0.05)
Combined population (n = 1,085)			
C-16X2GPR50T	1.18 (1.13–1.23)	1.32 (1.25–1.39)	<0.001
Ins501Del	1.20 (1.16–1.24)	1.31 (1.24–1.37)	<0.01
A1582G (T528A)	1.20 (1.16–1.24)	1.31 (1.24–1.37)	<0.01
G1804A (V602I)	1.22 (1.17–1.27)	1.30 (1.25–1.36)	<0.05

Significance was determined using mixed-model analysis under a recessive model testing for associations between the mutant homozygous group and carriers of the wild-type allele. Data are means adjusted for age, sex, and body mass index (BMI). n, number of individuals used in this analysis whose phenotypes were recorded at least once; in the majority of cases, phenotypes were recorded at baseline and then again at follow-up. ns, not significant ($P > 0.05$). Ninety-five percent confidence intervals are presented in parentheses after the geometric mean triglyceride levels.

age disequilibrium exists between Ins501Del and A1582G (T528A), strong linkage disequilibrium exists between Ins501Del and C-16X2GPR50T, and weak linkage disequilibrium exists between Ins501Del and G1804A (V602I). Linkage disequilibrium between C-16X2GPR50T, Ins501Del, and A1582G (T528A) is illustrated by a population frequency of the mutant alleles at each of these three loci within a *cis*-phase haplotype that greatly exceeds expectation.

Table 4 provides the results from an association study between *GPR50* polymorphisms and subject triglyceride levels; although no associations were observed for BMI, evidence for a statistically significant association between *GPR50* SNPs and fasting triglyceride levels was obtained. Because *GPR50* is X-chromosomal, we tested whether the significances observed were the result of a phenotypic effect in either males alone or females alone. Statistical calculations were performed as described above, although

TABLE 6. Frequencies of observed haplotypes in *GPR50*

Haplotype	Men		Women	
	n	freq	n	freq
1111	352	0.448	537	0.474
1112	82	0.104	111	0.098
1221	100	0.127	127	0.112
1222	168	0.214	218	0.192
2111	12	0.015	7	0.006
2112	2	0.003	11	0.01
2221	—	—	4	0.004
2222	70	0.089	112	0.099

The haplotype is formed in 5' to 3' orientation across *GPR50*, as follows: C-16X2GPR50T—Ins501Del—A1582G (T528A)—G1804A (V602I). n, number of observations; freq, haplotype frequency.

without correction for sex. C-16X2GPR50T, Ins501Del, and G1804A were tested. We observed a statistically significant association in males at Ins501Del ($P < 0.05$) and in females at C-16X2GPR50T ($P < 0.05$). These results indicate that the genotype-phenotype relationship between *GPR50* polymorphisms and fasting triglyceride levels are not sex-specific and that both male and female triglyceride levels are affected by genomic variation at this locus. Failure to obtain significance ($P < 0.05$) at each of the three loci in both sexes separately is most likely related to the decrease in power that results from dividing the entire data set into two approximately equal halves.

Carriers of only the mutant allele (hemizygous 2 males and homozygous 2,2 females) at each of the four loci were associated with significantly higher triglyceride levels ($P < 0.05$) compared with carriers of the wild-type allele (hemizygous 1 males, heterozygous 1,2 and homozygous 1,1 females of the mutant allele) (Table 4). Using Ins501Del as an example, carriers of two copies of the 501Del allele showed circulating triglyceride levels of 1.31 mM (1.24–1.37), in contrast to Ins501 allele-bearing individuals, with mean triglyceride levels of 1.20 mM (1.16–1.24). The consistent pattern of association between *GPR50* polymorphisms and triglyceride levels is in agreement with the observed pattern of linkage disequilibrium between the loci.

We genotyped an additional 585 individuals to test whether this association might be seen in another population and obtained statistically significant evidence for an association to fasting triglyceride levels in the replica-

TABLE 5. Quantitative trait characteristics within test and replication population data sets

Trait	Test Set	Replication Set	<i>P</i>
n	500	585	
Age (years)	54.5 (53.4–55.5)	55.2 (54.4–56.0)	ns (>0.05)
BMI (kg/m ²)	26.1 (25.8–26.5)	26.9 (26.5–27.3)	0.01
HDL (mmol/l)	1.49 (1.45–1.53)	1.50 (1.46–1.53)	ns (>0.05)
LDL (mmol/l)	4.00 (3.91–4.10)	3.98 (3.90–4.06)	ns (>0.05)
Fasting insulin (pmol/l)	41.9 (39.6–44.3)	39.9 (38.0–41.9)	ns (>0.05)
Fasting triglycerides (mmol/l)	1.25 (1.20–1.31)	1.24 (1.19–1.29)	ns (>0.05)

Data are arithmetic or geometric (fasting insulin and fasting triglycerides) means, with 95% confidence intervals in parentheses. n, number of observations; ns, not significant ($P > 0.05$). *P* values represent significance of differences in test and control data set phenotypic distributions.

TABLE 7. Haplotypic associations between the combined cohort and fasting triglyceride levels

	1111	1112	1221	1222	2222	<i>P</i>
Fasting triglycerides (mM)	1.22 (1.18–1.27)	1.12 (1.04–1.20)	1.19 (1.11– 1.27)	1.24 (1.17–1.31)	1.34 (1.24–1.44)	<0.05

Fasting triglycerides were adjusted for age, sex, and BMI. Triglyceride values in each haplotype grouping are presented as geometric means, with 95% confidence intervals in parentheses. *P* value represents significance of the associations of haplotype with fasting triglycerides levels.

tion set ($P < 0.05$) with C-16X2GPR50T, Ins501Del, and A1582G (T528A) (Table 4). Carriers of two copies of the mutant alleles at these loci were once again shown to have significantly higher circulating triglyceride levels compared with their wild-type-bearing counterparts. Using Ins501Del as an example, carriers of two copies of the 501Del allele showed circulating triglyceride levels of 1.30 mM (1.22–1.39), in contrast to Ins501 allele-bearing individuals, with mean triglyceride levels of 1.19 mM (1.13–1.25).

Importantly, we obtained replication of the preliminary associations using an identical genetic model to our initial study and obtained highly consistent absolute values for fasting plasma triglyceride levels in the genotype groupings for the polymorphisms in the two studies.

In the combined data set, we also observed highly statistically significant associations between C-16X2GPR50T ($P < 0.001$), Ins501Del ($P < 0.01$), A1582G (T528A) ($P < 0.01$), and G1804A (V602I) ($P < 0.05$) and fasting plasma triglyceride levels (Table 4). Interestingly, we also observed evidence for an association between C-16X2GPR50T and HDL-cholesterol in the combined data set, with the subgroup of our cohort carrying the increased fasting plasma triglyceride haplotype also having reduced plasma HDL-cholesterol levels [1.39 mM (SEM, 0.03 mM)] relative to their control group [1.47 mM (SEM, 0.01 mM)] ($P < 0.01$). No significant associations were seen with BMI, LDL-cholesterol, and fasting insulin levels in either the initial or the combined data set (Table 5).

Table 6 provides frequencies of haplotypes occurring in males and females separately in the combined cohort. Frequencies were determined from the 959 individuals in whom all genotypes at the four polymorphic loci had been successfully recorded. Haplotype (multipoint) associations were sought between fasting triglyceride levels and the commonly occurring (population frequency of >5%) haplotypes (Table 7). A significant association was observed with triglyceride levels ($P < 0.05$), consistent with the single-point association results shown in Table 4. However, there was insufficient additional information to identify which of the polymorphic loci within *GPR50* was functionally responsible for the metabolic effect.

DISCUSSION

In this study, we examined whether variants in *GPR50* (melatonin-related receptor) were responsible for a monogenic or polygenic contribution to certain obesity and metabolic phenotypes. Beyond our knowledge of its pattern of expression (2) and of its close structural homology to the melatonin receptor (3), little more is known about this


orphan G protein-coupled receptor. In a previous genetic study, polymorphisms in *GPR50* were considered to be associated with bipolar affective disorder susceptibility (6).

Although we did not identify a novel monogenic syndrome of obesity in the course of this study, or an association between SNPs within *GPR50* and BMI, we have demonstrated a strong association between genetic variants in *GPR50* and fasting circulating triglyceride and HDL-cholesterol levels.

The possibility that this is a type I error is significantly diminished by the fact that, having made the observations in a cohort of 500 English Caucasian subjects, we then went on to test the association in another cohort of 585 subjects from the same geographic area. It was notable that the results obtained were statistically significant and of the same direction and magnitude as those seen in the first study. Of course, it will be important to study these relationships in other independent populations before asserting the generalizability of this phenomenon. Assuming that the association is real and not a statistical quirk, how might these variants influence lipid levels? One possibility is that the variants are in linkage disequilibrium with other genes on the X chromosome that may themselves have a direct influence on plasma lipids, although there are no obvious candidates for such an effect. A direct effect of *GPR50* itself on lipid levels is not implausible. There is an increasing realization of the importance of efferent autonomic pathways from the hypothalamus in the acute and chronic control of intermediary metabolism. It is notable that, although *GPR50* has not yet been deorphanized, its closest relative is the melatonin receptor.

It is well known that perturbations to the circadian internal clock (and hence melatonin levels) have been shown to have marked effects on lipid metabolism, most notably on plasma triglyceride levels (7–10), via the sympathetic nervous system (9). It is also quite plausible that the effects of variation at the *GPR50* locus on lipid metabolism could be unrelated to hypothalamic mechanisms but directly related to actions in peripheral tissues that have roles in lipoprotein production (such as the intestine) or uptake (kidney, adrenal) and in which *GPR50* expression has been documented in mouse (2).

In conclusion, we have conducted the first mutational screen of the melatonin-related receptor (*GPR50*) in relation to obesity and identified four common variants. We have subsequently found a surprising association of these SNPs with fasting triglyceride levels. However, in a replication cohort, an effect of the same direction and magnitude was observed. These results should provoke studies in other populations. At present, they raise the possibility

of an intriguing link between the hypothalamus and the control of plasma triglyceride and HDL levels, and between shared neuronal mechanisms underlying key neurological and metabolic processes in health and disease. 

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