

Studies of the Peptide YY and Neuropeptide Y2 Receptor Genes in Relation to Human Obesity and Obesity-Related Traits

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Peptide-YY (PYY) is secreted from endocrine L-cells of the gastrointestinal tract in response to caloric ingestion and may mediate postprandial satiety through the hypothalamic neuropeptide Y2 receptor (Y2R). We examined whether variants in the genes encoding PYY and Y2R might be associated with obesity-related phenotypes in humans. Among 101 subjects with severe early-onset obesity and a history of hyperphagia, we found two rare sequence variants—L73P and IVS2 + 32delG—in *PYY* and three rare missense mutations—L40F, F87I, and A172T—in *Y2R*. Although none of these were found in 100 normal-weight white control subjects, L73P in *PYY* and F87I and A172T in *Y2R* did not segregate with obesity in family studies, and family data were unavailable for *IVS2* + 32delG in *PYY* and L40F in *Y2R*. Two common single nucleotide polymorphisms (SNPs), R72T and IVS3 + 68C>T, in *PYY* were in tight linkage disequilibrium but showed no association with BMI in a large white population. In the *Y2R*, two SNPs, 585T>C and 936T>C, were found and were in tight linkage disequilibrium. Men, homozygous for the rarer variant, had significantly lower BMI ($P = 0.017$), waist-to-hip ratio ($P = 0.013$), and, surprisingly, higher non-esterified fatty acid levels ($P = 0.01$). In conclusion, mutations in *PYY* and *Y2R* are not commonly found in humans with severe early-onset obesity. The relationship between common variants in *Y2R* and obesity-related traits deserves further exploration in other populations. *Diabetes* 53:2461–2466, 2004

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Received for publication 30 April 2004 and accepted in revised form 9 June 2004.

Additional information for this article can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

GOOS, Genetics of Obesity Study; MS-PCR, mutagenically separated PCR; NEFA, nonesterified fatty acid; NPY, neuropeptide Y; RFLP, restriction fragment-length polymorphism; POMC, proopiomelanocortin; PYY, peptide-YY; SNP, single nucleotide polymorphism; Y2R, Y2 receptor.

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There has been considerable interest recently in the role of gut hormones that may provide short-term information about hunger and satiety and their potential as targets for pharmacological suppression of appetite in humans. Peptide-YY (PYY) is a 36-amino acid peptide secreted from the L-cells of the gastrointestinal tract postprandially in proportion to the calorie content of a meal and then cleaved by the enzyme dipeptidyl peptidase-IV to yield the major circulating form, PYY₃₋₃₆ (1–3). Peripheral injection in rats and mice inhibits food intake, and infusion of postprandial concentrations of PYY₃₋₃₆ in humans has been shown to significantly decrease food intake at a buffet meal (4). PYY₃₋₃₆ is a selective agonist for the Y2 receptor (Y2R) (5), which is highly expressed on neuropeptide Y (NPY) neurons in the arcuate nucleus of the hypothalamus, an area known to be involved in the regulation of food intake (6).

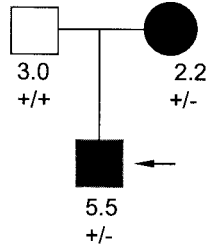
In addition, Batterham et al. (7) showed that endogenous fasting and postprandial levels of PYY₃₋₃₆ are significantly lower in obese compared with lean individuals; fasting PYY₃₋₃₆ levels measured using an in-house assay correlated negatively with BMI. If PYY₃₋₃₆ and Y2R are physiologically important components of the gut-hypothalamic pathway, then genetic defects in the *PYY* and/or *Y2R* genes might result in hyperphagia and severe obesity in humans. It is also possible that common genetic variants in the *PYY* or *Y2R* genes might influence adiposity or obesity-related traits in the general population. To test these hypotheses, we screened the coding regions of the two genes in 101 patients with severe early-onset obesity by nucleotide sequencing. Several rare and common variants were identified in each gene. We went on to assess whether rare variants segregated with severe obesity in extended family pedigrees and whether common variants in these genes were associated with obesity-related phenotypes in large ethnically homogenous population-based cohorts.

RESEARCH DESIGN AND METHODS

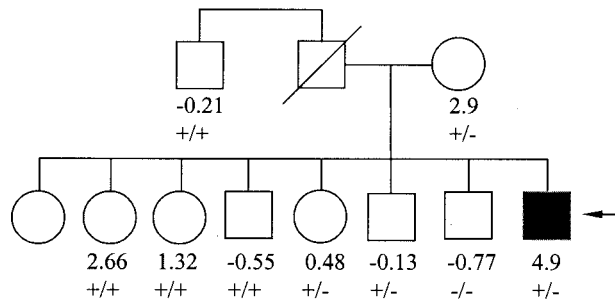
In the initial mutation screen, 101 unrelated, hyperphagic individuals were randomly selected from a larger cohort of children who were affected by severe, early-onset (<10 years of age) obesity. The cohort is also known as the U.K. Genetics of Obesity Study (GOOS). A total of 90% of the cohort is U.K. Caucasian. BMI (weight in kilograms divided by the square of height in meters) SD scores (SDSs) were calculated using the U.K. 1990 growth reference data (8). The mean BMI SDS of probands was 4.2 ± 0.8 .

The MRC Ely Study is a prospective, population-based cohort study of the

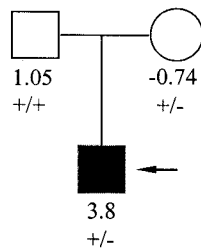
IVS2+32delG in PYY



L73P in PYY



F87I in Y2R



A172T in Y2R

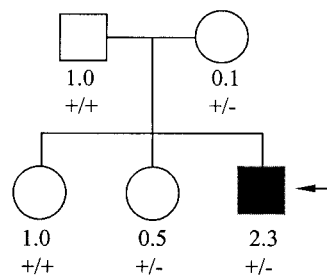


FIG. 1. Families with rare variants in the Y2R or PYY genes. Variants in each family. Circles, female family members; squares, male family members; slashes, deceased; open symbols, unaffected family members; filled symbols, family members with early-onset obesity (defined as weight above the 98th percentile and onset of obesity before 10 years of age). The arrow indicates the proband of each family. Known genotypes are indicated below each symbol: -, variants on one allele; +, normal genotype on one allele.

pathogenesis of type 2 diabetes and related metabolic disorders in individuals aged between 40 and 65 years in the U.K. (9,10). Participants were invited to attend for follow-up studies 5 years later. It is an ethnically homogeneous white population. This cohort was recruited from a population sampling frame with a high response rate (74%), making it representative of the general population for this area of eastern England. A total of 1,056 individuals, with a mean BMI SDS of 26.6 ± 4.4 , were studied for the R72T variant in PYY and the 585T>C single-nucleotide polymorphism (SNP) in Y2R. Genotyping was successful for 952 individuals for the R72T variant and for 1,005 for the 585T>C SNP.

South African Zulu participants were included after identification of the L73P mutation in a young Zulu boy with early-onset obesity and hyperphagia from the GOOS cohort. The boy resided in the northern part of the province of KwaZulu-Natal, and the boy's mother, paternal uncle, six siblings, and 100 healthy control subjects from the same geographic area, who had previously formed part of a cohort of 1,021 subjects who enrolled for a study of the prevalence of diabetes in rural KwaZulu-Natal, were included in the present study. Control subjects were selected randomly from the diabetes prevalence cohort and were required to have normal glucose tolerance (World Health Organization 1998 criteria) and genomic DNA available. According to these criteria, 54 female and 46 male subjects were included with a mean (\pm SD) age of 34 (\pm 11) years and mean (\pm SD) BMI of $23.97 (\pm 5.3)$ kg/m².

PCR and sequencing. To amplify the entire coding region of the Y2R gene from genomic DNA, we used Y2R1F (5'-TAG GTT GTA GAC TCT TGT GCT GG-3') and Y2R4R (5'-CTG GTC AGA ATT CAT CCA TAC AT-3') in a PCR. PCR was performed using BioTaq (Biolone, London, U.K.) and carried out under standard conditions. Thirty-five cycles of 30 s at 95°C, 30 s at 65°C, and 60 s at 72°C were performed using a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, MA). The PCR product was sequenced on both strands to determine the nucleotide sequence of Y2R. Eight primers (Y2R1R, 5'-TTG GCA ATG AAA AAG TTG GTT AC-3'; Y2R2F, 5'-GGT GAT CCA TGT GGT GAT CAA AT-3'; Y2R2R, 5'-GAA GAT GGC CAG GGG ACT TGC CA-3'; Y2R3F, 5'-ATT ATT GGC TTG GCC TGG GGC AT-3'; Y2R3R, 5'-TGG CTG TCA ATG TCA ACG GCA AG-3'; and Y2R4F, 5'-TGT TTG CGG TCA GCT GGC TGC CT-3'), including Y2R1F and Y2R4R, were used for sequencing Y2R. PYYFor (5'-CCC GCC GTG TAG GGT CGA GGC TT-3') and PYYRev (5'-GTG CGT ATG CAA ATG ACG TGG GC-3') were used to amplify the coding region of the PYY gene. PCR was carried out under standard conditions, and 35 cycles of 30 s at

95°C, 30 s at 69°C, and 30 s at 72°C were performed. PYYFor, PYYRev, and two additional primers (PYY2F, 5'-CCA GAT CTG ACC ACG CTC TTC CC-3'; and PYY2R, 5'-GGG AAG AGC GTG GTC AGA TCT GG-3') were used for determining the sequence of PYY on both strands. Sequencing reaction was carried out using BigDye Terminator chemistry (Perkin-Elmer, Foster City, CA) and analyzed on an ABI 377 automated DNA sequencer (Perkin-Elmer). **Genotyping Y2R and PYY variants.** The PYY IVS2 + 32delG variant was genotyped by mutagenically separated PCR (MS-PCR). All of the other variants were genotyped by PCR-restriction fragment-length polymorphism (RFLP) assays. Details for methods and primers used in MS-PCR and PCR-RFLP assays are described in the online appendix (available at <http://diabetes.journals.org>).

Statistical analysis. Linkage disequilibrium test was performed using the expectation-maximization algorithm, and Hardy-Weinberg equilibrium test was undertaken using a χ^2 "goodness-of-fit" test. The associations between the phenotype and the genotype, adjusted for appropriate covariates (see RESULTS for details), were tested using general linear model in SPSS for Windows 11.0.1. The adjusted means were provided. $P < 0.05$ was deemed significant.

RESULTS

PYY. The human PYY gene (accession no. NM_004160), on chromosome 17q21.1, is composed of four exons and three introns that span ~1.2 kb (11). Exons 2 and 3 and part of exon 4 contribute to the coding of the preproPYY protein. Posttranscriptional cleavage of the preproPYY molecule gives rise to the PYY or PYY₍₃₋₃₆₎ protein products. A total of 101 unrelated individuals with hyperphagia and severe early-onset obesity were screened for mutations in the PYY gene by direct nucleotide sequencing. Two rare sequence variants, IVS2 + 32delG (deletion of the guanine at nucleotide 32 of intron 2) and L73P (CTT->CCT) were found. The IVS2 + 32delG intronic variant was found in a 6-year-old boy of mixed-race parentage (Afro-Caribbean-Caucasian) with a BMI SDS of 5.5 (Fig. 1). The variant

TABLE 2
Association studies of the 585>T variant of the Y2R gene with obesity-related phenotypes in men from a U.K. Caucasian population-based cohort

Response	P					
	T/T	T/C	C/C	T/T vs. T/C vs. C/C	T/T + T/C vs. C/C	T/T + T/C vs. C/C (adjusted for age and BMI)
n	111	230	80			
BMI (kg/m ²)	26.96 (26.24–27.69)	27.10 (26.61–27.61)	25.92 (25.09–26.76)	NS	NS	—
Waist-to-hip ratio	0.97 (0.96–0.99)	0.97 (0.96–0.98)	0.94 (0.93–0.96)	0.044	NS	—
Fasting NEFA (mmol/l)	0.43 (0.39–0.47)	0.45 (0.42–0.48)	0.51 (0.47–0.56)	0.029	NS	0.007
Fasting triglycerides (mmol/l)*	1.27 (1.16–1.40)	1.37 (1.28–1.46)	1.35 (1.20–1.50)	NS	NS	NS
Fasting insulin (pmol/l)*	45.42 (39.69–51.99)	42.69 (38.86–46.85)	40.37 (34.54–47.18)	NS	NS	NS
Fasting plasma glucose (mmol/l)*	5.07 (4.93–5.21)	5.09 (4.99–5.19)	5.06 (4.90–5.22)	NS	NS	NS
Insulin increment (pmol/l insulin per nmol/l glucose)*	28.88 (25.43–32.82)	29.49 (26.98–32.20)	29.70 (25.64–34.43)	NS	NS	NS

Data are means (95% CI). Means were adjusted for age. *Geometric means.

Three silent polymorphisms, 159C>T (L53L; *n* = 3), 585T>C (I195I), and 936T>C (I312I) were identified. Among the three heterozygous 159C>T variant carriers, two were South African Zulu and one was of Afro-Caribbean origin; thus, this variant is likely to be ethnic group specific. The 585T>C variant, a silent change in codon 195 (ATT->ATC), was found in heterozygous form in 53 (52.5%) and homozygous form in 17 (16.8%) individuals, with an allele frequency of 0.43. The 936T>C variant, a silent change in codon 312 (ATT->ATC), was in almost complete genotypic concordance with the 585T>C variant, whereas only one individual had the major T variant at 585 and the minor C variant at 936.

Although both common Y2R variants are silent changes, it is possible that they associate with some obesity-related phenotypes indirectly through linkage disequilibrium with other functional variants in or near the Y2R gene. To investigate the relationship between different Y2R genotypes and obesity-related phenotypes, we genotyped 1,005 individuals (male/female: 421/584) from the Ely Study for the 585T>C variant by PCR-RFLP assays. Because the 585T>C and 936T>C variants are in tight linkage disequilibrium, only one variant was genotyped in the association study. The frequency of the 585T allele in the Ely Study was 0.54. The distribution of genotypes is consistent with the Hardy-Weinberg prediction. Men who were homozygous for the 585T>C variant had significantly lower BMI (*P* = 0.017) and waist-to-hip ratio (*P* = 0.013) and higher nonesterified fatty acid (NEFA) levels (*P* = 0.01; Table 2). Table 2 also shows the results adjusted for BMI, demonstrating that the effect of the genotype on fasting NEFA is independent of BMI (*P* = 0.007). There were no significant associations between any of the phenotypes examined and the sequence variants in women (data not shown).

DISCUSSION

PYY₃₋₃₆ is a credible candidate gene for human obesity because it has been shown to suppress appetite and energy intake acutely in both rodents and humans (4,7). Furthermore, fasting PYY₃₋₃₆ levels are lower in obese individuals, and thus it has been suggested that deficiency of PYY₃₋₃₆ may contribute to the pathogenesis of common human obesity. However, as no model of complete PYY deficiency has been reported to date, the extent to which PYY₃₋₃₆ is required for postprandial satiety and long-term energy balance is still unclear.

We have undertaken, to our knowledge, the first study that examines variation in the genes encoding the gut hormone PYY and its central receptor Y2R in relation to human obesity. We found two rare variants in the PYY gene, IVS2 + 32delG and L73P, which were not found in 100 white control subjects, although the IVS2 + 32delG variant was found in 4 of 100 Zulu control subjects, suggesting that this is an ethnicity-specific polymorphism. The L73 variant is highly conserved in rodents (although not in zebrafish) (human PYY sequence [NM_004160] compared with mouse PYY [NM_145436]) and has potential functional significance because of its close proximity to the dibasic cleavage site of PYY. Brakch et al. (13) showed that disruption of the helical structure COOH-terminal to the dibasic cleavage site preserved in prohormone peptides impairs the efficiency of proteolytic

processing by prohormone convertase 1/3. It is possible that the introduction of a proline residue at this position might break the helical structure and result in lowered cleavage efficiency and thus lead to lowered PYY levels. However, in our studies, the L73P variant did not cosegregate with obesity in a single family of Zulu origin. One of the lean siblings in that family was actually homozygous for the variant. The L73P variant was subsequently shown to be relatively common (18%) in an ethnically matched control population and was not associated with BMI or with any intermediate obesity-related phenotype. The common PYY variant found in white individuals (R72T) lies five residues downstream of the cleavage site. R72T might associate with obesity-related phenotypes directly through influences on cleavage efficiency or indirectly through linkage disequilibrium with other functional variants. We performed an association study in a large white population-based cohort and found no evidence of any association with obesity-related phenotypes. Given the observation that obese individuals might have low PYY levels, it would be of interest to study the relationship between these common variants and PYY levels.

The major neuronal populations that are responsible for integrating peripheral signals, such as PYY₃₋₃₆, are the orexigenic agouti-related protein/NPY neurones and the anorectic proopiomelanocortin (POMC)/CART (cocaine- and amphetamine-regulated transcript) neurones in the arcuate nucleus. Morphologic and electrophysiological studies have revealed direct synaptic contact between NPY terminals and neighboring POMC cell bodies and dendrites; NPY exerts an inhibitory tone over the POMC anorectic projections, most likely a Y1R-mediated effect (14,15). NPY itself is coexpressed with the presynaptic inhibitory Y2R (6) and thus acts as a feedback regulator of NPY signaling and indirectly of melanocortin signaling. Peripheral administration of PYY₃₋₃₆ in rodents decreases hypothalamic NPY expression and results in reduced food intake (4). Central administration of an Y2R selective agonist also reduces food intake, and peripheral administration of PYY₃₋₃₆ has no effect in fasted Y2R knockout mice, implicating Y2R in mediating the effects of PYY₃₋₃₆ on food intake. Thus, gut-derived PYY₃₋₃₆ may access a subpopulation of Y2R-expressing NPY neurones in the mediobasal hypothalamus and inhibit NPY release through Y2R and also decrease the tonic GABA-mediated inhibition of POMC neurons, leading to a reduction in food intake. However, other NPY receptors, with various affinities to PYY, also play a role in mediating feeding behavior, and studies in knockout animals support the existence of redundant or compensatory mechanisms. Naveilhan et al. (16) showed that Y2R null mutant mice had slightly increased food intake and body weight and manifest an attenuated response to leptin administration. Conditional deletion of hypothalamic Y2R in mice had only transient effects on body weight and food intake, and compensatory mechanisms operated to maintain homeostasis (17). Thus, the precise physiological pathways that mediate the effects of PYY and the role of specific NPY receptors in the regulation of energy homeostasis in humans requires further clarification.

We have undertaken the first studies of the human Y2R gene in a cohort with severe early-onset obesity and found

no major pathogenic mutations. Two common SNPs, 585T>C and 936T>C, were found and were in tight linkage disequilibrium. Male individuals who were homozygous for the rarer variant had significantly lower BMI and waist-to-hip ratio and higher NEFA levels, which could reflect increased lipolysis in these individuals. However, these observations require further investigation in other large populations on whom detailed metabolic phenotypes have been collected.

In conclusion, our studies do not support a major role for genetic abnormalities in PYY or Y2R in severe early-onset obesity. The importance of common variants in these genes and the relationship to PYY levels remain to be explored.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust (to I.S.F., N.J.W., and S.O.R.) and the Medical Research Council (to S.O.R. and N.J.W.). The Ely Study was funded by Diabetes U.K.

We are grateful to the subjects, referring physicians, and the staff of the GOOS and Ely Study research clinics.

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