Variants in the 5' region of the neuropeptide Y receptor Y2 gene (*NPY2R*) are associated with obesity in 5,971 white subjects

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Abstract

Aims/hypothesis The gene encoding neuropeptide Y receptor Y2 (*NPY2R*) is widely expressed in the central nervous system, with particularly high abundance in the hypothalamus, which is known to be important for appetite regulation. We tested whether variations in *NPY2R* are associated with obesity.

Methods The coding region of *NPY2R* was analysed for mutations in 48 obese Danish white subjects and two silent substitutions were identified: SNPs 1 and 2 (rs1047214 and rs2880415). SNP1 and additional reported variants (SNPs 3–6 [rs11099992, rs12649641, rs2342676 and rs6857530]) located in the 5' region were examined in 5,971 Danish white subjects. Since SNPs 1–2 and 4–6, respectively, were in tight linkage disequilibrium large-scale analyses of genetic epidemiology were restricted to SNPs 1, 3 and 4.

Results Homozygous carriers of the minor A allele of SNP4 were more common among obese subjects; the AA frequency was 15.9 (95% CI 15.2–16.6) among 4,837 non-obese subjects (BMI <30 kg/m²) vs 19.0 (95% CI 17.2–20.8)

among 960 obese subjects (BMI ≥30 kg/m²), odds ratio 1.24 (95% CI 1.04–1.48), $p=0.02$. SNPs 1–3 were not associated with obesity.

Conclusions/interpretation Common variants rs12649641, rs2342676 and rs6857530 in the 5' region of *NPY2R* are associated with obesity in Danish white subjects.

Keywords Genetic/epidemiology · Genetics of type 2 diabetes · Hormone receptors · Human · Weight regulation and obesity

Abbreviations

GATA-3 GATA-binding factor 3
LD linkage disequilibrium
NPY2R neuropeptide Y receptor Y2
PYY peptide YY

The neuropeptide Y receptor Y2 (*NPY2R*) is a seven-transmembrane receptor that signals by G-proteins when activated by peptide YY (PYY) [1]. *NPY2R* is widely produced in the central nervous system with particularly high abundance in the hypothalamus, which is known to be important for appetite regulation [2]. *NPY2R* knockout mice are hyperphagic and have significantly increased body weight and fat deposition compared with wild-type mice [3]. The ligand of *NPY2R*, PYY, is released postprandially from L cells in the distal part of the intestine in proportion to the ingested amount of food [4, 5]. Peripheral injection of PYY inhibits food intake and reduces weight gain but fails to do so in *NPY2R* knockout mice [6]. Interestingly, obese subjects have lower plasma PYY levels and a relatively smaller increase in circulating PYY levels after a meal compared with lean subjects [7], suggesting that gene variants in *PYY* may alter the level of

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circulating PYY. Indeed, in a study of 5,965 subjects we previously reported that the *PYY* Arg72 variant is associated with overweight and type 2 diabetes and that Arg72Arg carriers have lower circulating PYY levels [8]. In a study of 167 Pima Indian men variants in both *PYY* and *NPY2R* were reported to be associated with severe obesity [9]. Furthermore, in a study of 1,800 North American, 1,030 Polish and 240 Scandinavian subjects several variants in the 5' region of *NPY2R* were reported to be more common among obese men compared with lean men [10]. In contrast, no association with early onset obesity was found for variants in *PYY* and *NPY2R* among 101 children from UK [11]. In a study of 421 white men, homozygous carriers of the *NPY2R* variant Ile195 (rs1047214), had, however, a significantly lower BMI ($p=0.02$) and waist-to-hip ratio ($p=0.01$) [11]. In contrast, in a recent study of 479 Swedish white men homozygous carriers of the same variant had increased BMI [12]. In the present study of a relatively large study sample of Danish white subjects we tested the hypothesis that variants in *NPY2R* are associated with common subsets of obesity and related quantitative traits.

Subjects and methods

Subjects

All study participants were Danish white subjects by self-report. Informed written consent was obtained from all study participants. The studies were in accordance with the Helsinki Declaration II and were approved by the local Ethical Committee of Copenhagen.

Mutation detection

The screening for genetic variants was performed on genomic DNA extracted from human leucocytes from 48 unrelated overweight or obese subjects (age 65 ± 10 years [mean $\pm$ SD] and BMI 31.3 ± 5.1 kg/m²). All subjects were recruited at the Steno Diabetes Center.

Obesity

The studies of the association with obesity were done in the Inter99 cohort, which is a population-based randomised non-pharmacological intervention study for prevention of cardiovascular disease conducted at the Research Centre for Prevention and Health [13]. The analyses were performed in subjects of Inter99 who did not have known type 2 diabetes. However, the subjects with screen-detected type 2 diabetes were included in the analyses as these subjects were not treated for diabetes. Genomic DNA from 5,971

Danish white subjects from this cohort was available for genotyping. These subjects were divided into two groups: (1) 4,987 subjects with BMI <30 kg/m² (age 46 ± 8 years, BMI 24.7 ± 2.8 kg/m²); and (2) 984 obese subjects with BMI ≥ 30 kg/m² (age 48 ± 8 years, BMI 33.9 ± 3.9 kg/m²).

Type 2 diabetes

The association study of diabetes included 1,408 patients with type 2 diabetes and 4,897 subjects with fasting normoglycaemia and normal glucose tolerance in accordance with World Health Organization (1998) criteria [14]. Of the 1,408 diabetic patients (61% men, 39% women), 1,060 were recruited from the outpatient clinic at the Steno Diabetes Center and 348 were recruited from the Inter99 study cohort [13]. Diabetes was diagnosed in accordance with the World Health Organization (1998) criteria [14]. The phenotypic characteristics of the type 2 diabetic patients were: age 57 ± 10 years, age at clinical onset 52 ± 10 years, BMI 29.7 ± 5.3 kg/m², HbA_{1c} $7.8\pm 1.7\%$. The control group comprised 4,523 subjects from the Inter99 cohort and 374 subjects who were randomly recruited from Copenhagen County and examined at the Steno Diabetes Center (age 47 ± 9 years, BMI 25.6 ± 4.0 kg/m², 46% men, 54% women).

Quantitative traits

The genotype-quantitative trait studies were performed in the 5,797 subjects (normal glucose-tolerant, IFG, IGT and screen-detected type 2 diabetes subjects) of the Inter99 who did not have known type 2 diabetes.

Biochemical and anthropometrical measurements

Blood samples for analyses of biochemical variables were drawn in the morning after an overnight fast. Plasma glucose, serum-specific insulin (excluding des(31,32)-proinsulin and intact proinsulin) and serum cholesterol and triglycerides were analysed using Steno Diabetes Center standard methods. Height and weight were measured in light indoor clothes and without shoes, and BMI was calculated as weight in kg/(height in m)² [13]. Waist circumference was measured in the standing position midway between the iliac crest and the lower costal margin, and hip circumference at its maximum.

Mutation analysis and genotyping

Mutation detection in the coding region of *NPY2R* (NCBI accession no. U42766 [1/7-2005]) was performed by bidirectional nucleotide sequencing (MWG, Ebersberg, Germany) on genomic DNA extracted from human leuco-

cytes with subsequent analysis for variants applying Seq-Scape Software version 2.0 (Applied Biosystems, Foster City, CA, USA). Primers for mutation detection are available on request. SNP1 and four additional variants (SNPs 3–6 [Table 1]) located in the 5' region and previously shown to be associated with obesity [10] were genotyped by Taqman allelic discrimination (KBioscience, Hoddesdon, Herts, UK). The genotyping success rate for all variants was >95% and among 851 replicates samples the discrepancy rate was <0.35%. All genotype distributions obeyed Hardy–Weinberg equilibrium.

Statistical and in silico analyses

Pair-wise linkage disequilibrium (LD) was estimated using R^2 . Logistic regression with adjustment for sex, age and BMI (where appropriate) was applied to test for significant differences in genotype distribution in the case–control studies. Differences in quantitative phenotypes between the genotype groups were tested using a general linear model which included genotype and sex as fixed factors and age as covariate. All analyses were performed using Statistical Package for Social Science (SPSS) version 12.0.

The Inter99 study population was divided into case and control groups stratified by increasing BMI thresholds, and the case homozygosity frequency (AA) of *NPY2R* SNP4 was calculated for every other BMI unit. Case groups with less than ten case individuals homozygous for the SNP4 A allele were excluded, which accounted for case groups with BMI >42 kg/m². All subjects in control groups had a BMI <30 kg/m². Fisher's exact test was applied to test for significant differences in homozygosity frequency between cases and control subjects for a given BMI threshold.

The expectation–maximisation algorithm was used to infer haplotype frequencies. Haploview was used to place SNPs in haplotype blocks of *NPY2R*. Association studies were carried out for the categorical variables of obesity and type 2 diabetes, and for the quantitative traits of BMI and

waist circumference using a general linear model implemented in the *haplo.stats* R package, RGui version 2.1 [15].

Interaction between *PYY* Arg72Thr and *NPY2R* SNP4 was modelled as described [16]. A general linear model was used where the parameters for the marginal effects were chosen according to the significant finding, i.e., a recessive model for *NPY2R* SNP4 and a dominant model for *PYY* Arg72Thr [8]. The epistatic parameter was estimated using the individuals for whom both risk alleles were present.

A *p* value of less than 0.05 was considered significant.

In silico identification of SNPs was done by using the NCBI (<http://www.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org>) databases.

By applying MatInspector we performed in silico studies of the potential impact of *NPY2R* SNPs 4–6 to alter transcription factor binding sites in the 5' region of *NPY2R* (<http://www.genomatix.de/products/MatInspector/index.html>).

Results

Forty-eight unrelated obese individuals were examined for variants in the coding region of *NPY2R*. Two synonymous polymorphisms, which were in perfect LD ($R^2=1.00$), were identified at codons 195 (SNP1 [rs1047214]) and 312 (SNP2 [rs2880415]) (Table 1). SNP1 and four additional and previously reported variants [10] (SNPs 3–6 [rs11099992, rs12649641, rs2342676 and rs6857530]) (Table 1) located in the 5' region of *NPY2R* were genotyped. Tight linkage disequilibrium (LD) ($R^2=0.98$) was observed between SNPs 4–6 (Table 1). The six SNPs can therefore be represented by SNPs 1, 3 and 4 and these three SNPs were analysed for association with obesity. SNPs 1 and 3 were not associated with obesity, whereas the minor A allele of SNP4 was associated with obesity in the subjects of the Inter99 population without known type 2 diabetes, $p=0.02$, odds

Table 1 *NPY2R* SNP identification and estimation of LD between SNPs

SNP identification				R^{2a}					
SNP no.	SNP ID	Nucleotides	Location	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6
SNP1	rs1047214	T>C	Ile195		1.00	0.07	0.08	0.08	0.09
SNP2	rs2880415	T>C	Ile312			0.07	0.08	0.08	0.09
SNP3	rs11099992	A>G	–4271 ^b				0.66	0.66	0.66
SNP4	rs12649641	C>A	–4448 ^b					0.99	0.98
SNP5	rs2342676	A>G	–5077 ^b						0.98
SNP6	rs6857530	A>G	–627 ^b						

^a Calculated in the Inter99 study population, except for SNP2 which was found to be in perfect LD with SNP1 in the initial mutation screening of 48 obese subjects

^b Relative to the first nucleotide in exon 1

ratio 1.24 (95% CI 1.04–1.48) (Table 2). This finding was also significant when only men were analysed ($p=0.04$), but not in women only ($p=0.08$; results not shown). In genotype-quantitative trait studies of 5,797 subjects without known type 2 diabetes the SNP4 was not associated with BMI ($p=0.1$), waist circumference ($p=0.1$) or other obesity related quantitative metabolic variables (Table 3).

The homozygosity frequency AA of SNP4 increased with increasing BMI thresholds of the Inter99 study population (Fig. 1). Since the SNP4 AA homozygosity frequency increased from BMI ≥ 28 kg/m² we divided the genotype-quantitative trait analysis into two groups; BMI < 28 and BMI ≥ 28 kg/m². In the group with BMI ≥ 28 kg/m² homozygous carriers of the SNP4 A allele had significantly higher BMI ($p=0.03$), whereas there was no association with BMI in the group of BMI < 28 kg/m² ($p=0.08$). SNP4 was also investigated for association with extremes of BMI by dividing the subjects of Inter99 into two groups: (1) 5th–12th percentile of BMI (407 subjects); and (2) 90th–97th percentile of BMI (407 subjects). However, no association with obesity was found ($p=0.2$).

SNPs 1, 3 and 4 were not associated with type 2 diabetes (results not shown). The genotypic information of the six variants (SNPs 1–6) was used to determine the haplotype structure of the gene locus. SNPs 3–6 represent one haplotype block within the *NPY2R* locus involving three haplotypes. None of the haplotypes were, however, associated with obesity (results not shown).

Since the SNP4 A allele was shown to be associated with obesity we examined for a potential interaction between this SNP and the *PYY* Arg72Thr polymorphism previously shown to be associated with overweight [8]. No interactions or additive effect between the two variants with impact on risk of overweight or obesity were, however, found (results not shown).

Discussion

We present the results of a mutation analysis of the coding region of *NPY2R* and a relatively large-scale epidemiological study of identified polymorphisms and additional previously reported variants located in the 5' region of *NPY2R*. Our study of 5,971 subjects demonstrates that homozygous carriers of the SNP4 A allele, rs12649641, which is in tight LD with rs2342676 and rs6857530 in the 5' region of *NPY2R*, have an increased prevalence of obesity. The present finding of variants upstream of *NPY2R* that are associated with obesity in white subjects extends two reports: that of Ma et al., who in a study of 167 Pima Indian men found that several other variants in *NPY2R* were associated with severe obesity [9], and that of Campbell et al. of 1,800 North American, 1,030 Polish and 240 Scandinavian white subjects of both sexes, showing that SNPs 3–6 are associated with obesity in men [10].

SNP4 was associated with obesity as a dichotomous trait in Inter99, and with BMI as a quantitative trait only in the subjects of Inter99 with BMI ≥ 28 kg/m². This inconsistency may be due to a variable interaction of *NPY2R* SNP4 with other genetic and environmental factors with regulatory effects on BMI.

In the present study we did not find any association of SNP1 with decreased risk of obesity, in contrast to the findings of Hung et al., who showed a modest association with lower BMI in 421 UK white men [11]. However, as a study of 479 Swedish white men showed increased risk of obesity for SNP1 [12] it remains unresolved whether SNP1 is associated with BMI and in which direction. With a study sample of 939 obese subjects and 4,767 non-obese subjects we have a statistical power of 97% to show a relative risk of obesity of 1.3. Therefore, it is unlikely that SNP1 confers a major risk of obesity among the Danish white subjects studied.

Table 2 Association studies of the *NPY2R* variants with obesity in Danish white subjects

	<i>n</i> (men/women)	Wt (%)	He (%)	Ho (%)	MAF (95% CI)	<i>p</i> value		
						Wt vs He vs Ho	Wt vs He+Ho	Wt+He vs Ho
SNP1 (rs1047214)		TT	TC	CC				
BMI<30 kg/m ²	4,767 (2,363/2,404)	1,444 (30.3)	2,407 (50.5)	916 (19.2)	44.5 (43.5–45.5)			
BMI≥30 kg/m ²	939 (471/468)	277 (29.5)	472 (50.3)	190 (20.2)	45.4 (43.1–47.6)	0.8	0.5	0.7
SNP3 (rs11099992)		AA	AG	GG				
BMI<30 kg/m ²	4,867 (2,420/2,447)	2,361 (48.5)	2,073 (42.6)	433 (8.9)	30.2 (29.3–31.1)			
BMI≥30 kg/m ²	961 (484/477)	453 (47.1)	417 (43.4)	91 (9.5)	31.2 (29.1–33.2)	0.7	0.6	0.5
SNP4 (rs12649641)		CC	CA	AA				
BMI<30 kg/m ²	4,837 (2,401/2,436)	1,778 (36.8)	2,292 (47.4)	767 (15.9)	39.5 (38.6–40.5)			
BMI≥30 kg/m ²	960 (483/477)	340 (35.4)	438 (45.6)	182 (19.0)	41.8 (39.6–44.0)	0.05	0.5	0.02

p values were calculated using logistic regression with sex and age as covariates and describe the significance levels comparing obese and non-obese subjects

Wt wild-type; He heterozygous; Ho homozygous; MAF minor allele frequency; OR odds ratio

Table 3 Association studies of the *NPY2R* SNP4 (rs12649641) with obesity-related quantitative traits in 5,797 Danish white subjects without known type 2 diabetes

Trait	Wt (CC)	He (CA)	Ho (AA)	<i>p</i> value		
				Wt vs He vs Ho	Wt vs He+Ho	Wt+He vs Ho
<i>n</i> (men/women)	2,118 (1,067/1,051)	2,730 (1,344/1,386)	949 (473/476)			
Age (years)	46 (46–46)	46 (46–46)	46 (46–47)			
BMI (kg/m ²)	26.2 (26.0–26.3)	26.2 (26.0–26.4)	26.4 (26.1–26.7)	0.3	0.5	0.1
Waist (cm)	86.5 (86.0–87.0)	86.3 (85.9–86.7)	87.0 (86.3–87.7)	0.3	1.0	0.1
Waist/hip ratio	0.86 (0.85–0.86)	0.86 (0.85–0.86)	0.86 (0.85–0.86)	0.5	0.3	0.9
Fasting serum insulin (pmol/l) ^a	41.5 (40.3–42.6)	40.7 (39.7–41.8)	43.0 (41.2–44.8)	0.05	0.9	0.05
Fasting serum cholesterol (mmol/l) ^a	5.52 (5.48–5.57)	5.55 (5.52–5.59)	5.51 (5.45–5.60)	0.4	0.4	0.5
Fasting serum triglycerides (mmol/l) ^a	1.33 (1.27–1.39)	1.35 (1.30–1.40)	1.28 (1.19–1.36)	0.3	1.0	0.2
Fasting plasma glucose (mmol/l) ^a	5.53 (5.50–5.57)	5.55 (5.52–5.58)	5.50 (5.46–5.55)	0.3	0.7	0.2
HOMA-IR ^a	10.5 (10.1–10.8)	10.3 (10.0–10.6)	10.8 (10.3–11.3)	0.1	0.7	0.08

Data are means (95% CI). Means were adjusted for the effect of age and sex

Phenotypic differences between the genotype groups were tested with a general linear model which included genotype and sex as fixed factors and age as covariate factor

Wt wild-type; He heterozygous; Ho homozygous; HOMA-IR homeostasis model assessment for insulin resistance

HOMA-IR was calculated as fasting serum insulin (pmol/l) multiplied by fasting plasma glucose (mmol/l) and divided by 22.5

^a The traits were logarithmically transformed before analyses

Whether SNPs 4–6 are causative in the pathogenesis of obesity or whether they are markers in tight LD with yet unidentified functional variants within or near the *NPY2R* locus is yet to be settled. However, an *in silico* analysis

Fig. 1 Homozygosity frequencies of case groups of the *NPY2R* SNP4 (rs12649641) A allele with increasing BMI thresholds of the Inter99 study population. The left-hand side y-axis denotes the homozygosity frequency and the right-hand side y-axis denotes the odds ratio for association with obesity. Open circles, AA homozygosity frequency (95% CI); black squares, odds ratio for obesity. Asterisks indicate the significance levels of the difference between case and control groups for a given BMI (kg/m²) threshold. **p*<0.05, ***p*<0.01. Number of cases (*n*) for BMI (kg/m²) thresholds: BMI=28 (1821), BMI=30 (960), BMI=32 (649), BMI=34 (385), BMI=36 (221), BMI=38 (128), BMI=40 (85)

showed that SNP5 disrupts a GATA-binding factor 3 (GATA-3) consensus site. GATA-3 is essential for brain development and is abundantly expressed in the central nervous system [17–19]. Consequently, disruption of the GATA-3 site might influence the promoter activity of the *NPY2R* leading to less appetite inhibition and risk for development of obesity.

In contrast to the single variant analyses, the analyses of the haplotypes in *NPY2R* did not show any association with obesity. This finding may obviously be related to the fact that the statistical power of haplotype analyses decreases when the haplotype groups contain fewer subjects compared with the individual variant groups and to the possibility that one of the SNPs in the haploblock is the causative variant.

In conclusion, common variants, rs12649641, rs2342676 and rs6857530, in the 5' region of *NPY2R* are associated with obesity in Danish white subjects.

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