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Genetic studies of neuropeptide Y and neuropeptide Y receptors Y1 and Y5 regions in morbid obesity

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Summary Synthesis and release of neuropeptide Y (NPY) are both regulated by leptin binding to its hypothalamic receptor mediating some of the effects of leptin on food intake. Moreover, NPY administration is a powerful stimulant of feeding behaviour. Thus, we investigated the potential implication of NPY, NPY-Y1 and -Y5 subtype receptors [rNPY-Y1/-Y5] in the development of human obesity. Two complementary genetic approaches were used: 1) linkage analyses between obesity and polymorphic markers located nearby NPY and rNPY-Y1/-Y5 genes (respectively on chromosomes 7p15.1 and 4q[31.3–32]) in 93 French Caucasian morbidly obese families; 2) single strand conformation polymorphism (SSCP) scanning of the coding region of the NPY and rNPY-Y1 genes performed in 50 unrelated obese patients ascertained on the basis of a body mass index of 27 kg/m^2 or more and a family history of obesity. No evidence of linkage between morbid obesity or obesity-related quantitative traits and NPY and rNPY-Y1/Y5 regions was found in this population. Moreover, SSCP scanning revealed no mutation in the coding region of NPY and rNPY-Y1 genes among obese subjects. These results suggest that NPY and NPY-Y1/Y5 receptors are unlikely to be implicated in the development of human morbid obesity, at least in the French Caucasian population. [Diabetologia (1997) 40: 671–675]

Keywords Obesity, genetic, body mass index, neuropeptide Y, neuropeptide Y receptors, leptin.

Obesity is a disorder of energy balance due to chronic disequilibrium between energy intake and expenditure. Obesity is strongly associated with a number of common diseases that have a major impact on morbidity and mortality, including non-insulin-dependent diabetes mellitus, hypertension, cardiovascular diseases, and hyperlipidaemia [1]. Studies in twins, adoptees, and families suggest that from 40% to as much as 80% of the variance in body mass index (BMI) can be ascribed to genetic factors. In rare cases, human obesity is the consequence of single gene disorders (Bardet Biedl, Prader Willi, Ahlstrom, Cohen syndromes) where syndromes are well characterized by specific clinical phenotypes leading to their identification [2]. In human obesity, it is believed that a limited number of genes interact with the environment to produce the final phenotype, but such genes remain mostly unidentified. On the other hand, recent data in rodents have allowed the identification of a 'satiety-factor', leptin, which is secreted by adipose cells. Leptin binding to its specific receptor in the hypothalamus regulates feeding behaviour in rodents [3]. More recently, attention has switched to neuropeptide Y (NPY), the synthesis and release of which is inhibited by leptin [4].

NPY is a 36-amino peptide with a C-terminal amide and is known to be an important regulator in

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Abbreviations: SSCP, Single strand conformation polymorphism; LOD, maximum logarithm of odds; RH, radiation hybridization; IBD, identity by descent.

the central and peripheral nervous systems [5]. Moreover, NPY is a powerful stimulant of food intake. When injected into the central nervous system of rats, NPY results in an obesity syndrome closely resembling the phenotype of either leptin deficient *ob/ob* mice or leptin resistant *db/db* mice [6–8]. In these animals, genetic alterations of the satiety effect of leptin within the hypothalamus result in an overexpression of NPY leading to a complex syndrome including hyperphagia, increased fat storage and obesity. NPY displays a remarkable degree of structural conservation during evolution such that NPY sequences of human, rabbit, rat and mouse are identical and only differ from the porcine sequence by one amino acid [9]. The human NPY gene spans approximately 8 kilobase pairs (kbp) on chromosome 7p15.1 and is divided into four exons, the first one only containing non-translated DNA [10, 11]. Investigation on the World Wide Web and Genetic Location Database (GLD, 27) revealed a location of the NPY gene close to the D7S665 marker.

Binding studies with peptide fragments of NPY have found five rNPY subtypes, belonging to the family of the G protein-coupled transmembrane proteins [12]: Y1 [13], Y2 [14], Y3 [15], Y4 [16] and Y5[17]. The rNPY-Y1 is expressed in many tissues including the arcurate nucleus in the hypothalamus and administrating a selective agonist [Leu ^{31}, Pro ^{34}] of NPY increases appetite and the secretion of luteinizing hormone [18, 19]. Intracerebroventricular injection of a selective antagonist for rNPY-Y1 (1229U91) significantly suppresses physiological feeding behaviour after overnight fasting [20]. The rNPY-Y1 profile closely resembles that reported for the recently cloned Y5 receptor, which also presents a pattern of expression in the brain. A weak Y5-selective agonist [D-Tyr^{34}]NPY produced a statistically significant increase in food intake when injected in rats [17]. These observations led to the hypothesis that, in murine models, rNPY-Y1 and -Y5 may account for a major control of feeding behaviour. The deduced aminoacid sequence of rNPY-Y5 displays a very low level of transmembrane domain identity with other rNPY-Y types. Intriguingly, human Y1 and Y5 receptor genes map to the same *locus* in an opposite orientation [17] but the structure of the rNPY-Y5 is not known. The rNPY-Y1 gene contains three exons and is localized on chromosome 4q(31.3–32) [13].

Until now, despite strong evidence for a role of the NPY pathway in the brain's regulation of feeding behaviour, no genetic study has been conducted on this candidate gene. This prompted investigation of the potential implications of NPY, rNPY-Y1 and rNPY-Y5 in the pathogenesis of human obesity. Here we report the results of familial linkage analyses of these loci as well as SSCP of NPY and rNPY-Y1 in French Caucasian families with morbid obesity.

Subjects, materials and methods

Patients and families. Through multimedia recruitment in France or through the department of Nutrition, Hotel-Dieu Hospital in Paris, DNA and clinical data from 93 families were collected. Probands were ascertained on the basis of BMI 40 kg/m^2 or more and families were included in the study if probands had a least one sibling with a BMI of 27 kg/m^2 or more. Additional family members were ascertained and genotyped when possible. Both parents were available for genotyping in 23% of the families, and one parent was available in 34% of the families and 10% of subjects have diabetes. We selected 278 sibling pairs for the linkage analysis. For SSCP scanning, 50 unrelated patients (BMI ≥ 27 kg/m^2) with at least one consecutive generation with an obese subject available in the family were ascertained. Two unrelated subjects randomly selected with no family history of diabetes and obesity were used as controls.

DNA was prepared by standard procedures [21] from whole-blood samples.

Mapping rNP-Y1/-Y5 genes. A Genethon radiation hybrid (RH) panel was used to map the rNPY-Y1 gene. The presence of rNPY-Y1 in cell lines was determined by polymerase chain reaction (PCR) using the following primers designed for the amplification of the third exon of the gene: 5′ TTT GCT ACT TCA AGG TAA GAA AAC 3′ and 5′ GTC CGC CCT TTT AAA ATC AAA CAC 3′. The PCR reaction was performed as previously described [23]. After reaction, 4μl of the PCR was checked on gels containing 1% Seakem and 3% NuSieve agarose in TBE (89 mmol/l TRIS, 89 mmol/l Borate, 2 mmol/l EDTA, pH 7) buffer stained with ethidium bromide. Gels were scored for the presence or absence of amplified fragments. Results were submitted for calculations using the Radiation Hybridation MAP package [24] allowing the determination of LOD scores for rNPY-Y1 PCR product compared to markers from the Genethon linkage map.

For the localization of rNPY-Y5, radiation hybridation mapping data was presented by Rumberger et al. [25].

Typing genetic markers. Markers close to the *locus* of interest were amplified using the published sequences described in Table 1 as primers. The forward primer of each pair was labelled with one of the three fluorescent dyes, TET, FAM or HEX (Applied Biosystems, Foster City, Calif., USA). DNA amplification was performed using the PTC100 thermocycler (MI Research, San Francisco, Calif., USA). The final volume was 25 μl using 100 ng of DNA, 100 ng of each primer and 1 U of AmpliTaq DNA Polymerase (Perkin Elmer, Branchburg, N.J., USA). Amplified fragments were subjected to electrophoresis on a ABI 377 automatic sequencer, using a 4% Long Range denaturing gel (FMC Bioproducts, Rockland, Calif., USA) and analysed using Applied Biosystem's GeneScan 2.0.

Scanning for mutation (PCR-SSCP). For all exons of NPY and rNPY-Y1 genes, primer pairs and PCR conditions were available from authors.

DNA amplification was performed using the PTC100 thermocycler (MI Research). The final volume was 25 μl using 100 ng of DNA, 100 ng of each primer and 1 U of AmpliTaq DNA Polymerase (Perkin Elmer). *Reaction* products were checked on 2% agarose gels (SeaKem CTG; FMC Bioproducts, Rockland, ME, USA) stained with ethidium bromide (Sigma, St. Louis, Mo., USA).

For analysis of NPY and exon 1 of rNPY-Y1 genes, SSCP was performed using the non-isotopic Pharmacia Phast System as described previously [22]. The denatured PCR products

Table 1. Primer pairs and PCR conditions used for amplification of selected markers

Markers	Primer pairs	PCR conditions
D4S393	5' GAA TCC CCA AGC ACA ATG TC 3' 5' TGG CTG TTT TTG GAT GCT AC 3'	1.5 mmol/l MgCl 5% DMSO 50 °C
D4S413	5' TCT GAA TAT AGT GCT CCA GAA A 3' 5' CAA TCA GTG GGT TTT TGA A 3'	1.5 mmol/l $MgCl_2$ 5% DMSO 50 °C
D7S665	5' CCT GCA CTA ACA TTA ACG ATT T 3' 5' GAA GAG TGA GTG AGG TGT ATA TGA G 3'	1.5 mmol/l $MgCl_2$ 55 °C
D7S2506	5' CAG CAG GGC TTG AAA TGA AC 3' 5' ACA CAG TGG AGC TGG CAT AG 3'	1.5 mmol/l $MgCl_2$ 5% formamide 58 °C

DMSO, Dimethyl sulphoxide

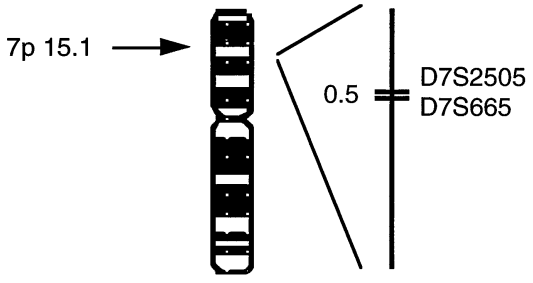

Fig. 1. Chromosome 7p markers and centimorgan spacing in the NPY region used in linkage test

were separated on precast polyacrylamide gels (PhastGel homogeneous 20%) with PhastGel native buffer strips. Preruns were performed at 400 V, 5 mA, 1 W for 10 Vh. Runs were performed for 150 Vh at specific exon temperatures: NPY- exon 1 and exon 2 at 12 °C; NPY- exon 3, exon 4 at 10 °C and rNPY-Y1- exon 1 at 14 °C.

Because this method enables reproductive detection of mutation in PCR fragments that do not exceed 150 bp, SSCP analysis of exon 2 and exon 3 of rNPY-Y1 gene was performed as follows: samples were re-amplified using nested primers (sequences are available from authors) and dUTP-TAMRA (Applied Biosystems). Reaction conditions were 95 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s followed by a final extension step of 72 °C for 6 min. Labelled PCR products were then purified using P60 columns (BioRad, Richmond, Calif., USA). After precipitation and drying, samples were resuspended in formamide loading buffer and subjected to electrophoresis on a automated ABI 377 sequencer (Applied Biosystems), using 6% MDE gel (FMC Bioproducts) and 0.3% glycerol at 30 °C, for 10 h. Analysis was done using Applied Biosystem's Genescan 2.0.

Sequence analysis of SSCP variants. When SSCP revealed an abnormal pattern of migration compared with negative controls, a non-labelled PCR was performed of the corresponding fragment. After purification using P60 columns (BioRad), fragments were sequenced following the ABI protocol for Taq-Dye Terminator and Taq-Dye Primer cycle sequencing on an automated ABI 377 sequencer. Sequencing was always performed on both strands.

Data analysis. The method of Haseman and Elston [26] was used for estimation of the proportion of alleles that shared identity by descent (IBD). For the analysis, qualitative (BMI ≥ 30 kg/m^2 and BMI ≥ 35 kg/m^2) and quantitative traits adjusted for age, sex and diabetic status; Z score (reflecting variation between BMI of the patient and the mean BMI of the French population weighed by age and sex; its formula being BMI–BMI mean/variation [BMI mean]) were tested. Among affected and discordant siblings, a significant excess of shared alleles (greater than 0.5) in affected sibling-pairs indicates evidence of linkage whereas the expected value under the null hypothesis was equal to 0.5. A significant decrease in alleles-sharing IBD in discordant sibling-pairs strengthens the evidence of linkage. Moreover, differences in quantitative traits between descendants of the same family have been compared to the IBD status. In this case, it is expected that differences in BMI between two siblings with identical genotype are less than differences in BMI observed between two siblings with different genotypes.

Results

Mapping genes. According to the NPY physical map, we selected two markers close to NPY on the basis of their high heterozygocity: DS2505 and D7S665. In our families, selected markers are ordered with the VITESSE program [28] as shown in Figure 1. For rNPY-Y1, the precise localization of the gene was performed by RH mapping. Linkage analysis co-localized rNPY-Y1 STS and microsatellite markers D4S1588 and D4S413 with a LOD score of 11.29 and 11.92, respectively. The estimated distance between D4S1588 and rNPY-Y1 was 0.31 cR3000 and between D4S413 and rNPY-Y1 was 0.39. Assuming an average of 200 kbp per cR3000, the physical distance between D4S1588 and rNPY-Y1 is estimated to be 62 kbp and is estimated to be 78 kbp between D4S413 and rNPY-Y1. In the same way, precise localization of an STS from rNPY-Y5 has also been done by RH mapping. rNPY-Y5 has been mapped at the same *locus* [17], with an estimated distance less than 2 Mb between rNPY-Y5 and the marker D4S393. In our families, three microsatellite markers (D4S413; D4S1588 and D4S393) flanking the rNPY-Y1/Y5 region were ordered with the LINKAGE program [28] as shown in Figure 2.

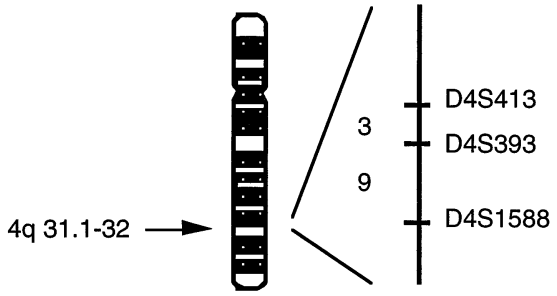

Fig. 2. Chromosome 4q markers and centimorgan spacing in the rNPY-Y1/-Y5 region used in linkage test

Sibling-pair analysis. Selected markers flanking NPY and rNPY-Y1/-Y5 genes were genotyped in our 93 French obese families. Two different thresholds for obesity were evaluated for linkage analyses: BMI $\geq$ 27 kg/m^2 (obese phenotype) and BMI $\geq$ 35 kg/m^2 (extreme obesity phenotype). Table 2 summarizes the linkage analysis results. No significant evidence for linkage was obtained for any thresholds for obesity. For quantitative traits tested, a *T* value testing the regression to the mean IBD probability and the corresponding *p* value have been calculated. Results for the BMI adjusted for age, sex and diabetic status and for the Z score are presented in Table 3. As no significant *p* value was obtained, our results demonstrated no significant evidence for linkage in quantitative traits with any tested markers.

Scanning for mutation by SSCP. SSCP was performed on all exons of the NPY and rNPY-Y1 genes. Alterations of the SSCP-band pattern compared to the negative controls have been found in NPY exon 2 for two probands and in nested fragment 1 of rNPY-Y1 exon 2 for 3 probands. Direct sequencing on both strands of the DNA fragment corresponding to these abnormal patterns revealed no sequence abnormality (data not shown), suggesting false positive results.

Discussion

This study has shown no evidence for a direct implication of NPY and rNPY-Y1/Y5 in human obesity in a French Caucasian population. Indeed, in our families no linkage was found between morbid obesity and highly polymorphic markers in the vicinity of NPY and rNPY-Y1/-Y5 regions, leading to the conclusion that these *loci* do not play a major role in human obesity. Moreover, mutation screening for NPY and rNPY-Y1 shows no mutation in the coding region of these two genes. Because SSCP is very sensitive for variant detection, the lack of mutation confirms the close sequence conservation of NPY and rNPY-Y1 genes among species. However, it remains possible that SSCP scanning failed to detect variants in a few obese subjects. In addition, we could not exclude that mutations in the rNPY-Y5 coding region were present in some obese families. The screening for mutations by SSCP for rNPY-Y5 will be done as soon as the genomic sequence is available. However, according to our familial linkage analysis results, we could not ascertain a possible minor effect of these undiagnosed mutations on obesity. In contrast to these negative data, a leptin gene effect on extreme obesity (BMI $\geq$ 35 kg/m^2) has been recently described [29] in the same family structure and affected sibling pairs, suggesting that the leptin pathway is involved in the development of human morbid obesity. Our final

Table 2. Proportion of alleles sharing identity by descent (IBD) for NPY and for rNPY-Y1/Y5 regions for concordant obese-obese sibling pairs

Locus	BMI $\geq$ 27 kg/m^2			BMI $\geq$ 35 kg/m^2		
	Mean IBD	Standard deviation	*P*-value	Mean IBD	Standard deviation	*P*-value
D7S665	0.4700	0.2867	NS	0.4946	0.2092	NS
D7S2506	0.5130	–1.2901	NS	0.4960	0.3631	NS
D4S1588	0.4895	0.3364	NS	0.4655	0.2834	NS
D4S393	0.4931	0.3123	NS	0.5530	0.2627	NS
D4S413	0.5009	0.3057	NS	0.55195	0.3154	NS

Table 3. *T* and *p* values in quantitative traits for NPY and rNPY-Y1/Y5 regions

Locus	Z score			BMI		
	Nbr pairs	*T* values	*p* values	Nbr pairs	*T* values	*p* value
D7S665	300	1.1297	NS	225	1.6621	NS
D7S2506	234	0.4878	NS	291	0.2277	NS
D4S1588	222	0.2095	NS	215	–1.5114	NS
D4S393	194	1.3926	NS	187	1.6941	NS
D4S413	216	1.6210	NS	210	0.9385	NS

Nbr, Number of pairs

data suggest that the current analysis is powerful enough to detect linkage and that the leptin gene effect is not mediated by NPY and NPY-Y1/Y5. This result is in keeping with the published work on NPY knock-out mice [30]: in this model, animals are lean and only decreased their food intake and lost weight when treated with recombinant leptin, suggesting that compensatory mechanisms can substitute for NPY in the leptin pathway. Our data are also consistent with results presented by Sipols [31] in baboons. NPY central administration was reported not to increase feeding behaviour of treated animals, suggesting that NPY is not effective in primates. In the course of identification of genes implicated in the development of human obesity, genome-wide searches could be successful for identifying multiple predisposing loci with unknown functions, as has been described in diabetes [32]. It is also likely that other neuropeptides showing regulatory effects via specific hypothalamic receptors on feeding behaviour (melanocyte-stimulating hormone, cholecystokinine, galanin, GPL-1 etc) and their receptors remain candidate genes for human obesity.

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