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Human neuropeptide Y signal peptide gain-of-function polymorphism is associated with increased body mass index: possible mode of function

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Abstract

Neuropeptide Y (NPY) has been implicated in the control of food intake and energy balance based on many observations in animals. We have studied single nucleotide polymorphisms (SNPs) within the regulatory and coding sequences of the human *NPY* gene. One variant (1128 T>C), which causes an amino acid change from leucine to proline at codon 7 in the signal peptide of NPY, was associated with increased body mass index (BMI) in two separate Swedish populations of normal and overweight individuals. In vitro transcription and translation studies indicated the unlikelihood that this signal peptide variation affects the site of cleavage and targeting or uptake of NPY into the endoplasmic reticulum (ER). However, the mutant, and to a lesser extent the wild-type, signal peptide by themselves markedly potentiated NPY-induced food intake, as well as hypothalamic NPY receptor signaling. Our findings in humans strongly indicate that the NPY signaling system is implicated in body weight regulation and suggest a new and unexpected functional role of a signal peptide.
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Keywords: Neuropeptide Y; Polymorphism; Association; Obesity; Function

1. Introduction

Obesity is a complex disorder of appetite regulation and energy metabolism controlled by specific biological factors. In clinical practice, body fatness is assessed by body mass index (BMI), which is defined as weight in kilograms divided by square of the height in meters. A World Health Organization (WHO) expert committee has proposed the classification of overweight and obesity and defines obesity as a BMI above 30 kg/m^2, while a BMI of 25–30 is referred to as overweight. Many studies have indicated that obesity is a highly heritable trait, with genetic variation estimated to account for 40–70% of the interindividual variation in body mass [1]. In Sweden, although the prevalence of obesity is lower than in the United States, it is increasing at an alarming rate, especially in children [2].

Neuropeptide Y (NPY) is an abundant and well-characterized 36-amino acid neuromodulator that is secreted by neurons in the central and peripheral nervous system. The *NPY* gene is located on chromosome 7q15.1 and is

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about 8 kilobases (kb) in length with four exons separated by three introns of approximately 965, 4300, and 2300 bp [3,4]. The gene produces a precursor protein that includes a signal peptide, mature NPY, and a carboxyl-terminal flanking peptide with no known function [5]. Since its discovery in 1982, NPY has been shown to play a critical role in the regulation of satiety, reproduction, the central endocrine and cardiovascular systems, and many other physiological processes, such as potent stimulation of food intake and associated weight gain in animal models [6]. In 1998, Karvonen et al. first reported the identification of a common single nucleotide polymorphism (SNP), i.e., 1128 T>C, which causes an amino acid change from Leucine to Proline (Leu7Pro), in the signal peptide of NPY, and presence of this Pro7 allele was associated with high serum cholesterol and LDL cholesterol levels [7]. Since the publication of this paper, a vast majority of studies linking this polymorphism to diseases have been followed. This Leu7Pro polymorphism was further found to be associated with enhanced carotid atherosclerosis in elderly patients with type 2 diabetes [8], retinopathy in type 2 diabetes [9], birth weight and serum triglyceride concentration in pre-school aged children [10], alcohol consumption and alcoholism [11,12]. A two base pair TG insertion/deletion (I/D) variant at the position −880 in the promoter region was found to be associated with body mass and fat patterning in nonobese Mexican Americans [13]. In an indirect study on functional consequences of this polymorphism by comparing plasma NPY concentration between Leu/Pro genotype subjects and Leu/Leu genotype controls after physical exercise, the authors found that subjects with Leu/Pro genotype had 42% higher maximal increases in the plasma concentration than did subjects with Leu/Leu genotype [14]. These findings suggest that alterations of NPY could affect energy metabolism and body fat accumulation. We here initially asked the questions (1) to what extent the *NPY* gene is polymorphic in the Swedish population, and 2) whether such polymorphism might contribute to phenotypic parameters related to overweight or obesity.

2. Materials and methods

2.1. Subjects

Population 1 (n=572) was recruited from the greater Stockholm area using a registry containing all permanent residents (of Swedish origin) in Stockholm County. They are all 50-year-old men without a history of cardiovascular disease, severely impaired renal function, arteritis, collagenosis, diabetes mellitus, a history of alcohol abuse, or other forms of addiction. Population 2 (n=674) consisted of adult women who were either healthy control subjects (n=398), without a medical history and with varying BMI (BMI<30 kg/m^2), or referrals of the hospital's unit for treatment of uncomplicated obesity (n=278, BMI≥30 kg/m^2). The subjects were recruited by local advertisement from the hospital's obesity clinic. All subjects were healthy except for obesity, and none was on regular medication. All individuals were from communities nearby Huddinge University Hospital, Sweden. This study was approved by the Karolinska Institutet Ethics Committee.

2.2. Mutation screening

The genomic sequences containing the *NPY* gene (GenBank accession no. BC029497) were identified with the *NPY* mRNA sequence (GenBank accession no. AC004485) by performing a BLAST homology search on the human genome sequence. PCR products corresponding to part of promoter region and the complete known human *NPY* coding sequence plus neighboring intronic sequences were amplified from 30 randomly selected Swedish samples. PCR and sequencing primers are listed in Table 1.

2.3. SNP genotyping

Genotyping of SNPs was performed using Dynamic Allele Specific Hybridization (DASH) [15]. The sequences of the forward and reverse primers are 5′-biotin-GCGA-GAGTCAGTCCAGACAGCC-3′ and 5′-GCAGATGCTA-GGTAACAAGTGA-3′, respectively. The probe used for DASH is 5′-CAAGTGAC*T*GGGGCTGT-3′ or 5′- CAAG-TGAC*C*GGGGCTGT-3′. The SNPs may be found in the

Table 1
PCR and sequencing primers used for *NPY* SNP identification in 30 Swedish individuals

Fragment	Primer sequence (5′→3′)	Product size (bp)
Promotor and exon 1	F[a]-CAGGAGCATTCATTCACGG	1312
	R[b]-GAGTGGAGCGCGATCATC	
	FN[c]-TCTTGCATATTCATTCAACAGG	
	FN[c]-CAGAACCCACATTCTCAACG	
	FN[c]-AGTTGCCTCACTCCAACAGC	
	RN[d]-CGTCTCTAGCAAGTTCGC	
	RN[d]-GCCGCACGAGTGTCGACTTA	
	RN[d]-CTTAGAACAAGTCGGAGGCA	
Exon 2 and flanking sequence	F[a]-CCACTCCTGGGTTCTCTCTG	508
	R[b]-GAGTGTGCCCTGTACAAACATT	
	FN[c]-CTGGGACGAGAGCGGATT	
	RN[d]-TACTGTCCTGCCCTGGGATA	
Exon 3 and flanking sequence	F[a]-ATGCTTCATACACCTAGCTTGC	380
	R[b]-TCCTCTGCCTGCTTCTTCA	
	FN[c]-AGTTTTCATATCCCAAATAGGAGAC	
	RN[d]-CTCTGACTTCCCTCCCCTG	
Exon 4 and 3′UTR[e]	F[a]-CTTTTCAACAGTTCCCGGTC	457
	R[b]-TCTTTCATAGCCGCTGTTTT	
	FN[c]-CCCTTGCTCATACCTCAGGA	
	RN[d]-GAAACGAACCCTGAATCTGC	

[a] Forward primer.
[b] Reverse primer.
[c] Forward nesting sequencing primer.
[d] Reverse nesting sequencing primer.
[e] 3′ untranslated region.

HGVbase database (http://hgvbase.cgb.ki.se/) under the respective IDs shown in Fig. 1.

2.4. In vitro translocation

*Xba*I and *Nde*I restriction sites were introduced by PCR at the 5′ and 3′ ends of the human NPY cDNA. Site-directed mutagenesis was performed to mutate one single nucleotide (T>C) at the second position in codon 7 by PCR amplification. Two mutants with signal peptide sequence and entire NPY cDNA were produced, respectively. The *Xba*I and *Nde*I restricted PCR fragments were cloned into a pGEM1-derived vector containing the P2 domain (codon 81–323) of *Escherichia coli* protein leader peptidase (Lep) preceded by an *Nde*I site. The constructs in pGEM1 were transcribed by SP6 RNA polymerase for 1 h at 37 °C in a transcription mixture composing of 1–5 μg DNA template, 5 μL 10×SP6 H-buffer (400 mM HEPES/KOH, pH 7.4, 60 mM magnesium acetate, 20 mM spermidine hydrochloride), 5 μL BSA (1 mg/mL), 5 μL m7G(5′)ppp(5′)G (10 mM), 5 μL dithiothreitol (50 mM), 5 μL gNTP mix (10 mM ATP, 10 mM CTP, 10 mM UTP, 5 mM GTP), 18.5 μL water, 1.5 μL RNase inhibitor (50 units), and 0.5 μL SP6 RNA polymerase (20 units). Translation was performed in reticulocyte lysate in the presence of dog pancreas microsomes. The translation products were analyzed by SDS/PAGE.

2.5. Cloning

The wild-type signal peptide (SP), SP-NPY, and SP-NPY-Cpon were cloned into the EGFP-N3 vector (Clontech) by conventional techniques at the *Eco*RI and *Bam*HI sites. Mutants were generated using the mutagenic oligonucleotides: mutant nucleotides are indicated in parentheses. Sense, 5′-GGAATTCACCATGCTAGGTAACAAGCGCC(C)GGGGCTGTCCGGA-3′ and antisense, 5′-CGGGATCCCGCCTCGGCCAGCGCACC-3′ for mut-SP; sense, 5′-GGAATTCACCATGCTAGGTAACAAGCGCC(C)GGGGCTGTCCGGA-3′ and antisense, 5′-CGGGATCCATATCTCTGCCTGGTGAT-3′ for mut-SP-NPY; sense, 5′-GGAATTCACCATGCTAGGTAACAAGCGCC(C)GGGGCTGTCCGGA-3′ and antisense 5′-CGGGATCCCCACATTGCAGGGTCTTC-3′ for mut-SP-NPY-Cpon. All wild-type and mutants generated were completely sequenced in both directions to confirm the presence of the target sequence and to rule out any additional undesired changes. PC12 cells were cultured in DMEM with 10% fetal calf serum (FCS), 5% horse serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. To analyze the subcellular distribution of the constructs, PC12 cells were transfected using Lipefectamine 2000 Reagent (GibcoBRL), and 24 h later, cells were fixed with 3.7% paraformaldehyde and mounted. GFP fluorescence was visualised in a Zeiss LSM510 confocal microscope.

2.6. In vivo animal injection

Male Sprague–Dawley rats ranging from 280 to 320 g were stereotaxically implanted with a guide cannula at the following coordinates taken from bregma according to the atlas of Franklin and Paxinons: AP, 1.0 mm; ML, 1.3 mm; and DV, 4.0 mm. The rats were individually housed in cages for a week's recovery after surgery. Seven groups with various number of rats were divided for saline and NPY and/or plus two types of signal peptides. When monitoring food intake, rats were individually housed in polypropylene cages with metal grid floors. Animals had free access to a standard rat diet and tap water at any time, and rats were housed at a temperature of 21±1 °C and 60% of humidity. Animals in good condition had 3 to 4 days' washout and were then

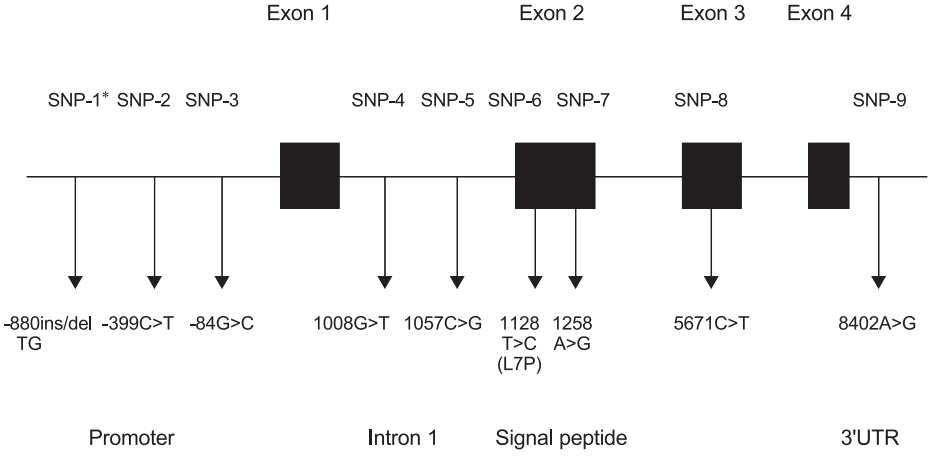

Fig. 1. Schematic illustration of the human *NPY* gene. The 9.6 kb of the NPY locus is illustrated. One ~1.3-kb and three ~500-bp regions were amplified by PCR in 30 unrelated Swedes. These regions were sequenced and found to contain eight SNPs. The SNP positions are based on the nucleotide sequence of the human *NPY* identified by Minth et al. [3] and the GenBank sequence (accession no.AC004485). 3′-UTR, 3′-untranslated region; *SNP-1 (-880 ins/DelTG), two base pair TG insertion (I) or deletion (D). Data are obtained from Ref. [13]. The corresponding HGVbase database IDs for the SNPs found are as follows: SNP-2, ID SNP001745758; SNP-3, ID SNP001745759; SNP-4, ID SNP001026460; SNP-5, ID SNP001026461; SNP-6, ID SNP000003321; SNP-7, ID SNP000008952; SNP-8, ID SNP000008045; SNP-9, ID SNP000008942.

divided into groups at random for the feeding experiments. Rats had intracerebroventricular (i.c.v.) brain injections with saline, 9 μg of mammalian neuropeptide Y, 2.5 and 7.5 μg of mutant and wild-type signal peptides alone and/or coinjected with wild-type or mutant signal peptides. Food intake was monitored after i.c.v. injection. Food spillage at the bottom of cage and food left in the cage were collected and weighed after 30 min, 1 h, 2 h, and 4 h.

2.7. Radioligand binding experiments

Rats were decapitated, and the hypothalamus was dissected out from the brain and homogenized in cold binding buffer. The homogenate was centrifuged at 1000×*g* for 10 min at 4 °C. The precipitated nucleic fraction was discarded, and the supernatant was centrifuged at 50,000×*g* for 40 min at 4 °C. The membrane pellet was resuspended in cold binding buffer and frozen at a protein concentration of 1–4 mg/mL. SK-N-MC cells were grown adherent and maintained in minimum essential medium (MEM) with Earle's salts, supplemented with nonessential amino acids, containing 10% fetal calf serum, penicillin (50 U/mL), streptomycin (50 μg/mL), L-glutamine (2 mM) at 37 °C in 5% CO_2/95% air. Cells were collected with a cell scraper. Harvested cells were washed twice with ice-cold PBS and once with ice-cold Tris–HCl buffer, 50 mM, pH 7.4. The cells were then resuspended in cold binding buffer and homogenized on ice with an Ultra-Turrax, 3×10 s at full speed, and thereafter treated as above. Competition experiments were performed in a final volume of 300 μL with 5 μg of protein, 250 pM [^{125}I]-PYY 2200 Ci/mmol (DuPont NEN, Stockholm, Sweden), and 12 increasing concentrations (0.3 pM-1 μM) of NPY were used with and without 3 nM wild-type or mutant signal peptide. Three different concentrations (3, 30, and 300 nM) of the signal peptides were used without NPY. The peptides were synthesized and HPLC-purified (>97%) by Peptide Specialty Laboratories (Heidelberg, Germany). Nonspecific binding was determined in the presence of 1 μM NPY. After 120 min incubation at room temperature, samples were filtered through Whatman GF/C filters (Filtermat A, Wallac, Gaithersburg, MD, USA), which had been presoaked in 0.3% polyethyleneimine using a TOMTEC (Orange, CT, USA) cell harvester. The filters were washed with 6 mL of 50 mM Tris (pH 7.4) at 4 °C and dried in a microwave oven. Radioactivity retained on the filter was determined in a Wallac 1450 Microbeta counter using Meltilex A (Wallac) melt-on scintillator sheets. Protein concentration was determined by the method of Bradford, with bovine serum albumin used as standard.

2.8. Binding of [^{35}S]GTPγS

Membranes used in [^{35}S]GTPγS binding experiments were obtained in the same way as in the radioligand experiments. To investigate if NPY could induce [^{35}S]GTPγS binding, we incubated the membrane with increasing concentrations of the nucleotide GDP in the presence of one high concentration of NPY (100 nM) to establish the optimal concentration of GDP and to obtain the best ratio of agonist-stimulated to basal [^{35}S]GTPγS binding. To investigate if addition of wild-type or mutant signal peptide could modulate NPY-induced [^{35}S]GTPγS binding, we incubated the membranes with increasing concentrations of NPY (1 nM to 30 μM) in the presence or absence of signal peptide (3 nM). Three different concentrations (3, 30, and 300 nM) of the signal peptides alone were also tested. The binding experiments were performed in 96-well plates at 30 °C. The incubation mixture contained, in a final volume of 300 μL, 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 μM 1–10 phenanthroline, 0.1% bovine serum albumin, 0.03% bacitracin, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.1 nM [^{35}S]GTPγS, and 50 μg membrane protein. The nonspecific binding was measured in the presence of 0.1 mM Gpp(NH)p. Incubations were terminated by rapid filtration of samples through Whatman GF/C filters (Filtermat A, Wallac) which had been presoaked in a buffer with 50 mM Tris–HCl, 1 mM EDTA, 5 mM $MgCl_2$, and 100 mM NaCl followed by one 6 mL wash with the same buffer using a TOMTEC cell harvester and dried in a microwave oven. Radioactivity retained on the filter was determined in the same way as in the binding experiments.

2.9. Statistical analyses

The allelic frequency distribution was tested for Hardy–Weinberg equilibrium by the χ^2 test with one degree of freedom. Associations of polymorphisms with clinical parameters were estimated using analysis of variance (ANOVA). *P* values less than 0.05 were interpreted as statistically significant. Statistical computations were done using the StatView software 5.0 (SAS Institute, Cary, NC, USA). In vivo data were analyzed by one-way ANOVA. All the binding data were analyzed by nonlinear regression analysis, and statistical analyses were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Resequencing and SNP discovery of the NPY gene

Using a direct PCR product sequencing approach, we sequenced the *NPY* gene from 30 randomly selected Swedish healthy subjects. A total of 1649 bp of the 9.6-kb sequence were read in each individual. Eight single-nucleotide polymorphisms (SNPs) were found (Fig. 1). This yielded an average of 1 SNP/183 nucleotides sequenced. The polymorphisms consisted of five transitions (five of eight, 62.5%) and three transversions (three of eight,

Table 2
Allele frequencies for *NPY* Leu7/Pro7 polymorphism in two Swedish populations

Genotype	Population 1+population 2			Population 1			Population 2		
	Nonobese	Obese	Total	Nonobese	Obese	Total	Nonobese	Obese	Total
N	907	339	1246	510	62	572	396	278	674
Leu7/Leu7	839	306	1145	473	54	527	365	253	618
Leu7/Pro7	68	32	100	37	8	45	31	24	55
Pro7/Pro7	0	1	1	0	0	0	0	1	1
Leu7	0.963	0.950	0.959	0.964	0.935	0.961	0.961	0.953	0.958
Pro7	0.037	0.050	0.041	0.036	0.065	0.039	0.039	0.047	0.042

Nonobese: BMI<30 kg/m^2; Obese: BMI≥30 kg/m^2.

37.5%). These SNPs contain two variants in the promoter region (SNP-2 and -3), synonymous base pair changes within exon 2 (SNP-7) and exon 3 (SNP-8), and three single base pair substitutions within intron 1 (SNP-4 and -5), and the 3′ untranslated region (3′-UTR; SNP-9). One non-synonymous SNP (SNP-6, Leu7Pro) was found in the signal peptide part of pre-pro-NPY within exon 2.

3.2. Association analysis

The potentially functional nonsynonymous SNP (Leu7-Pro) was chosen for further screening in the two independent study populations. There was no deviation from the Hardy–Weinberg equilibrium for the Leu7Pro polymorphism in both populations (χ^2=0.96, P=0.33 for population 1, and χ^2=0.04, P=0.85 for population 2) (Table 2).

We first independently analyzed the effect of the Leu7Pro polymorphism on obesity and metabolic parameters in population 1. We found an association of Leu7Pro with BMI (Table 3). No individual with genotype Pro7/Pro7 was found in population 1. Subjects with the Pro7 allele had higher mean BMI values (26.9±2.8 compared with 25.9±3.2, P=0.039, ANOVA) in the entire study sample (n=572). When the data were analyzed separately by obesity status, in all individuals with BMI below 30 kg/m^2 (n=510), subjects with Pro7 also had higher mean BMI values (25.9±2.0 compared with 25.1±2.4, P=0.043, ANOVA), but no significant association was found in individuals with BMI above 30 kg/m^2 (n=62). Plasma triglyceride, cholesterol, VLDL cholesterol, LDL cholesterol, and HDL cholesterol, as well as systolic and diastolic blood pressures, did not differ between the NPY genotype groups in all subjects nor in obese and nonobese subjects analyzed separately. To ensure that our genotyping data were reliable, we performed classical PCR-RFLP analysis for this SNP in population 1. The results showed identical genotypes using the DASH approach.

To further investigate the relationship between the Leu7Pro polymorphism and BMI, a second population (n=674) consisting of adult women with wide range BMI was further genotyped. Among the genotyped subjects, one was homozygote (genotype Pro7/Pro7) with BMI 39.2 kg/m^2. She was included in the Leu7/Pro7 group for the association analysis. A significant association was found in nonobese individuals (n=396, BMI<30 kg/m^2, P=0.020), but no significant association was found in obese individuals (n=278, BMI≥30 kg/m^2, P=0.345; Table 3).

Taking populations 1 and 2 together and partitioning by obesity status, we found that healthy people (BMI<30 kg/m^2) carrying the Pro7 allele show a significantly increased BMI (P<0.01; Table 3) with no gender bias. However, this effect is only observed in nonobese subjects, which suggests that other genetic or environmental factors might be involved and might override the effect in obese subjects.

3.3. In vitro translation

To ascertain whether the Leu7Pro polymorphism in the signal peptide has any direct effects on targeting to and translocation across the endoplasmic reticulum (ER) membrane, we carried out in vitro translation in the absence and presence of dog pancreas rough microsomes of two different constructs: one where the NPY wild-type or Leu7Pro signal peptide (residues Glu-Ala) was fused to a reporter domain (P2) carrying one acceptor site for N-linked glycosylation, and one where the entire NPY (including the signal peptide) was fused to the reporter domain. Both the wild-type and Leu7Pro constructs gave rise to a product of slightly higher

Table 3
Mean values for BMI by obesity status in two Swedish populations

	Population 1+population 2 (N=1246)			Population 1 (N=572)			Population 2 (N=674)		
	Leu7/Leu7	Leu7/Pro7[a]	P	Leu7/Leu7	Leu7/Pro7	P	Leu7/Leu7	Leu7/Pro7[a]	P
Total	27.985±7.183	28.965±6.275	0.185	25.861±3.206	26.882±2.757	0.039	29.795±8.931	30.643±7.688	0.492
Nonobese	24.303±2.842	25.296±2.439	0.005	25.122±2.376	25.935±1.984	0.043	23.227±3.027	24.538±2.735	0.020
Obese	38.078±5.642	36.525±4.802	0.129	32.337±3.027	31.263±0.930	0.149	39.271±5.424	38.214±4.270	0.345

The values are mean±S.D. Nonobese: BMI<30 kg/m^2; Obese: BMI≥30 kg/m^2.
[a] Including one subject carrying Pro7/Pro7 genotype.

molecular weight when translated in the presence of microsomes (Fig. 2A, lanes 1–4). When translation was carried out in the presence of a peptide inhibitor (AP) of the oligosaccharyl transferase enzyme, a new band of lower molecular weight than the product obtained in the absence of microsomes was seen (lanes 5–6). This latter band represents nonglycosylated molecules lacking the signal peptide, while the upper band represents glycosylated molecules lacking the signal peptide. Thus, both the wild-type and mutant signal peptides support efficient targeting to and translocation across the ER membrane.

3.4. ER translocation

In a separate experiment using green fluorescent protein (GFP) tagging of the NPY precursor, there was no difference in the distribution of GFP between wild-type and mutant signal peptide constructs transfected into PC-12 cells (Fig. 2B). GFP fluorescence was as expected detected in the Golgi apparatus and in dots in the cytoplasm, corresponding to vesicles.

3.5. In vivo animal experiment

To test our hypothesis that the NPY signal peptide might exert another effect after being cleaved from the parent prepro-NPY, both wild-type and mutant NPY signal peptides, as well as NPY, were synthesized and initially tested in rat food intake assays. We found that the cointracerebroventricular (i.c.v.) administration of NPY, and mutant signal peptide (tested at two doses, 2.5 and 7.5 μg) markedly elevated overall food intake over a 4-h period (Fig. 3A). In particular, within 30 min after i.c.v. injection of the mutant signal peptide, the action of NPY was sharply elevated. Mutant signal peptide injected alone was also found to elevate food intake, but only in the first 30 min after injection in contrast to the prolonged actions of NPY (Fig. 3B), possibly implying that the signal peptide is more rapidly metabolized than NPY. Overall, in terms of cumulative food intake and combination of NPY and mutant signal peptide, wild-type signal peptide showed only a modest, however significant, potentiating effect on NPY-induced feeding and no effect per se.

3.6. Binding assay

To investigate the mechanism behind the orexigenic effect of the signal peptide observed in the animal experiments, we performed NPY receptor binding experiments using membranes from rat hypothalamus and [I^{125}]PYY as radioligand. Neither the mutant nor the wild-type signal peptide (1 nM–300 nM) modified the affinity of NPY, nor did they directly affect [I^{125}]PYY binding (data not shown). Since the effect of the signal peptide observed in the in vivo experiments may occur via other pathways than those detected in binding experiments or may occur via other receptors than those detected with [I^{125}]PYY, we also examined the effect of the signal peptides on NPY-induced binding of [^{35}S]GTPγS in membranes from rat hypothalamus. Analysis of the dose–response curve for NPY (Fig. 4A) gave an estimated EC_{50} value of 7.6 nM [95% confidence interval (CI)=6.0–9.7]. Interestingly, the addition of 3 nM mutant signal peptide significantly (P=0.019, one-way ANOVA) changed the EC_{50} value to 3.1 nM (95%

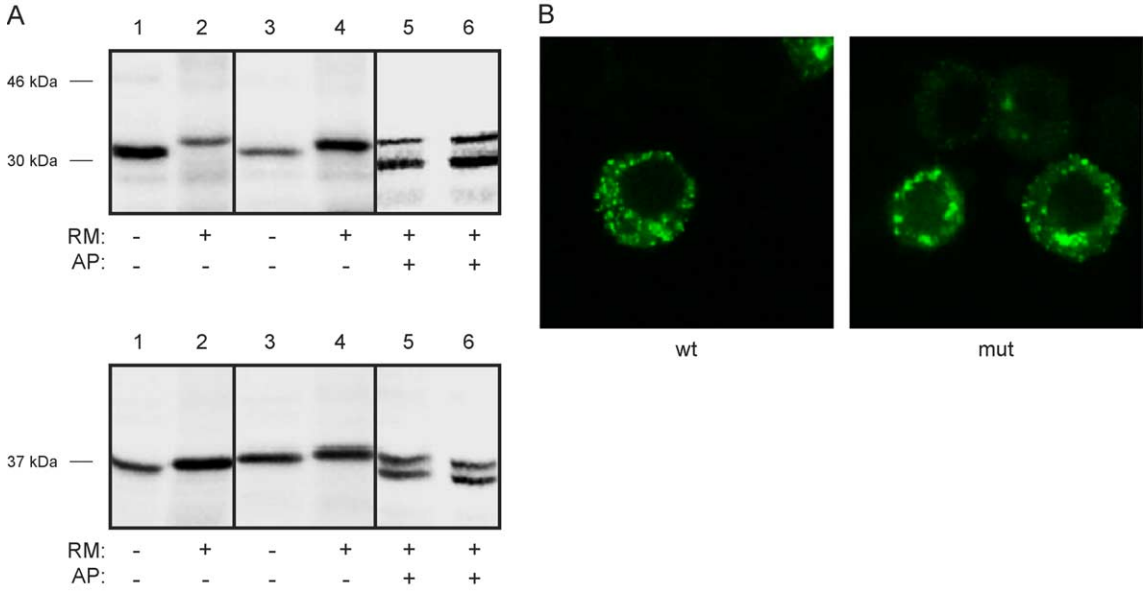

Fig. 2. In vitro function assays. (A) ER translocation assay in the wild-type and mutant NPY. Top panel, lanes 1,2: SP (wt)-P2; lanes 3,4: SP (mut)-P2; lane 5: SP (wt)-P2; lane 6: SP (mut)-P2. Bottom panel, lanes 1,2: NPY (wt)-P2; lanes 3,4: NPY (mut)-P2; lane 5: NPY (wt)-P2; lane 6: NPY (mut)-P2. RM: dog pancreas rough microsomes; AP: acceptor peptide (inhibits glycosylation of the protein). (B) PC-12 cells expressing NPY-GFP (wt) and NPY (Leu7Pro)-GFP(mut). wt, wild-type; mut, mutant.

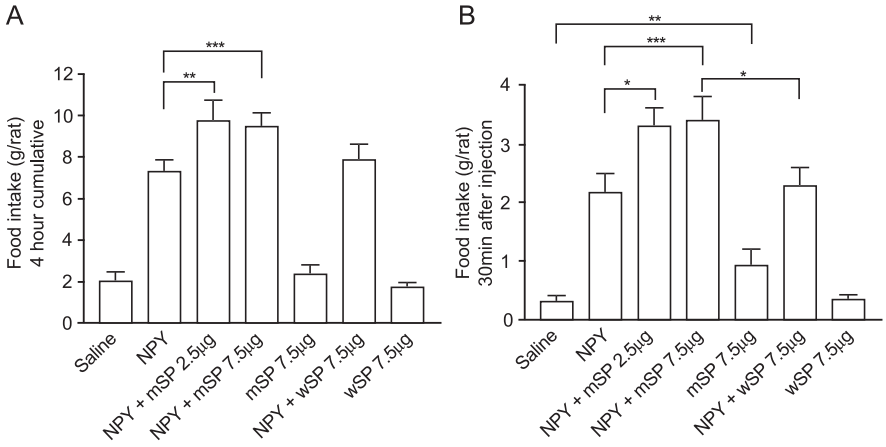

Fig. 3. Effect of NPY, NPY plus wild-type, and mutant signal peptides on food intake in rats. (A) Effect of single coinjection of NPY (9 μg/rat) with and without mutant (2.5 and 7.5 μg/rat) and wild-type signal peptides (7.5 μg/rat) on food intake postinjection of the peptides during 4 h. Data are expressed as mean±S.E.M. Number of rats in each group is indicated. *P<0.05, **P<0.01, and ***P<0.001 vs. the corresponding NPY alone or NPY plus wild-type signal peptide injected. (B) Effect of single coinjection of NPY (9 μg/rat) with and without mutant (2.5 and 7.5 μg/rat) and wild-type signal peptides (7.5 μg/rat) on food intake during the first 30 min period after i.c.v. injection. All others are the same as (A).

CI=1.9–5.0), whereas the addition of the wild-type signal peptide did not change the EC_{50} value (6.2, 95% CI=4.4–8.8; Fig. 4A). Neither the mutant nor the wild-type signal peptide increased the [^{35}S]GTPγS binding by themselves at 3 nM, but at higher concentrations, both the mutant and the wild-type signal peptide significantly increased the [^{35}S]GTPγS binding (Fig. 4B). To investigate whether the mechanism might be universal to all NPY receptors, we performed the same experiment using membranes from SK-N-MC neuroblastoma cells, which express endogenous NPY receptors [6]. Using these membranes, NPY dose-dependently increased [^{35}S]GTPγS binding with an estimated EC_{50} value of 1.3 nM (95% CI=0.6–2.7), but there was no change in the NPY potency with the addition of 3 nM mutant or wild-type signal peptide; the estimated EC_{50} values were 2.1 (95% CI=1.1–4.1) and 2.0 (95% CI=1.2–3.6), respectively. The mutant or wild-type signal peptides given alone to SK-N-MC cell membranes (3, 30, and 300 nM) were also ineffective on [^{35}S]GTPγS binding (data not shown). Thus, hypothalamus appears to differ from neuroblastoma cells with respect to NPY signal peptide action.

4. Discussion

Bray et al. [13] identified a −880 ins/del TG polymorphism that was associated with body fat patterning in nonobese Mexican Americans. To test if this insertion/deletion exists in Swedish populations, we also studied population 1 with respect to this locus. Our data showed that the insertion frequency is 0.75, which is similar to that in Mexican Americans [13]. However, we did not find any

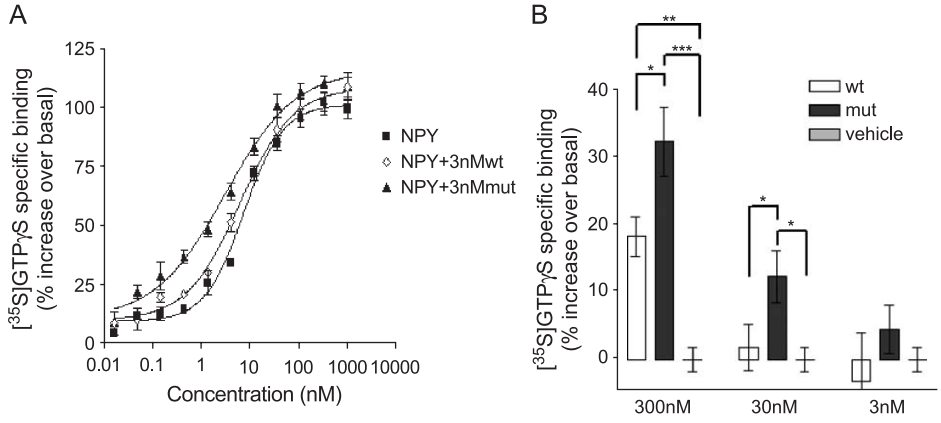

Fig. 4. Binding assays. (A) Dose–response curves for NPY-induced specific [^{35}S]GTPγS binding to homogenates of rat hypothalamus (■), in the presence of 3 nM wild-type signal peptide (◇) and in the presence of 3 nM mutant signal peptide (▲). Results are expressed as percent increase over basal. Means of three experiments with duplicates are shown. EC_{50} values were (mean and 95% confidence interval) NPY alone 7.6 (6.0–9.7), NPY with 3 nM wild-type signal peptide 6.2 (4.4–8.8), and NPY with 3 nM mutant signal peptide 3.1 (1.9–5.0). Ten micromolar GDP was present in all these experiments. (B) Signal peptide induced specific [^{35}S]GTPγS binding to homogenates of rat hypothalamus. Results are expressed as percent increase over basal. Means of three experiments with duplicates are shown (10 μM GDP was present in all these experiments). Significance test was done by one-way ANOVA with Newman–Keuls multiple comparison test (***P<0.001, **P<0.01, *P<0.05).

association between the -880 ins/del TG and BMI. Interestingly, they did not observe the Leu7Pro polymorphism in the 20 Mexican American sequenced from Starr County, TX [13]. It is most likely that this is due to the population characteristic difference.

Karvonen et al. [7] originally identified the NPY Leu7Pro polymorphism and found this polymorphism was associated with high serum cholesterol and LDL cholesterol levels in Finnish (n=138) and Dutch (n=93) obese and Finnish normal-weight subjects (n=64), but not in normal weight Dutch subjects (n=263). They did not observed the effect on obesity or parameters related to energy metabolism, including body weight, body mass index, waist-to-hip ratio, basal metabolic rate, or respiratory quotient. This is likely due to the small number of subjects which is likely to reduce the power to detect significant associations given the fact of low Pro7 allele frequency (~5%). Given α level 0.05 and the sample size (total 1246) in this study, we would have >95% power to identify the association if a true association exists. Association studies require significant numbers of cases to be adequately powered to study disease genetics. Because the modest effects is likely to be contributed by disease genes, the presence of locus heterogeneity, the tendency to analyze subgroups of the patient population and the characterization of allele frequencies at markers distant and with uncertain linkage disequilibrium from the functional variant. Studies incorporated 100 or fewer cases and a similar number of controls, and it is now thought to be likely that such studies are possible only if done with much larger patient samples [16].

By the end of October 2003, the reported number of genes, markers, and chromosomal regions that have been associated or linked with human obesity phenotypes is above 430 [17], which reflects complex of obesity and indicates many possible genetic factors could attribute to obesity. This may explain the modest effect we observed in this study.

The Leu7Pro polymorphism results in an amino acid change in the signal peptide of NPY from leucine that has a hydrophobic aliphatic side chain amino acid to proline that has a cyclic structure. We therefore assumed that this change will lead a marked structure alteration. Indeed, computer modeling simulation indicated that the introduction of proline at position 7 disrupts the local conformation of the peptide by altering the packaging of the helical bundle and diminishes helix propensity for the sequence (data not shown).

A signal sequence is responsible for targeting secretory proteins and membrane proteins to the protein-conducting channel and subsequent translocation across or insertion into the ER membrane. During transport into the ER lumen, the signal sequence is often cleaved from the precursor protein [18]. Several studies have revealed that signal sequences have multiple functions and that they contain specific information for performing distinct functions in targeting and membrane insertion and even for roles after their cleavage from the parent protein [19–22]. Signal sequences can discriminate between different targeting pathways, mediate translocation of the N- or the C-terminus across the membrane, allow variable membrane translocation such that the protein either remains in the cytosol, is inserted into the membrane, or is translocated into the lumen, or can even have a further function after cleavage from parent protein [18]. However, our functional data suggested that the NPY signal peptide instead might exert another effect after being cleaved from the parent prepro-NPY.

Our ER translocation experiment indicated that the NPY Leu7Pro polymorphism does not affect translocation or intracellular trafficking of the NPY precursor. This is consistent with bioinformatic efforts [23], which indicate that the localization of the polymorphism is unlikely to affect conventional signal peptide function.

We have attempted but failed several times to raise antisera to the NPY signal peptide for possible use in localization and release studies.

Notwithstanding the fact that the polymorphic signal peptide potentiated NPY-induced feeding, many issues remain to be worked out. Further extensive studies demonstrating that the signal peptides indeed are bioavailable, intra- or extracellularly, and that the effects of the signal peptide on feeding indeed are specifically related to NPY mechanisms are warranted.

In conclusion, we have found that a polymorphism in the *NPY* gene that occurs at a 5% allele frequency is associated with an increased BMI in two large Swedish populations consisting of men and women, respectively. Our findings lead strong support to the hypothesis that the NPY system is involved in human body weight control and validate the concept pursued by many that modulation of NPY signaling can provide a new therapeutic principle to suppress appetite and achieve weight reduction. We also demonstrate a previously unknown and nonconventional action of the NPY signal peptide. Finally, our study demonstrates that association studies in human can guide functional studies, even within previously well-characterized biological systems.

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