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

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Received 17 June 2004; accepted 21 October 2004

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/m², 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 preschool 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 oxygen uptake 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/m²), or referrals of the hospital’s unit for treatment of uncomplicated obesity (n=278, BMI≥30 kg/m²). The subjects were recruited by local advertisement from the hospital’s obesities 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-GCGACATTCCATCAGG and 5’-GCAGATGCTAAGTTCGCA-3’, respectively. The probe used for DASH is 5’-CAAGTGACGAGGGCTGTG-3’, and 5’-CAAGTGACGAGGGCTGTG-3’. The SNPs may be found in the

Table 1

<table>
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<tr>
<th>Fragment</th>
<th>Primer sequence (5’→3’)</th>
<th>Product size (bp)</th>
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<td>Promoter and exon 1 primer</td>
<td>F³-CAGGAGCATCAGGCGA</td>
<td>1312</td>
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<tr>
<td></td>
<td>R³-GAGTTGAGCGGAGGAGC</td>
<td></td>
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<tr>
<td></td>
<td>FN³-TCTTCAGACAGACATC</td>
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<tr>
<td></td>
<td>FN³-GAGAACACCATCTCAAAGC</td>
<td></td>
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<td>RN³-GGTAACAAGTGA-CAAGGC</td>
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<td></td>
<td>RN³-CTTAGAACAGTGCGGGC</td>
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<td>508</td>
</tr>
<tr>
<td></td>
<td>R³-GAGTTGAGCCTCTGATCAAAACATT</td>
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<td>FN³-GTCGAGCAGAGGAGGATT</td>
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<td></td>
<td>RN³-TACTGTGTGCCCTGGGATA</td>
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</tr>
<tr>
<td>Exon 3 and flanking sequence</td>
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<td></td>
<td>FN³-AGGTTTCACTTCAAATAGGAC</td>
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<td>RN³-CTTCGACTCTCCCTCCTGGT</td>
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<tr>
<td>Exon 4 and 3’UTR</td>
<td>F³-CTTTCACAGTTCCCGGTC</td>
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<tr>
<td></td>
<td>R³-TCTTTCATACCGCCTGGTTT</td>
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<tr>
<td></td>
<td>FN³-CCCTTGCTCATACCTCAGGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RN³-GAAAACGGACCTCTGACCTG</td>
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</tbody>
</table>

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

XbaI and NdeI 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 XbaI and NdeI 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 NdeI 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 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 EcoRI and BamHI sites. Mutants were generated using the mutagenic oligonucleotides: mutant nucleotides are indicated in parentheses. Sense, 5'-GGAATTCACCATGCTAGGTAAACAAGCGCC(C)GGGGCTGTCCGGA-3' and antisense, 5'-CGGGATCCCATATCTCTGCTTGGTGAT-3' for mut-SP; sense, 5'-GGAATTCACCATGCTAGGTAAACAAGCGCC(C)GGGGCTGTCCGGA-3' and antisense, 5'-CGGGATCCCATATCTCTGCTTGGTGAT-3' for mut-SP-NPY; sense, 5'-GGAATTCACCATGCTAGGTAAACAAGCGCC(C)GGGGCTGTCCGGA-3' and antisense 5'-CGGGATCCCATATCTCTGCTTGGTGAT-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 Lipfectamine 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

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**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 co-injected 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 1–4 mg/mL. NPY containing 10% fetal calf serum, penicillin (50 U/mL), Earle’s salts, supplemented with nonessential amino acids, dithiothreitol, 100 mM NaCl, 0.1 nM [35S]GTPγS binding, we incubated the membrane with increasing concentrations of the nucleotide GDP 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 MgCl2, 1 mM 1–10 phenanthroline, 0.1% bovine serum albumin, 0.03% bacitracin, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.1 nM [35S]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 MgCl2, 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 χ2 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,
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 pro-pre-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 ($\chi^2$=0.96, $P=0.33$ for population 1, and $\chi^2$=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 (27.5%) compared with those with the Leu7 allele. The association was only significant in nonobese subjects ($n=396$, BMI<30 kg/m$^2$, $P=0.020$), but no significant association was found in obese individuals ($n=278$, BMI$\geq$30 kg/m$^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 molecular weight.

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**Table 2**

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Population 1</th>
<th>Population 2</th>
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<tbody>
<tr>
<td></td>
<td>Nonobese</td>
<td>Obese</td>
</tr>
<tr>
<td>N</td>
<td>907</td>
<td>339</td>
</tr>
<tr>
<td>Leu7/Leu7</td>
<td>839</td>
<td>306</td>
</tr>
<tr>
<td>Leu7/Pro7</td>
<td>68</td>
<td>32</td>
</tr>
<tr>
<td>Pro7/Pro7</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

| Leu7      | Nonobese     | Obese        | Total      |
|-----------|--------------|--------------|
| 0.963     | 0.950        | 0.959        |
| 0.964     | 0.935        | 0.961        |
| 0.306     | 0.065        | 0.309        |
| 0.037     | 0.050        | 0.041        |

| Pro7      | Nonobese     | Obese        | Total      |
|-----------|--------------|--------------|
| 0.037     | 0.050        | 0.041        |

Nonobese: BMI<30 kg/m$^2$; Obese: BMI$\geq$30 kg/m$^2$.

**Table 3**

Mean values for BMI by obesity status in two Swedish populations

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>Leu7/Leu7</td>
<td>Leu7/Pro7</td>
<td>P</td>
</tr>
<tr>
<td>Total</td>
<td>27.985±7.183</td>
<td>28.965±6.275</td>
<td>0.185</td>
</tr>
<tr>
<td>Nonobese</td>
<td>24.303±2.842</td>
<td>25.296±2.439</td>
<td>0.005</td>
</tr>
<tr>
<td>Obese</td>
<td>38.078±5.662</td>
<td>36.525±4.802</td>
<td>0.129</td>
</tr>
</tbody>
</table>

The values are mean±S.D. Nonobese: BMI<30 kg/m$^2$; Obese: BMI$\geq$30 kg/m$^2$.

* 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 [125I]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 [125I]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 [125I]PYY, we also examined the effect of the signal peptides on NPY-induced binding of [35S]GTPγS in membranes from rat hypothalamus. Analysis of the dose–response curve for NPY (Fig. 4A) gave an estimated EC50 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 EC50 value to 3.1 nM (95%
CI=1.9–5.0), whereas the addition of the wild-type signal peptide did not change the EC50 value (6.2, 95% CI=4.4–8.8; Fig. 4A). Neither the mutant nor the wild-type signal peptide increased the [35S]GTPγS binding by themselves at 3 nM, but at higher concentrations, both the mutant and the wild-type signal peptide significantly increased the [35S]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 [35S]GTPγS binding with an estimated EC50 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 EC50 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 [35S]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. 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).

Fig. 4. Binding assays. (A) Dose–response curves for NPY-induced specific [35S]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. EC50 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 [35S]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<0.0001, ***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.

Acknowledgements

We thank Prof. Dan Larhammar, Department of Medical Pharmacology, Uppsala University, Uppsala, Sweden for providing the human NPY cDNA plasmid. We thank Michael Sundström and Martin Norin at Biovitrum, Stockholm, for support regarding the peptide structure predictions.

References


