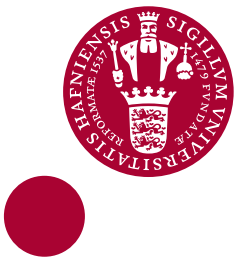

Association of the leucine-7 to proline-7 variation in the signal sequence of neuropeptide Y with major depression

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Objective: There is clear evidence of a genetic component in major depression, and several studies indicate that neuropeptide Y (NPY) could play an important role in the pathophysiology of the disease. A well-known polymorphism encoding the substitution of leucine to proline in the signal peptide sequence of NPY (Leu7Pro variation) was previously found to protect against depression. Our study aimed at replicating this association in a large Danish population with major depression.
Method: Leu7Pro was studied in a sample of depressed patients and ethnically matched controls, as well as psychiatric disease controls with schizophrenia. Possible functional consequences of Leu7Pro were explored *in vitro*.
Results: In contrast to previous studies, Pro7 appeared to be a risk allele for depression, being significantly more frequent in the depression sample (5.5%, $n = 593$; $p = 0.009$; odds ratio, OR: 1.46) as compared to ethnically matched controls (3.8%, $n = 2912$), while schizophrenia patients (4.1%, $n = 503$) did not differ. *In vitro*, the Pro7 substitution appeared to be associated with reduced levels of NPY without affecting its mRNA level.
Conclusion: The Leu7Pro variation may increase the risk of major depression, possibly by affecting the biosynthesis of NPY.

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Keywords: candidate gene approach; case-control association study; NPY Leu7Pro; major depression

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Significant outcomes

- The Leu7Pro variation of NPY was associated with depression, but not schizophrenia, in a large Danish sample.
- The 'Pro7 allele' was associated with reduced levels of NPY in cell cultures.
- The study is consistent with a role for NPY in the aetiology of depression.

Limitations

- Swedish and Dutch studies reported lower frequency of the "Pro7 allele" in depressed patients opposite to the finding in the present Danish sample. However, the former studies used different sampling procedures and control populations.

Introduction

Depression is a complex, severe psychiatric disorder with a lifetime prevalence of at least 10% with clear evidence for a genetic component; thus twin studies indicate heritability of 40–50% (1,2). Both clinical and animal studies suggest that neuropeptide Y (NPY) plays an important role in depression (3). For instance, the synthesis of NPY is decreased in the hippocampus in rodent models of depression (4–6). Conversely, treatment with antidepressant drugs (7–9) and electroconvulsive stimulation increase hippocampal NPY and NPY receptor expression (10–12) as well as release of NPY (11). In addition, intracerebral administration of NPY has antidepressant-like activity in rodents (13,14) and depressed patients have decreased concentrations of NPY in the cerebrospinal fluid (CSF) (15,16), suggesting a possible link between shortage of NPY and predisposition to depression. NPY also causes anxiolytic-like effects in rodents (17,18). Consistent with this concept, certain haplotypes of the *NPY* gene have been implicated in the regulation of emotions and stress responses in humans (19).

The most thoroughly investigated polymorphism (rs16139) of several in the *NPY* gene (20–23) is located at position 20 T > C from the transcription start (*A* in AUG; in the literature sometimes referred to as 1128 T > C) on chromosome 7p15.1. The 20 T > C polymorphism causes a substitution of amino acid residue number 7 leucine with proline in the signal peptide of NPY (Leu7Pro variation) (20). The Leu7Pro variation has been associated with high serum cholesterol and low-density lipoprotein (LDL) cholesterol levels (20), carotid atherosclerosis (24), retinopathy in type 2 diabetes (25), high alcohol consumption and alcoholism (26,27). In one study with alcoholism, association with depression was examined, but no association was found (26). Later, a small study with only 51 patients suggested that the 'Pro7 allele' protects against depression (15), and this was also suggested in two recent papers (28,29).

The present study aimed at replicating the association between the Leu7Pro variation with major depression in a large sample of Danish patients. To determine whether effects were specific for depression, we also studied the occurrence of Leu7Pro in patients diagnosed with schizophrenia (psychiatric disease controls). Moreover, as the functional consequences of carrying the 'Pro7 allele' are not clear, we also explored the effect on levels of NPY peptide and mRNA *in vitro* in two different cell lines.

Materials and methods

Subjects

Control samples. Two groups of controls were examined: screened controls and population controls. *Screened controls* ($n = 287$) consisted of healthy subjects of whom 51 were employees or students from Rigshospitalet University Hospital, and the rest were control samples collected in earlier studies (30–32). These were all questioned about the occurrence of personal or family history (including grandparent) of depression, anxiety, or other psychiatric disorders, and only persons without such history were included. The mean age of the screened controls was 48 years (range = 19–84 years, SD = 19) for males ($n = 109$) and 48 years (range = 19–85 years, SD = 18) for females ($n = 178$). *Population controls* ($n = 2625$) consisted of 374 physically healthy blood donors collected in the Copenhagen area in 2005; a random sample of 451 unrelated individuals from the adult Danish population (age unknown) collected during the late 1980s in the Copenhagen area; and a large group of 1804 individuals randomly selected from a random sample of individuals aged 30–60 years living in the Copenhagen area participating in a non-pharmacological intervention study for prevention of ischaemic heart disease (33). All population controls were anonymous, thus psychiatric clinical state was unknown. The mean age of the population controls was 48 years (range = 30–66 years, SD = 9) for males ($n = 1254$) and 47 years (range = 19–65 years, SD = 9) for females ($n = 1371$).

Patients with depression. Blood was sampled from patients diagnosed with major depression independently by two trained psychiatrists ($n = 593$; ICD-10 diagnoses F32-33) that were admitted to psychiatric departments at hospitals in the Copenhagen area. The sample consisted of patients collected within the period 2002–2008 ($n = 389$; The Danish Psychiatric Biobank) and 1993–1998 ($n = 204$; described elsewhere (30,31,34)). The mean age of the depressed patients was 50 years (range = 19–89 years, SD = 15) for males ($n = 210$) and 48 years (range = 18–93 years, SD = 19) for females ($n = 383$), respectively. Additional information on comorbid anxiety disorder (F40 and F41 in the ICD-10 diagnostic criteria) was present for a subset of patients ($n = 253$; 95 males and 158 females).

Psychiatric disease controls. Blood samples from patients with schizophrenia were also analysed ($n = 503$; ICD-10 diagnosis F20). They were sampled in the Copenhagen area within the period 2002–2007 (The Danish Psychiatric Biobank; $n = 302$) and in the Aarhus area in 2002–2005 (Opus Project; $n = 201$). Of these, 292 were men (mean age = 37, range = 19–77, SD = 11) and 211 were women (mean age = 36, range = 18–84, SD = 12). The patients were described in more details elsewhere (35).

The subjects of the present study were Danish Caucasians. Data on the origin of the depressed patients were available in 277 cases. The majority of these (90.6%) had both parents born in Denmark. A minor fragment had one grandparent (7.6%) born outside Denmark (mainly Germany or Sweden) while 1.8% had no information on birthplace of grandparents. More details regarding origin were also available for blood donor population controls, with a majority (94%) having both parents born in Denmark (36). A minor fraction had one parent (1.5%) or both parents (0.5%) born outside Denmark in other European countries while there was no information in 4% with regard to place of birth of both parents.

The Scientific-Ethical Committees in Denmark as well as the Danish Data Protection Agency approved the present study, and all participants gave informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Genotyping (rs16139)

Genomic DNA was extracted from peripheral blood lymphocytes using QIAamp® DNA Blood Mini kit or FlexiGene DNA kit (Qiagen, Albertslund, Denmark) according to the manufacturer's instructions. For 528 of the samples, the *NPY* exon 2 was analysed at the denaturing high-performance liquid chromatography (D-HPLC) wave system (Transgenomic, Omaha, NE, USA). Fragments showing mutations were analysed on an ABI PRISM DNA Sequencer (ABI310 or ABI3730) (Applied Biosystems, Foster City, CA, USA). The rest of the samples were analysed by real-time PCR using TaqMan Assay-by-design genotyping assay (Applied Biosystems). Each 96-well plate contained two control samples (one heterozygote (CT) and one homozygote (CC)) as well as one negative control (no template). The primer and probe sequences, and temperatures used at the D-HPLC for each fragment are available on request. The success rate was 99.7%; only subjects with successful samples were included in the study. The genotype for 3.2% of the samples was reanalysed resulting in 100% concordance.

Plasmids, cell cultures and transient DNA transfections

The pGEM-1-NPY plasmid, containing the human *NPY* gene, including signal peptide, NPY and C-terminal peptide of NPY (CPON), was a kind gift from Professor Thue W. Schwartz, University

of Copenhagen. The pGEM-1-NPY plasmid was digested with *Eco*RI and *Bam*HI and the *NPY* coding region was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), generating pcDNA3.1-NPY-Leu7. Mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the instructions from the manufacturer, generating pcDNA3.1-NPY-Pro7. Both constructs (the CMV promoter, *NPY* cDNA and the poly-A sequence) were verified by sequencing.

The Syrian hamster beta-cell line HIT-T15 (LGC Standards AB, Boras, Sweden) and human neuroblastoma SK-N-MC cells (ATCC, Manassas, VA, USA) were cultured at 10% CO_2 and 37 °C in Dulbecco's modified Eagle's medium (with Glutamax, 5 mM glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). One day before transfection, 8×10^5 cells were seeded in 6-well culture dishes. Cells were transfected with 8 µg pcDNA3.1, pcDNA3.1-NPY-Leu7 or pcDNA3.1-NPY-Pro7 using FuGENE 6 (Roche A/S, Hvidovre, Denmark) according to the manufacturer's instructions. Forty-eight hours later, 5 ml culture medium was recovered, and the cells were harvested in PBS/EDTA, pelleted and resuspended in 1-ml sterile H_2O. Transfection series were performed in triplicates and carried out at least three times.

To ensure that transfection efficiency did not vary significantly between cells transfected with pcDNA3.1-NPY-Leu7 and pcDNA3.1-NPY-Pro7, quantitative PCR was performed as follows: transfected HIT-T15 and SK-N-MC cells were harvested as above in PBS/EDTA, and washed once in PBS. Nucleospin Tissue kit (Macherey-Nagel, Düren, Germany) was subsequently used for DNA purification according to the manufacturer's instructions. The amount of purified plasmid DNA encoding NPY was measured in 96-well plates on an Icycler (Bio-Rad, Grenaa, Denmark). Each well contained a mixture of plasmid DNA, SYBR Green I and 750 nM of forward and reverse primers. The plasmid DNA was quantified by two sets of primers recognising the CMV promoter and ampicillin resistance gene, both located on the transfection plasmid. Relative quantification was achieved by comparing each sample to in-plate reference gene controls (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). Primer sequences are available on request.

NPY radioimmunoassay

HIT-T15 and SK-N-MC cells were transfected as described above. The subsequent procedure for radioimmunoassay was previously described (37). Cells and culture media were boiled for 20 min and cell debris was removed by centrifugation. Extracts were analysed by radioimmunoassay using anti-NPY antibody (#6730-0204, Biogenesis, Kidlington, UK) and ^{125}I-labelled NPY (#IM170, GE Healthcare, Hillerød, DK). The NPY antibody recognised primarily the mid-portion of the molecule, with little or no cross-reactivity for N-terminal NPY1-9 or C-terminal NPY 31-36 (Biogenesis).

NPY mRNA determination

Transfected HIT-T15 and SK-N-MC cells (two individual transfection series run in triplicates) were harvested as above in PBS/EDTA, and washed once in PBS. Total RNA was purified using RNeasy spin columns and on-column DNAse treated (Qiagen), to remove plasmid DNA encoding NPY, which would otherwise contribute to the final measurement of NPY mRNA levels. Total RNA (1 µg) was reverse transcribed using TaqMan RT master mix (Applied Biosystems) and random hexamers in a 100-µl reaction. Relative quantification of the cDNA was performed in 96-well plates on the iCycler (Bio-Rad). Each well contained 2 µl of cDNA mixed with 10 µl of SYBR Green I master mix (Bio-Rad), 0.2 µl of uracil-*N*-glycosylase (Epicentre Biotechnologies, Madison, WI, USA) and 750 nM forward and reverse primers (MWG Biotech, Ebersberg, Germany). All samples were run in duplicates. Collected data were analysed using the iCycler software, and the cycle threshold (Ct; i.e., the number of cycles necessary for the studied gene to be linearly expanded) for each sample was determined. Relative quantification was achieved by subtracting each Ct sample with the in-plate Ct of a reference control gene (delta Ct). The smaller the delta Ct value the more the gene is expressed compared with in-plate tested reference gene.

A group of reference genes, that is, GAPDH, beta-actin, TATA-box binding protein (TBP) and HPRT, was tested. GAPDH and TBP were found to be the least regulated genes and were consequently used for subsequent quantification of mRNA levels. Water controls and genomic DNA controls were run simultaneously with the samples on all plates and did not differ significantly from background. Primer sequences are available on request.

Data analysis

Genotype and allele frequencies of the 20 T > C polymorphism were analysed using Fisher's exact test (http://www.graphpad.com/quickcalcs/contingency1.cfm) or chi-squared test. OR and 95% confidence interval (CI 95%) were calculated using the website www.hutchon.net/ConfidOR.htm. Logistic regression

was used to include age and gender as covariates in the analysis. Other data were analysed using Mann-Whitney U test, when relevant, following significant Kruskal-Wallis analysis of variance by ranks. NPY peptide data and delta Ct values of quantitative PCR are presented as mean ± standard error of the mean (SEM). The level of significance was $p < 0.05$.

Results

Genotyping

In contrast to previous studies, the Leu7Pro variation was found to be significantly more frequent in patients with major depression as compared to the combined control group ($p < 0.0004$; Table 1). This effect did not depend on gender. Thus increased frequencies of the Leu7Pro variation were detected in both males ($p = 0.02$) and females ($p = 0.002$) (data not shown). Similarly, a significantly higher 'Pro7 allele' frequency (i.e., MAF, minor allele frequency) was seen in depressed patients as compared to the combined control groups (5.5% vs. 3.8%; $p = 0.009$; OR = 1.46, 95% CI = 1.10–1.93). This was also seen when we compared the depressed sample with the screened controls ($p = 0.01$; OR = 2.02, 95% CI = 1.16–3.53) and population controls ($p = 0.02$; OR = 1.41, 95% CI = 1.06–1.88), respectively. Comparing the combined cases and controls including schizophrenia disease controls also resulted in overall significance ($p = 0.033$). No significant differences in the frequency of the Leu7Pro variation were found between patients with schizophrenia and control individuals (Table 1). Gender or age did not vary between genotype groups ($p > 0.95$ and $p > 0.22$, respectively; data not shown).

Consistent with the association of the Leu7Pro variation with major depression, the genotype frequencies of depressed male or female patients were not in Hardy-Weinberg equilibrium, also when including both sexes ($p < 0.005$, $\chi^2 = 11.53$, df = 2). The genotypic distributions for the control group and psychiatric disease controls were all in Hardy-Weinberg equilibrium.

When cases ($n = 253$) were divided into groups according to comorbid anxiety (F40 or F41 in the ICD-10 system), no differences were found in the distribution of the Leu7Pro variation between patients with or without comorbid anxiety (MAF 6.4 and 6.3%, respectively; $p = 0.98$). Significantly more women (56.3%) showed comorbid anxiety as compared to men (30.5%; $p < 0.001$).

Transfections and measurements of NPY and its mRNA

To study the potential functional implications of Leu7Pro, HIT-T15 and SK-N-MC cells were transfected with plasmids encoding either the NPY 'Leu7 allele' or the mutant 'Pro7 allele'. HIT-T15 cells transfected with the 'Leu7 allele' synthesised and released substantial amounts of NPY peptide as compared to cells transfected with empty cDNA (Fig. 1a). In contrast, NPY peptide concentrations were significantly lower in cell extracts (68% reduction; $p = 0.0007$) and cell media extracts (48% reduction; $p = 0.003$) from HIT-T15 cells transfected with cDNA carrying the Pro7 substitution as compared to HIT-T15 cells carrying the Leu7 plasmid. In the SK-N-MC cell line, NPY peptide concentrations appeared substantially lower than in HIT-T15 cells in cell extracts where the NPY concentration was not consistently above the assay detection limit (data not shown). However, as in HIT-T15 cells, the NPY concentrations were found to be significantly lower (34% reduction; $p = 0.019$) in cell media extracts from SK-N-MC cells transfected with Pro7 mutant as compared to the Leu7 cDNA (Fig. 1c). As shown in Fig. 1a and c both cell types had a low level of endogenous NPY secretion.

Table 1. Distribution of the Leu7Pro variation (T/C) (rs16139)

		Genotype						
	n	L7/L7 (TT) *n* (%)	L7/P7 (TC) *n* (%)	P7/P7 (CC) *n* (%)	*p* value*	MAF† (C) %	*p* value‡	OR [95% CI]
Control samples								
Screened controls	287	271 (94.4)	16 (5.6)	0 (0.0)		2.8		
Population controls	2625	2420 (92.2)	203 (7.7)	2 (0.2)	0.38	3.9	0.21	ND
Case-control sample								
Combined controls	2912	2691 (92.4)	219 (7.5)	2 (0.1)		3.8		
Depressed cases	593	534 (90.1)	53 (8.9)	6 (1.0)	**0.0004**	5.5	**0.009**	**1.46 [1.10–1.93]**
Psychiatric controls								
Schizophrenia	503	462 (91.8)	41 (8.2)	0 (0.0)	0.70	4.1	0.36	ND

OR, odds ratio; CI, confidence interval; ND, not determined.

*Fisher's exact test between cases and controls, significant results are presented in bold.

†MAF: minor allele frequency (C-allele; Pro7 (P7) variant).

‡Chi-square test between cases and controls, significant results are presented in bold.

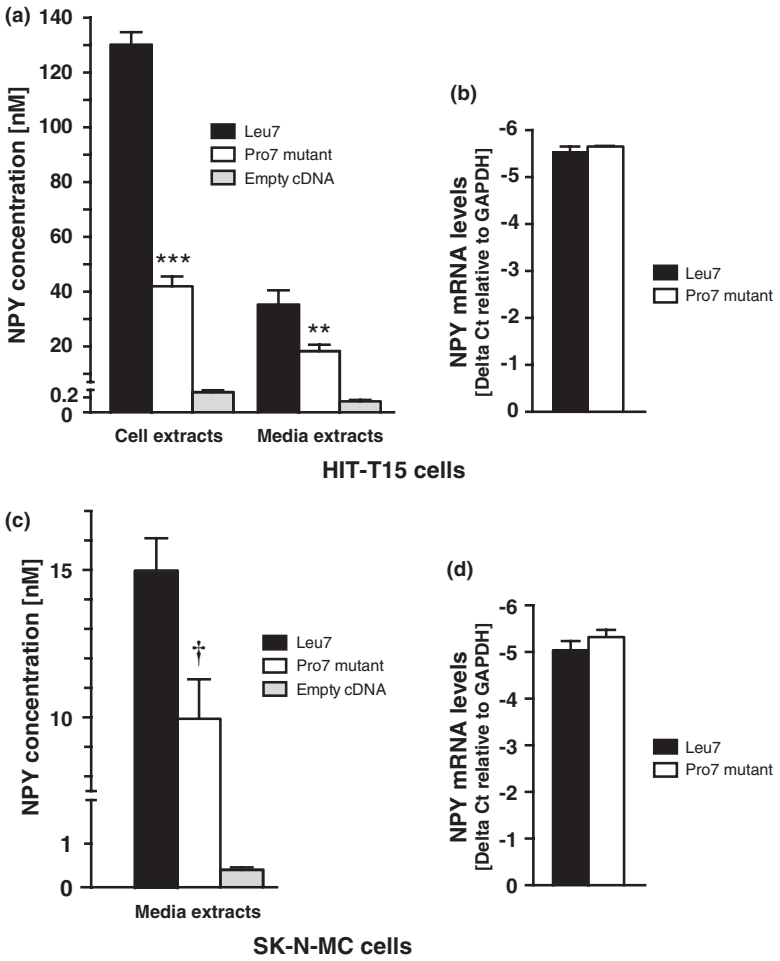

Fig. 1. Levels of NPY peptide and mRNA *in vitro* in cells transfected with the Leu7 *NPY* gene, the Pro7 mutant and cDNA empty of the *NPY* gene. NPY peptide concentrations as measured with radioimmunoassay (RIA) in cell and/or media extracts from (a) 800 000 HIT-T15 and (c) 800 000 SK-N-MC cells carrying the Pro7 mutant were significantly lower than in those carrying the Leu7 *NPY* gene. Levels of NPY peptide in SK-N-MC cell extracts are not shown as they were not consistently above the assay detection limit. In contrast, *NPY* mRNA levels measured using quantitative PCR did not differ between Leu7 and Pro7 transfected cells, neither in HIT-T15 (b) nor in SK-N-MC (d) cells. Data are mean (±SEM) from individual transfection series performed in triplicates ($n = 9$–12, RIA; $n = 6$, quantitative PCR). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{\dagger}p < 0.02$ versus WT, Mann-Whitney U test following significant Kruskal-Wallis analysis of variance by ranks.

In contrast, quantitative PCR using GAPDH as reference gene showed that NPY mRNA levels were similar in Pro7 and Leu7 transfected cells of both studied cell lines (Fig. 1b, d), indicating that the reduction in NPY levels in mutant transfected cells did not result from a decrease in *NPY* gene expression. Similar results were obtained using TBP as reference gene (data not shown). The fact that NPY mRNA levels were similar in Pro7 and Leu7 transfected cells would suggest that transfection efficiency was also similar. As further evidence that differences in NPY concentrations in Pro7 and Leu7 transfected cells did not result from a decrease in transfection efficiency, we also tested transfection efficiency in the cell lines by measuring levels of two DNA segments located on the transfection plasmid: the CMV promoter and ampicillin resistance gene. Quantitative PCR confirmed that there were, indeed, no significant differences in transfection efficiency in any of the two cell lines as revealed by the following delta Ct values (mean ± SEM; GAPDH as reference gene): CMV promotor wild-type (-9.3 ± 0.1) versus mutant (-9.0 ± 0.2; $p = 0.15$); ampicillin resistance gene wild-type (-9.0 ± 0.2) versus mutant (-8.6 ± 0.2; $p = 0.17$).

Discussion

The present study shows that the Leu7Pro variation may be associated with increased risk of major depression. The frequency of Leu7Pro was not significantly different from the control population in psychiatric disease control patients with schizophrenia. We also show in two cell lines *in vitro*, that the

Leu7Pro variation can be associated with decreased levels of NPY protein, apparently without accompanying changes in NPY mRNA levels.

Our results are not consistent with previous studies, reporting either no difference between depressed patients and controls (26) or decreased frequency of Leu7Pro variation in depression (15,28,29). Thus our study suggests Pro7 to be a risk allele as opposed to a protective allele in previous studies. The lack of agreement between the present and previous studies is puzzling. Smaller sample sizes could play a role. For instance, Lappalainen et al. (26), who reported no association, studied a smaller sample of 122 depressed patients and the frequency of Leu7Pro in this study was low in the control group (2.0%) compared to that of the present study (3.8%). Similarly, Heilig et al. (15) reported the opposite of the present study in an even smaller Swedish sample ($n = 51$) of treatment resistant depression. However, decreased frequency of the 'Pro7 allele' was also found in a larger depression sample ($n = 461$) from a Swedish population (28), and this was confirmed in a recent replication study using a Dutch population (GAIN; $n = 1862$) ($p = 0.034$; OR = 0.73) (29).

Differences in ethnic origin of the subjects could also contribute to apparent discrepancies between our study and previous studies. Thus the large Swedish population (28) was taken from a large cohort of Swedish citizens, in which up to 10% were born outside Sweden (38), with no information on place of birth, which might indicate that some of the cases were of non-Caucasian origin. In the large study by Bosker et al. the individuals were of Dutch or Western European origin (29). Given the north-to-south gradient of decreasing frequency of the 'Pro7 allele' with reported frequencies in Dutch (3.5%) and German (3.6%) versus Swedish (3.9–4.6%) and Finnish (5.8–6.9%) populations and, more importantly, the absence of the 'Pro7 allele' in non-Caucasian populations (39), it is important that ethnicity of patients and controls is similar. All our patients were Caucasians born in Denmark and only a minority of their parents were born outside Denmark in another European country. To clarify this issue further future studies might explore ethnicity in more depth by genetic stratification using ethnically specific marker genes.

Differences in sampling procedures could also be involved. Thus our patients were diagnosed by trained psychiatrists and recruited among patients hospitalised at psychiatric departments. In contrast, the Dutch cases (29) were recruited from mental health-care organisations, primary care and community samples, and the Swedish cases (28) were identified on the basis of self-reported questionnaires. Yet another explanation could be differences in diagnostic criteria in the different studies. We collected patients in our sample based on a diagnosis of major depression in hospitalised patients (F32 or F33 in the ICD-10 system), whereas the Dutch cases were included on the basis of a lifetime diagnosis of major depression (DSM-IV system) as diagnosed through the Composite International Diagnostic Interview (CIDI Version 2.1.12) (29). Moreover, the Swedish study (28), in addition to major depression, also included patients with dysthymia and mixed anxiety depression. We found no difference in genotype and allele frequencies between patients with comorbid anxiety as compared to patients without, suggesting that our association of Leu7Pro with depression is not influenced by comorbid anxiety.

Other more common variations in the *NPY* gene have been studied (19,22,34,40). The T allele in the promoter polymorphism −399C>T (rs16147) has been associated with decreased expression of NPY (22) and diplotypes containing the NPY-399C allele resulted in low expression of NPY and predicted emotional regulation in healthy individuals (19). The low expression diplotypes were overrepresented in depressed subjects and also found to increase neural responsivity to negative stimuli within the medial prefrontal and anterior cingulate cortices (41). However, the diplotype analysis did not include the Pro7 variant; therefore the impact of Leu7Pro was not analysed in the latter study.

The signal sequence in prepro-peptides has multiple functions in targeting and subsequent translocation or insertion of proteins into the membrane of the endoplasmatic reticulum (ER) (42). During transport into the ER lumen, signal sequences are often cleaved from the precursor protein. Secondary and tertiary structure of the signal sequence of preproNPY could be dramatically altered by the Leu7Pro substitution, as proline easily forms brakes and kinks in alpha-helical structures (43). Therefore, it could be expected that the Leu7Pro variation could lead to modifications in the formation, storage, and release of mature NPY. Consistent with this view, previous studies have reported that substitution of leucine with proline in the signal peptide may be associated with drastic decreases in expression of the mature protein. For instance, Lanza et al. (44) showed that leucine for proline substitution (also in position 7) in the signal peptide of platelet glycoprotein IX (GPIX) was associated with abolished GPIX synthesis. Likewise, leucine with proline substitution in the signal peptide of calcium-sensing receptor (CASR) resulted in markedly reduced levels of CASR that appeared to be due to a failure of insertion into the ER (45).

Studying the HIT-T15 and SK-N-MC cell lines, the present study shows that the 'Pro7 allele' could be associated with a decrease in both intra- and extracellular levels of NPY. A previous study using

PC12 cells did not find that the Leu7Pro variation affected the site of cleavage and targeting or uptake of NPY into the ER (46). In addition, another study found increased synthesis and secretion of NPY in the Pro7 mutant when co-transfected with 'Leu7 constructs' (47). The reason for these apparent discrepancies is not clear. Perhaps the effect of the Leu7Pro variation could be cell-type specific. Thus the effect of Leu7Pro appeared to be more pronounced in HIT-T15 as compared to SK-N-MC cells, and different cell types (PC12 cells, ArT-20 cells and mouse chromaffin cells) were used in the other studies (46,47). Nonetheless, Leu7/Pro7 heterozygous individuals had decreased concentrations of NPY in plasma (48,49) in accordance with the present *in vitro* findings of decreased intra- and extracellular NPY levels. However, an increase in plasma NPY concentrations has been reported during exercise of Leu7/Pro7 individuals as compared to Leu7/Leu7 controls (50). Since only heterozygous individuals were tested in the latter study, it is not directly comparable to our *in vitro* data. Thus the HIT-T15 and SK-N-MC cell lines are basically a 'homozygous situation', expressing only the Pro7 allele.

It is not known how the Leu7Pro variation might increase the risk of depression. However, the present finding that the Pro7 substitution was associated with impaired NPY synthesis and decreased extracellular levels *in vitro* is consistent with data from the literature, that central administration of NPY has actions that may antagonise key symptoms of depression, including orexigenic, sleep inducing, anti-stress and anxiolytic effects (3,51). In addition, central injection of NPY induces antidepressant-like effects in experimental animal models (14,52) and depressed patients have significantly lower concentrations of NPY in CSF (15,16). Conversely, several types of antidepressant treatments cause increases in *NPY* gene expression in rodents or in NPY concentration in CSF of human patients (11,12,53). Moreover, animal and human data indicate that NPY exerts widespread effects on the release of hypothalamic hormones implicated in the pathophysiology of depression, including corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and gonadotropins (3).

In conclusion, the present study shows that carrying the 'Pro7 allele' in the signal sequence of NPY may be a risk factor for major depression in the Danish population. Our results were based on a large cohort of patients, two types of controls, more narrow diagnostic criteria, which could account for the results that are in the opposite direction from previous studies. *In vitro*, the Leu7Pro variation showed functional consequences in the form of impaired NPY biosynthesis and reduced extracellular levels consistent with previously described antidepressant effect of NPY in the brain.

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References

1. Kessing LV. Gender differences in patients presenting with a single depressive episode according to ICD-10. Soc Psychiatry Psychiatr Epidemiol 2005;**40**:197–201.
2. Levinson DF. The genetics of depression: a review. Biol Psychiatry 2006;**60**:84–92.
3. Redrobe JP, Dumont Y, Quirion R. Neuropeptide Y (NPY) and depression: from animal studies to the human condition. Life Sci 2002;**71**:2921–2937.
4. Husum H, Vasquez PA, Mathe AA. Changed concentrations of tachykinins and neuropeptide Y in brain of a rat model of depression: lithium treatment normalizes tachykinins. Neuropsychopharmacology 2001;**24**:183–191.
5. Mathé AA, Jimenez PA, Theodorsson E, Stenfors C. Neuropeptide Y, neurokinin A and neurotensin in brain regions of Fawn-Hooded "depressed", Wistar, and Sprague-Dawley rats. Effects of electroconvulsive stimuli. Prog Neuropsychopharmacol Biol Psychiatry 1998;**22**:529–546.
6. Jimenez-Vasquez PA, Mathé AA, Thomas JD, Riley EP, Ehlers CL. Early maternal separation alters neuropeptide Y concentrations in selected brain regions in adult rats. Brain Res Dev Brain Res 2001;**131**:149–152.
7. Caberlotto L, Fuxe K, Overstreet DH, Gerrard P, Hurd YL. Alterations in neuropeptide Y and Y1 receptor mRNA expression in brains from an animal model of depression: region specific adaptation after fluoxetine treatment. Brain Res Mol Brain Res 1998;**59**:58–65.
8. Heilig M, Wahlestedt C, Ekman R, Widerlöv E. Antidepressant drugs increase the concentration of neuropeptide Y (NPY)-like immunoreactivity in the rat brain. Eur J Pharmacol 1988;**147**:465–467.
9. Mathé AA, Jousisto-Hanson J, Stenfors C, Theodorsson E. Effect of lithium on tachykinins, calcitonin gene-related peptide, and neuropeptide Y in rat brain. J Neurosci Res 1990;**26**:233–237.
10. Stenfors C, Theodorsson E, Mathé AA. Effect of repeated electroconvulsive treatment on regional concentrations of tachykinins, neurotensin, vasoactive intestinal polypeptide, neuropeptide Y, and galanin in rat brain. J Neurosci Res 1989;**24**:445–450.
11. Christensen DZ, Olesen MV, Kristiansen H, Mikkelsen JD, Woldbye DPD. Unaltered neuropeptide Y (NPY)-stimulated [35S]GTPγS binding suggests a net increase in NPY signalling after repeated electroconvulsive seizures in mice. J Neurosci Res 2006;**84**:1282–1291.
12. Mikkelsen JD, Woldbye DPD. Accumulated increase in neuropeptide Y and somatostatin gene expression of the

rat in response to repeated electroconvulsive stimulation. J Psychiatr Res 2006;**40**:153–159.
13. Husum H, Mikkelsen JD, Hogg S, Mathé AA. Mørk A. Involvement of hippocampal neuropeptide Y in mediating the chronic actions of lithium, electroconvulsive stimulation and citalopram. Neuropharmacology 2000;**39**:1463–1473.
14. Redrobe JP, Dumont Y, Fournier A, Quirion R. The neuropeptide Y (NPY) Y1 receptor subtype mediates NPY-induced antidepressant-like activity in the mouse forced swimming test. Neuropsychopharmacology 2002;**26**: 615–624.
15. Heilig M, Zachrisson O, Thorsell A et al. Decreased cerebrospinal fluid neuropeptide Y (NPY) in patients with treatment refractory unipolar depression: preliminary evidence for association with preproNPY gene polymorphism. J Psychiatr Res 2004;**38**:113–121.
16. Widerlöv E, Lindstrom LH, Wahlestedt C, Ekman R. Neuropeptide Y and peptide YY as possible cerebrospinal fluid markers for major depression and schizophrenia, respectively. J Psychiatr Res 1988;**22**:69–79.
17. Bannon AW, Seda J, Carmouche M et al. Behavioral characterization of neuropeptide Y knockout mice. Brain Res 2000;**868**:79–87.
18. Sørensen G, Lindberg C, Wörtwein G, Bolwig TG, Woldbye DPD. Different roles for neuropeptide Y Y1 and Y5 receptors in anxiety and sedation. J Neurosci Res 2004; **77**:723–729.
19. Zhou Z, Zhu G, Hariri AR et al. Genetic variation in human NPY expression affects stress response and emotion. Nature 2008;**452**:997–1001.
20. Karvonen MK, Pesonen U, Koulu M et al. Association of a leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels. Nat Med 1998;**4**:1434–1437.
21. Okubo T, Harada S. Polymorphism of the neuropeptide Y gene: an association study with alcohol withdrawal. Alcohol Clin Exp Res 2001;**25**(6 Suppl):59S–62S.
22. Itokawa M, Arai M, Kato S et al. Association between a novel polymorphism in the promotor region of the neuropeptide Y gene and schizophrenia in humans. Neurosci Lett 2003;**347**:202–204.
23. Mottagui-Tabar S, Prince JA, Wahlestedt C, Zhu G, Goldman D, Heilig M. A novel single nucleotide polymorphism of the neuropeptides Y (NPY) gene associated with alcohol dependence. Alcohol Clin Exp Res 2005;**29**: 702–707.
24. Niskanen L, Karvonen MK, Valve R et al. Leucine 7 to proline 7 polymorphism in the neuropeptide Y gene is associated with enhanced carotid atherosclerosis in elderly patients with type 2 diabetes and control subjects. J Clin Endocrinol Metab 2000;**85**:2266–2269.
25. Niskanen L, Voutilainen-Kaunisto R, Terasvirta M et al. Leucine 7 to proline 7 polymorphism in the neuropeptide Y gene is associated with retinopathy in type 2 diabetes. Exp Clin Endocrinol Diabetes 2000;**108**:235–236.
26. Lappalainen J, Kranzler HR, Malison R et al. A functional neuropeptide Y Leu7Pro polymorphism associated with alcohol dependence in a large population sample from the United States. Arch Gen Psychiatry 2002;**59**:825–831.
27. Kauhanen J, Karvonen MK, Pesonen U et al. Neuropeptide Y polymorphism and alcohol consumption in middle-aged men. Am J Med Genet 2000;**93**:117–121.
28. Sjöholm LK, Melas PA, Forsell Y, Lavebratt C. PreproNPY Pro7 protects against depression despite exposure to environmental risk factors. J Affect Disord 2009;**118**: 124–130.
29. Bosker FJ, Hartman CA, Nolte IM et al. Poor replication of candidate genes for major depressive disorder using genome-wide association data. Mol Psychiatry 2011;**16**: 516–532.
30. Kessing LV, Jørgensen OS. Apolipoprotein E-ε4 frequency in affective disorder. Biol Psychiatry 1999;**45**: 430–434.
31. Mellerup E, Bennike B, Bolwig T et al. Platelet serotonin transporters and the transporter gene in control subjects, unipolar patients and bipolar patients. Acta Psychiatr Scand 2001;**103**:229–233.
32. Haugboel S, Pinborg LH, Arfan HM et al. Reproducibility of 5-HT2A receptor measurements and sample size estimations with [18F]altanserin PET using a bolus/infusion approach. Eur J Nucl Med Mol Imaging 2007;**34**:910–915.
33. Jørgensen T, Borch-Johnsen K, Thomsen TF et al. A randomized non-pharmacological intervention study for prevention of ischaemic heart disease: baseline results Inter99. Eur J Cardiovasc Prev Rehabil 2003;**10**:377–386.
34. Lindberg C, Koefoed P, Hansen ES et al. No association between the – 399 C>T polymorphism of the neuropeptide Y gene and schizophrenia, unipolar depression or panic disorder in a Danish population. Acta Psychiatr Scand 2006; **113**:54–58.
35. Koefoed P, Hansen TVO, Woldbye DPD et al. An intron 1 polymorphism in the CCK-A receptor gene associated with schizophrenia in males. Acta Psychiatr Scand 2009; **120**:281–287.
36. Hansen T, Olsen L, Lindow M et al. Brain expressed microRNAs implicated in schizophrenia etiology. PLoS One 2007;**12**:e873.
37. Rehfeld JF. Radioimmunochemical analysis of neuropeptides based on general characteristics of the analyte. Trends Analyt Chem 1989;**8**:102–106.
38. Hällström T, Damström-Thakker K, Forsell Y, Lundberg I, Tinghög P. The Part study. A population based study of mental health in the Stockholm county: study design. 2003. http://www.folkhalsoguiden.se/upload/Psykisk%20H%c3%a4lsa/PARTs%20tekniska%20rapport.pdf [accessed on April 2011].
39. Ding B. Distribution of the NPY 1128C allele frequency in different populations. J Neural Transm 2003;**110**: 1199–1204.
40. Domschke K, Dannlowski U, Hohoff C et al. Neuropeptide Y (NPY) gene: impact on emotional processing and treatment response in anxious depression. Eur Neuropsychopharmacol 2010;**20**:301–309.
41. Mickey BJ, Zhou Z, Heitzeg MM et al. Emotion processing, major depression, and functional genetic variation of neuropeptide Y. Arch Gen Psychiatry 2011;**68**:158–166.
42. Hegde RS, Bernstein HD. The surprising complexity of signal sequences. Trends Biochem Sci 2006;**31**:563–571.
43. Fitches AC, Appleby R, Lane DA, De SV, Leone G, Olds RJ. Impaired cotranslational processing as a mechanism for type I antithrombin deficiency. Blood 1998;**92**: 4671–4676.
44. Lanza F, De La Salle C, Baas MJ et al. A Leu7Pro mutation in the signal peptide of platelet glycoprotein (GP)IX in a case of Bernard-Soulier syndrome abolishes surface expression of the GPIb-V-IX complex. Br J Haematol 2002;**118**:260–266.

45. Pidasheva S, Canaff L, Simonds WF, Marx SJ, Hendy GN. Impaired cotranslational processing of the calcium-sensing receptor due to signal peptide missense mutations in familial hypocalciuric hypercalcemia. Hum Mol Genet 2005;**14**:1679–1690.
46. Ding B, Kull B, Liu Z et al. Human neuropeptide Y signal peptide gain-of-function polymorphism is associated with increased body mass index: possible mode of function. Regul Pept 2005;**127**:45–53.
47. Mitchell GC, Wang Q, Ramamoorthy P, Whim MD. A common single nucleotide polymorphism alters the synthesis and secretion of neuropeptide Y. J Neurosci 2008;**28**: 14428–14434.
48. Kallio J, Pesonen U, Jaakola U, Karvonen MK, Helenius H, Koulu M. Changes in diurnal sympathoadrenal balance and pituitary hormone secretion in subjects with Leu7Pro. J Clin Endocrinol Metab 2003;**88**: 3278–3283.
49. Jaakkola U, Kuusela T, Jartti T et al. The Leu7Pro polymorphism of preproNPY is associated with decreased insulin secretion, delayed ghrelin suppression, and increased cardiovascular responsiveness to norepinephrine during oral glucose tolerance test. J Clin Endocrinol Metab 2005;**90**: 3646–3652.
50. Kallio J, Pesonen U, Kaipio K et al. Altered intracellular processing and release of neuropeptides Y due to leucine 7 to proline 7 polymorphism in the signal peptide of preproneuropeptide Y in humans. FASEB J 2001;**15**: 1242–1244.
51. Heilig M. The NPY system in stress, anxiety and depression. Neuropeptides 2004;**38**:213–224.
52. Ishida H, Shirayama Y, Iwata M et al. Infusion of neuropeptide Y into CA3 region of hippocampus produces antidepressant-like effect via Y1 receptor. Hippocampus 2007;**17**:271–280.
53. Nikisch G, Agren H, Eap CB, Czernik A, Baumann P, Mathé AA. Neuropeptide Y and corticotropin-releasing hormone in CSF mark response to antidepressive treatment with citalopram. Int J Neuropsychopharmacol 2005;**8**: 403–410.