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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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 Righospitalet 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 \((\text{range } 30–66, \text{SD } 19)\) for males \((n = 109)\) and 48 years \((\text{range } 30–85, \text{SD } 18)\) 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 \((\text{age unknown})\) 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 \((\text{range } 30–66, \text{SD } 19)\) for males \((n = 1254)\) and 47 years \((\text{range } 19–65, \text{SD } 9)\) for females \((n = 1371)\).

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

Psychiatric disease controls. Blood samples from patients with schizophrenia were also analysed \((n = 503; \text{ICD-10 diagnosis } F20)\). They were sampled in the Copenhagen area within the period 2002–2007 \((\text{The Danish Psychiatric Biobank}; n = 302)\) and in the Aarhus area in 2002–2005 \((\text{Opus Project}; n = 201)\). Of these, 292 were men \((\text{mean age } 37, \text{range } 19–77, \text{SD } 11)\) and 211 were women \((\text{mean age } 36, \text{range } 18–84, \text{SD } 12)\). 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 \((\text{mainly Germany or Sweden})\) 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 \((\text{Transgenomic, Omaha, NE, USA})\). Fragments showing mutations were analysed on an ABI PRISM DNA Sequencer \((\text{ABI310 or ABI3730})\) \((\text{Applied Biosystems, Foster City, CA, USA})\). The rest of the samples were analysed by real-time PCR using TaqMan Assay-by-design genotyping assay \((\text{Applied Biosystems})\). Each 96-well plate contained two control samples \((\text{one heterozygote } (CT) \text{ and one homozygote } (CC))\) as well as one negative control \((\text{no template})\). 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 \((\text{CPON})\), was a kind gift from Professor Thue W. Schwartz, University
of Copenhagen. The pGEM-1-NPY plasmid was digested with EcoRI and BamHI 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% CO2 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 × 10⁵ 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, 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 (Epicent 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/quickcalc/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 \( (p < 0.0004; \text{Table 1}) \). 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 \( (5.5\% \text{ vs. } 3.8\%; \ p = 0.009; \ OR = 1.46, 95\% \ CI = 1.10–1.93) \). 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 \( (p > 0.95 \text{ and } p > 0.22, \text{respectively; data not shown}) \).

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 \( (p < 0.005, \chi^2 = 11.53, \text{df } = 2) \). 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 \( (F40 \text{ or } F41 \text{ in the ICD-10 system}) \), 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 \( (68\% \text{ reduction; } p = 0.0007) \) and cell media extracts \( (48\% \text{ reduction; } p = 0.003) \) 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 \( (34\% \text{ reduction; } p = 0.019) \) 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)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>L7/L7 (TT) n (%)</th>
<th>L7/P7 (TC) n (%)</th>
<th>P7/P7 (CC) n (%)</th>
<th>( p ) value*</th>
<th>MAF (C) %</th>
<th>( p ) value†</th>
<th>OR [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Screened controls</td>
<td>287</td>
<td>271 (94.4)</td>
<td>16 (5.6)</td>
<td>0 (0.0)</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Population controls</td>
<td>2625</td>
<td>2420 (92.2)</td>
<td>203 (7.7)</td>
<td>2 (0.2)</td>
<td>0.38</td>
<td>3.9</td>
<td>0.21 ND</td>
</tr>
<tr>
<td>Case-control sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined controls</td>
<td>2912</td>
<td>2891 (92.4)</td>
<td>219 (7.5)</td>
<td>2 (0.1)</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed cases</td>
<td>593</td>
<td>534 (90.1)</td>
<td>53 (8.9)</td>
<td>6 (1.0)</td>
<td>0.0004</td>
<td>5.5</td>
<td>0.009 1.46 [1.10–1.93]</td>
</tr>
<tr>
<td>Psychiatric controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>503</td>
<td>462 (91.8)</td>
<td>41 (8.2)</td>
<td>0 (0.0)</td>
<td>0.70</td>
<td>4.1</td>
<td>0.36 ND</td>
</tr>
</tbody>
</table>

*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, †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 promoter 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 Cauca-

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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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