Studies of the neuromedin U-2 receptor gene in human obesity: evidence for the existence of two ancestral forms of the receptor

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Abstract

Central administration of neuromedin U (NMU) suppresses food intake acting through the NMU-2 receptor (NMU2R), which is expressed in the hypothalamus. We screened the NMU2R gene in 96 patients with severe early-onset obesity. A common variant haplotype was found (f-0·21). This common variant haplotype was unusual in nature, consisting of four non-contiguous missense changes in complete linkage disequilibrium, and across two separate exons. The variant haplotype resulted in four amino acid substitutions (S295T/F312L/P380L/M385V) and was present in several other Europid populations and in subjects of South Asian, East Asian and African American origin, but not in eleven African Pygmies. This variant haplotype was not associated with obesity or related traits in 500 subjects from a prospective population-based cohort.

In summary, we have identified two markedly different isoforms of the NMU-2 receptor, presumably arising through an ancient and complex mutational event; no genetic associations between this haplotype and obesity-related traits were, however, discerned. Further investigation of the pharmacogenomic consequences of NMU2R variation in humans is warranted.

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Introduction

Obesity is a heritable complex trait and recent progress in understanding the genetic aetiology of the disease has involved both classical monogenic and polygenic studies in rodents and humans (O’Rahilly et al. 2003). Whilst genetic screening of severely obese cohorts has been used to identify novel monogenic obesity syndromes, obesity polygenes have generally been sought through association and linkage studies in populations consisting of comparatively older and less severely obese individuals. Such studies have identified leptin, the leptin receptor, proopiomelanocortin-derived peptides and the melanocortin 4 receptor (MC4R) as forming the central hypothalamic axis regulating food intake in humans as well as rodents (Schwartz et al. 2000). In addition, other neuropeptides (and their receptors) have been implicated as anorexigenic signals including the cocaine-and amphetamine-regulated transcript and prolactin releasing peptide (Crowley et al. 2002). Although not supported by the same body of irrefutable evidence provided by genetic studies, these latter molecules have been shown to be expressed or present in the hypothalamus and to result in a robust reduction in food intake following intracerebroventricular (i.c.v.) administration.

Neuromedin U (NMU) acting through the NMU-2 receptor (NMU2R) has recently been added to this list of appetite regulatory molecules (Howard et al. 2000). Two subtypes of the neuromedin U receptor (NMU1R (FM-3) and NMU2R (FM-4)) exist with markedly contrasting patterns of tissue localization (Hosoya et al. 2000). NMU1R is expressed in a wide variety of peripheral tissues including the pancreas, testes, small intestine and liver. However, NMU2R is found almost exclusively in the brain, exhibiting a discrete pattern of expression most notably within known feeding centres (including the lateral arcuate nucleus and the paraventricular nucleus of the hypothalamus) (Howard et al. 2000). Consistent with its role as an anorexigenic peptide, hypothalamic NMU expression levels have been shown to reduce on fasting, and i.c.v. injection of NMU into the rodent brain produces a robust reduction in food intake (Howard et al. 2000).
2000). In order to examine whether sequence variation in the NMU2R gene might contribute to human obesity, we examined the coding region and intron/exon boundaries of the NMU2R gene in a cohort of 94 subjects with severe early-onset obesity. Having found some common single nucleotide polymorphisms (SNPs), we then examined the association of these variants with obesity-related phenotypes in a UK Caucasian population-based cohort. Although we did not find any evidence for association of NMU2R variants with obesity-related traits, we did find an unusual haplotype in the gene, which is of evolutionary interest and may have pharmacogenomic implications.

Materials and Methods

Study populations

Ninety-four unrelated individuals with hyperphagia and severe early-onset obesity from the Genetics of Obesity Study (G.O.O.S.) were used in the initial mutation screen (Faroqui & O'Rahilly 2004). In all probands within this cohort, obesity arose before the age of 10 years and their body mass index (BMI) was greater than 4 standard deviations above the population mean for their sex and age. Fifteen of these 94 individuals were of South Asian origin; the remaining 79 affected individuals in this dataset were European Caucasians.

The Isle of Ely study is a prospective population-based cohort study of the aetiology and pathogenesis of type 2 diabetes and related metabolic disorders (Wareham et al. 2000). It is an ethnically homogeneous Caucasian population and is of particular value since phenotypic data have been recorded on individuals both at the outset and again after 4–5 years. Subjects were all aged between 40 and 65 years at baseline.

DNA from 6 Vietnamese, 3 Taiwanese, 5 Chinese, 9 Japanese, 1 Korean, 6 African Biaka Pygmies and 5 African Mbuti Pygmies (Watkins et al. 2001) and from 17 white American Caucasian, 7 Hispanic American, 13 African American and 13 East Asian American obtained from the National Institute of General Medical Sciences Human Genetic Cell Repository (http://locus.umdnj.edu/nigms/) were scanned for the presence of mutations within NMU2R (Jorde et al. 2001).

PCR, sequencing and genotyping

Genomic DNA was isolated from whole blood using a QIAamp blood kit (Qiagen, London, UK). PCR was performed using BioTaq (Bioline, London, UK) and carried out as recommended by the manufacturer. Thirty-five cycles (30 s at 96 °C, 40 s at 60 °C, 40 s at 72 °C) were performed using a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA).

Following digestion, gel electrophoresis was performed using 3%(w/v) agarose gels (Gibco BRL, Paisley, UK) containing ethidium bromide and the pattern of bands was visualised and recorded following exposure of the gel to ultraviolet radiation.

Sequencing was carried out using BigDye terminator chemistry (Perkin-Elmer, Beaconsfield, Bucks, UK) and electrophoresed on an ABI 377 automated DNA sequencer (Perkin-Elmer, Foster City, CA, USA). Sequences were assembled and examined using Sequencher software (Gene Codes, Ann Arbor, MI, USA).

G884C (S295T), C936A (F312L), C1139T (P380L) and A1153G (M385V) were genotyped by PCR amplification of the fragment containing these polymorphisms and then digestion with restriction enzymes BglI, EarI, BstNI/NcoI and NcoI respectively (New England Biolabs, London, UK). Primers were designed to force the presence of a restriction site when no naturally occurring restriction sites that could be used to generate genotypes were formed by the changes.

NMU2R is a 412 amino acid protein encoded by a gene on chromosome 5q33·1 consisting of 4 exons. Primer sequences were designed from accession identifiers AF242874 and NT029289. The primers used in the PCR, sequencing and genotyping reactions are listed in Table 1.

Statistical methodology

Association analyses between genotype and phenotype were performed using the MIXED procedure in the Statistical Analysis System (SAS for Windows 8·2); this allows incorporation of unbalanced repeated data, which considerably improves the power of the study. The allowance for unbalanced repeated data allows the inclusion of individuals who contributed 1 as well as 2 measures of the phenotypic variables. Means were adjusted for age and sex, and P values were calculated for the comparison between the wild-type genotype grouping with haplotype carriers.

Results

In the 94 children with severe early-onset obesity, six different sequence variants were found (Table 2), two of which were silent (C483T and C963A). Four variants, all found at high frequency (16%), resulted in amino acid changes. S295T is a conservative change occurring between the sixth and seventh transmembrane domains of the receptor (Fig. 1). F312L, P380L and M385V are non-conservative changes with F312L located within the seventh transmembrane domain and both P380L and M385V in the 3′ tail of NMU2R. Notably S295T, F312L, P380L and M385V together formed a haplotype, with each mutation in complete linkage disequilibrium with one another. This haplotype was found in both Europeans and Asians within our obese cohort. It was found at a similar frequency in control subjects (15%) and is therefore containing ethidium bromide and the pattern of bands was visualised and recorded following exposure of the gel to ultraviolet radiation.

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highly unlikely to be the aetiological basis of the severe obesity present within our cohort. No rare missense changes were detected in the NMU2R gene.

To determine whether the NMU2R mutant haplotype might influence obesity-related phenotypes in the general population, we typed one of the polymorphisms forming the haplotype (C1139T) in 500 individuals drawn randomly from a population of 1085 UK Caucasians, in whom detailed phenotypic information was available. Ninety-six randomly selected individuals from this cohort were then selected and their sequence determined across exons 3 and 4 of NMU2R, to confirm that the 295T-312L-380L-385V haplotype occurred consistently with the genotypes obtained at C1139T. This was found to be the case. The mutant haplotype allele frequency was 0·21 in this cohort and there was no association with BMI, fasting glucose, insulin or triglyceride levels, waist–hip ratio, body fat or fat intake percentage (data not shown).

To begin to determine the evolutionary history of this highly unusual variant haplotype, we examined DNA obtained from multiple different Europid, Asian and African populations. We found the variant in subjects of European, South Asian, East Asian and African American ancestry but not in 11 African Pygmies (Table 3). While the size of this study is insufficient to make any definite statement regarding the likely evolutionary history of this variant, it is clearly widespread.

Discussion

There is a growing body of evidence that suggests that neuromedin U may be involved in the hypothalamic modulation of feeding and energy balance. Howard et al. (2000) demonstrated that NMU was expressed in the ventromedial hypothalamus, that its expression levels were reduced by fasting, and that i.c.v. administration of NMU reduced food intake. These authors also identified a novel receptor for NMU, the NMU2R, and demonstrated that it was expressed in several regions of the brain including the hypothalamus. Hanada and colleagues (2003) reported that in addition to reducing food intake, i.c.v. NMU infusion increased physical activity, body temperature and resting energy expenditure and thus concluded that NMU was likely to be a potent catabolic neuropeptide. Ivanov et al. (2002) showed that NMU (i.c.v.) activated neurons

Table 1 Primers used in the PCR, sequencing and genotyping reactions

<table>
<thead>
<tr>
<th>Primers</th>
<th>PCR</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>F1X1FM4PCR</td>
<td>TGAAACAGAGCCTCGTACC</td>
<td></td>
</tr>
<tr>
<td>R1X1FM4PCR</td>
<td>TCTGAACTTGTAGGCTTG</td>
<td></td>
</tr>
<tr>
<td>F2X1FM4PCR</td>
<td>TGAAACAGAGCCTCGTACC</td>
<td></td>
</tr>
<tr>
<td>R2X1FM4PCR</td>
<td>TCTGAACTTGTAGGCTTG</td>
<td></td>
</tr>
<tr>
<td>F1X2FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
<td></td>
</tr>
<tr>
<td>R1X2FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
<td></td>
</tr>
<tr>
<td>F2X2FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
<td></td>
</tr>
<tr>
<td>R2X2FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
<td></td>
</tr>
<tr>
<td>F1X3FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
<td></td>
</tr>
<tr>
<td>R1X3FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
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<tr>
<td>F2X3FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
<td></td>
</tr>
<tr>
<td>R2X3FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
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</tr>
<tr>
<td>F1X4FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
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<tr>
<td>F2X4FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
<td></td>
</tr>
<tr>
<td>R2X4FM4PCR</td>
<td>TTGCAATCTGAGCTGTG</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Variant sequences in NMU2R in 94 subjects with severe early-onset obesity

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism (nucleotide)</th>
<th>Coding change (amino-acid)</th>
<th>Type of change</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMU2R</td>
<td>C483T</td>
<td>L161L</td>
<td>Silent</td>
<td>14%</td>
</tr>
<tr>
<td></td>
<td>G884C</td>
<td>S295T</td>
<td>Conservative</td>
<td>16%</td>
</tr>
<tr>
<td></td>
<td>C936A</td>
<td>F312L</td>
<td>Non-conservative</td>
<td>16%</td>
</tr>
<tr>
<td></td>
<td>C963A</td>
<td>P321P</td>
<td>Silent</td>
<td>2%</td>
</tr>
<tr>
<td></td>
<td>C1139T</td>
<td>P380L</td>
<td>Non-conservative</td>
<td>16%</td>
</tr>
<tr>
<td></td>
<td>A1153G</td>
<td>M385V</td>
<td>Non-conservative</td>
<td>16%</td>
</tr>
</tbody>
</table>
**Figure 1** Multiple alignment of the human, mouse and rat forms of NMU1R with the human and rat forms of NMU2R. The amino acid changes that occur in the variant form of NMU2R are underlined in the human NMU2R row. Amino acid residues are shown for human NMU2R above each block of sequence and the alignment begins at residue 239 of human NMU2R. The sixth and seventh transmembrane regions of human NMU2R are emboldened. Below each block of sequence is an asterisk (*), a colon (:), a full point (.) or a ‘blank character’ which designates the conservation of transition in that column. An * indicates complete conservation of amino acid, : and : indicate strong and weak semi-conservative changes respectively and a blank character indicates that a non-conservative change has occurred in that column of the multiple alignment.
in the hypothalamus and brainstem, some of which were catecholaminergic. They also showed that fa/fa rats had decreased expression of NMU in several brain nuclei (Ivanov et al. 2002). Wren et al. (2002) injected NMU into specific hypothalamic nuclei and showed that injection into the paraventricular nucleus (PVN) was a particularly powerful inhibitor of food intake and also stimulated locomotor activity and plasma adrenocorticotrophin levels. Wren and colleagues also showed that hypothalamic explants exposed to NMU increased their release of corticotrophin releasing factor (Wren et al. 2002). Qiu et al. (2003) showed that NMU depolarises a subpopulation of PVN neurons via enhancement of the hyperpolarisation activated inward current.

Thus, while there is now extensive physiological data suggesting a possible role for NMU as a regulator of energy balance in the hypothalamus, there has, as yet, been no human genetic data to support this role. In this study, we sought to determine whether genetic variation in NMU2R might be associated with human obesity-related traits. While we found no evidence of this, it is possible that our study design was of insufficient power to detect such effects, and only through further studies with larger populations will we be able to categorically reject the hypothesis that genetic variation at the NMU2R locus influences body fat mass in humans.

In the course of this study we did, however, identify an unusual haplotype involving one missense amino acid change in exon 3 and three in exon 4 of the NMU2R. These exons are separated by a 3 kb intron. Both F312 L and P380 L occur at residues which are conserved in the rat NMU2R and also in the mouse, rat and human NMU1R genomic loci. This is a highly unusual haplotype, involving four nucleotide changes spread across two exons, each of which results in a change in an amino acid. This suggests a conversion event occurring at some time during the evolutionary history of the gene. The fact that both forms of the receptor are found in different Asian, European and African American populations indicates that the mutational event leading to these two isoforms must have occurred at least 100 000 years ago (Cavalli-Sforza & Feldman 2003). It is interesting that only one receptor type was found in our cohort of African Pygmies, but the group size is too small to make definitive statements about the absence of the variant in Sub-Saharan African populations.

G protein-coupled receptors are increasingly being targeted by the pharmaceutical industry. If NMU2R is to become a target for pharmacological intervention, it will be essential to investigate the functional consequences of this ubiquitous, common and highly polymorphic variant of the receptor.

In summary, while we found no evidence that genetic variation at the NMU2R locus influences obesity-associated traits in man, we identified two highly prevalent ancestral forms of the NMU2 receptor which, unusually, differ substantially in amino acid sequence, and may therefore ultimately turn out to have different functional properties.

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