Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8666–8670, August 1996
Neurobiology

Structure, tissue distribution, and chromosomal localization of the prepronociceptin gene

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Communicated by Jean-Pierre Changeux, Institut Pasteur, Paris, France, April 17, 1996

ABSTRACT **Nociceptin (orphanin FQ), the newly discovered natural agonist of opioid receptor-like (ORL_1) receptor, is a neuropeptide that is endowed with pronociceptive activity *in vivo*. Nociceptin is derived from a larger precursor, prepronociceptin (PPNOC), whose human, mouse, and rat genes we have now isolated. The PPNOC gene is highly conserved in the three species and displays organizational features that are strikingly similar to those of the genes of preproenkephalin, preprodynorphin, and preproopiomelanocortin, the precursors to endogenous opioid peptides, suggesting the four genes belong to the same family—i.e., have a common evolutionary origin. The PPNOC gene encodes a single copy of nociceptin as well as of other peptides whose sequence is strictly conserved across murine and human species; hence it is likely to be neurophysiologically significant. Northern blot analysis shows that the PPNOC gene is predominantly transcribed in the central nervous system (brain and spinal cord) and, albeit weakly, in the ovary, the sole peripheral organ expressing the gene. By using a radiation hybrid cell line panel, the PPNOC gene was mapped to the short arm of human chromosome 8 (8p21), between sequence-tagged site markers WI-5833 and WI-1172, in close proximity of the locus encoding the neurofilament light chain NEFL. Analysis of yeast artificial chromosome clones belonging to the WC8.4 contig covering the 8p21 region did not allow to detect the presence of the gene on these yeast artificial chromosomes, suggesting a gap in the coverage within this contig.**

Nociceptin (1) or orphanin FQ (2), a newly discovered neuropeptide of 17 amino acids, is the natural agonist of the opioid receptor-like (ORL_1) receptor, a G protein-coupled receptor whose human (3) and murine (4–10) cDNAs had been identified previously.

The ORL_1 receptor is most closely related to opioid receptors in terms of primary structure (≈60% homology), yet its *in vitro* pharmacological profile is clearly not opioid. When expressed in CHO cells, ORL_1 responds to high (micromolar) concentrations of the potent opiate agonist etorphine and inhibits adenylate cyclase (3). The distribution of ORL_1 mRNAs in murine brain (3–7, 9) is compatible with the receptor being involved in a number of important central functions, including nociperception. Indeed, repeated *in vivo* administration of an antisense oligonucleotide to ORL_1 mRNA induces analgesia in mice (1).

The endogenous ligand of ORL_1 was recently isolated as a component of brain tissue extracts that inhibits adenylate cyclase in recombinant cells expressing the orphan receptor (1, 2). It is a heptadecapeptide whose sequence, FGGFTGARKSARKLANQ, resembles to some extent that of the endogenous opioid peptide dynorphin A. The synthetic peptide exhibits nanomolar potency in inhibiting forskolin-induced accumulation of cAMP in recombinant CHO cells expressing ORL_1 and induces hyperalgia when administered intracerebroventricularly in mice (1, 2). The latter effect led us to name the peptide "nociceptin." Nociceptin is likely to be processed *in vivo* from a larger precursor, prepronociceptin (PPNOC), whose partial cDNA was isolated from a rat brain library (1). The mouse cDNA encoding PPNOC was recently reported as induced by dibutyryl cyclic AMP in the mouse NS20Y neuroblastoma cell line (11). Partial human cDNA sequences have also been identified among the expressed sequence tags present in the data bases (accession nos. R19874 and Z20405).

Here we report the complete coding sequences of the mouse, rat, and human PPNOC genes. The PPNOC gene is highly conserved in the three species. It encodes a single copy of nociceptin as well as of other potentially neuroactive peptides. The overall structure and organization of the PPNOC gene are strikingly similar to those of the precursors to endogenous opioid peptides. The PPNOC gene is located on human chromosome 8p and appears to be transcribed predominantly in the brain and spinal cord.

MATERIALS AND METHODS

Isolation of Human, Mouse and Rat PPNOC Genomic DNAs. A human genomic library constructed in the λDashII vector (no. 945201; Stratagene) and a mouse genomic library from the 129SV strain, constructed in the λFIXII vector (no. 946305; Stratagene) were screened by using the partial rat PPNOC cDNA clone RB101 (1). The human genomic library, as well as a rat genomic library constructed in the λDASH vector (no. 943501; Stratagene) were also screened with synthetic oligonucleotides corresponding to the 5′ end of the human and rat cDNA coding sequences, after analysis of the clones resulting from the first screening, and demonstration of the position of the intron splitting the coding region. The human oligonucleotide sequence (5′-GTGGAGCTTCTCCTGGCATGTGAGACAGTC-3′) was derived from the human expressed *tag* sequence (GenBank accession no. R19874) found to correspond to the 5′ untranslated region and the beginning of the coding sequence of the human PPNOC cDNA. The rat oligonucleotide (5′-GTGGAGCCTCTCCTGGCAGGTGAGGCAGTC-3′) was derived from the rat cDNA sequence (1). All clones were purified to homogeneity, their restriction map were determined, and the fragments hybridizing with the rat cDNA or the oligonucleotide probes were

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Abbreviations: ORL1, opioid receptor-like receptor; PPNOC, prepronociceptin; YAC, yeast artificial chromosome; UTR, untranslated region; cR, centiRay; Mb, megabase; PPENK, preproenkephalin; PPDYN, preprodynorphin; PPOMC, preproopiomelanocortin.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X97367–X97375).

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subcloned in pBluescript SK$^+$ (Stratagene) by using standard methods (12). Sequencing was performed on both strands on plasmid DNA, or after subcloning in M13 mp derivatives, using fluorescent primers and an automated DNA sequencer (Applied Biosystem model 370A). Sequence handling and data analysis was carried out using the DNASIS/PROSIS software (Hitachi), and the GCG software package (Genetics Computer Group).

Northern Blot Analysis of Rat PPNOC mRNA. Poly(A)$^+$ RNAs were isolated from adult rat tissues by using either the Fast Track kit (Invitrogen) for brain and peripheral organs or the minimessage maker kit from R & D Systems for brain regions. Glyoxal-treated RNA samples (5 μg for tissues, 3 μg for brain regions) were separated on 1% agarose gels in 10 mM sodium phosphate (pH 7) and blotted onto nitrocellulose filters according to Sambrook *et al.* (12). The filters were baked and then prehybridized for 4 h at 42°C in 50% formamide, 5× standard saline phosphate/EDTA (SSPE; 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll), 0.3% SDS, 200 μg of BSA per ml, and 250 μg of denatured salmon sperm DNA per ml. For hybridization, 10% dextran sulfate was added. The 926-bp fragment corresponding to the rat pronociceptin cDNA (1) was ^{32}P-radiolabeled by random priming with Klenow polymerase and used as probe. After a final washing step in 0.1× standard saline citrate (SSC), 0.1% SDS for 30 min at 65°C, the filters were autoradiographed with intensifying screens onto Fuji films for 24 h at −70°C. To verify integrity of the mRNA preparations the blots were also hybridized with a β-actin probe.

Chromosomal and Physical Mapping of the Human PPNOC Gene. The presence of the human PPNOC gene in the 93 clones of the GeneBridge 4 Radiation Hybrid Panel (13) was determined by polymerase chain reaction (PCR). The hybrid panel was obtained from Research Genetics (Huntsville, AL). PCR was performed by using a forward primer (5′-GCAGGAAGAGCCCGAGCC-3′) and a reverse primer (5′-GGGGCGGGGAGATGAATG-3′) located, respectively, in the coding and 3′-uncoding regions of the human gene. The PCR conditions were as follows: 93°C for 1 min, 57°C for 2 min, 72°C for 3 min for 30 cycles in a buffer containing 1 mM $MgCl_2$, 50 ng of genomic DNA, and 50 ng of each of the primers in a total volume of 50 μl. The reaction amplified a fragment of 356 bp from human genomic DNA, but not from hamster DNA. Integration of the PPNOC locus into the STS-based map of the human genome (14) was done by using the RHMAPPER computer package through the World Wide Web server of the Whitehead Institute (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl). The presence of the human PPNOC gene was further investigated by PCR on individual yeast artificial chromosome (YAC) clones belonging to the WC8.1, WC8.2, WC8.3, and WC8.4 contigs (14). The nonchimeric YAC selected within these contigs were 948D5, 755B1, 770C5, and 904D7 (WC8.1); 746H6, 900G12, and 810A1 (WC8.2); 734F10 and 820H6 (WC8.3); 741B6, 816B4, 949B8, 926H8, 803C1, 965E1, 808F9, 848A7, 757B1, 755B3, 791B9, 771G9, 750E9, 814E11, 896F4, 953H12, 901E10, 815E8, 788H6, 888D12, 761A2, 955C4, 849C2, 808C7, 802F7, and 716G9 (WC8.4).

RESULTS

Isolation of the Human, Mouse, and Rat Genes Encoding PPNOC. The rat partial cDNA clone (1) was used as a probe to screen a human genomic library. Two overlapping clones, HG61 and HG91, were isolated and a 5-kb *Eco*RI fragment from HG91 hybridizing with the rat cDNA probe was subcloned and partially sequenced. This fragment contained most (405 bp) of the coding sequence of the PPNOC gene and could be further aligned over a short region (47 bp) of the rat cDNA 3′ untranslated region (UTR). Another 3.6-kb *Xba*I fragment from HG91 was found to include a 414-bp sequence matching (89% identity) the rat cDNA 3′-UTR, up to the poly(A) tail (not shown), indicating that the 3′-UTR of the PPNOC gene is interrupted by an intron about 3.5 kb long. To isolate the missing 5′ part of the coding region, the library was screened again with a 30-mer synthetic oligonucleotide corresponding to the alignment in this region of the rat partial cDNA clone and the human expressed sequence tag sequence found in the data bases (GenBank accession no. R19874). A single clone (HGEX107) was isolated, a 3-kb *Eco*RI–*Sst*I fragment subcloned in pBluescript SK$^+$, partially sequenced and found to encode the remaining part of the coding sequence (126 bp). The expressed sequence tag was colinear with the genomic clone for only 23 bp ahead of the start codon, demonstrating the presence of at least one intron splitting the 5′-UTR of the message. The human PPNOC gene is therefore composed of at least four exons (Fig. 1*I*). Exon I (for which no genomic sequences have yet been obtained) contains exclusively 5′ noncoding sequences, exons II and III share the open reading frame of the gene, while exon IV contains most of the 3′ noncoding region of the message. Data that were gathered from the mouse and rat genes do fit into a similar structure for the PPNOC gene in these species (see below).

The rat cDNA was also used to probe a mouse genomic library. A single clone (MG43) was isolated. A 2.5-kb *Hin*dIII–*Xba*I fragment was subcloned, partially sequenced, and shown to contain exon III (483 bp) of the PPNOC gene, including 420 bp of coding sequence and 45 bp of 3′-UTR. A 1.3-kb *Bam*HI fragment, also hybridizing with the rat cDNA, contained exon II (150 bp), including 24 bp of 5′-UTR and the first 126 bp of coding sequence. The borders of the mouse exons were confirmed following the recent report of the mouse PPNOC cDNA (11). The distance between mouse exons II and III was estimated from restriction analysis to be 4.5 kb.

The 5′ missing part of the rat coding sequence was obtained by screening a rat genomic library using a synthetic oligonucleotide corresponding to the second exon. One clone (RGEX109) was found to contain both exons II and III. Exon II was subcloned as a 2.5-kb *Eco*RI–*Xba*I fragment and

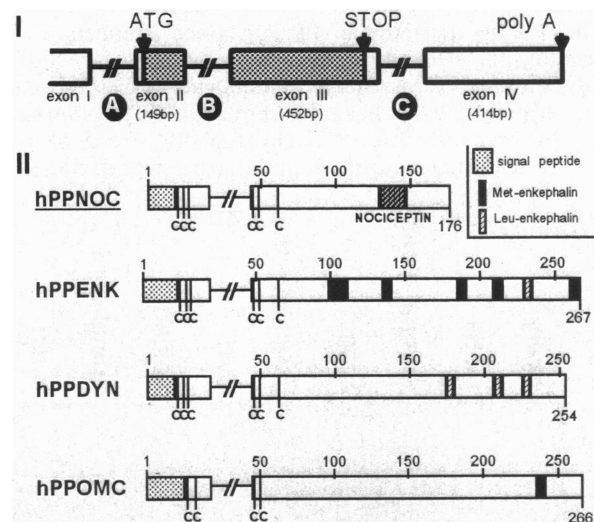

FIG. 1. (*I*) General organization of the PPNOC gene. The PPNOC gene consists of four exons (numbered I to IV) interspersed by three introns (A, B, and C). The filled boxes correspond to the coding region. ATG, STOP, and poly(A) are the transcription start, stop, and polyadenylation sites, respectively. (*II*) Comparison between the translated regions of the human prepronociceptin (hPPNOC), preproenkephalin (hPPENK), preprodynorphin (hPPDYN), and preproopiomelanocortin (hPPOMC) genes. Boxes indicate the coding frame and the interrupted lines materialize the introns.

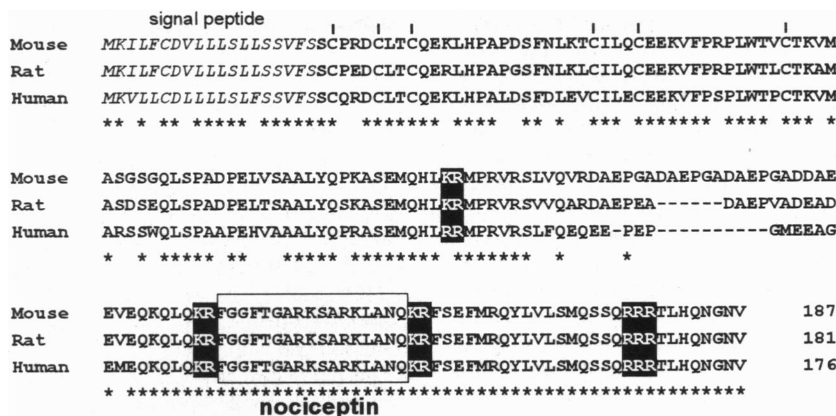

FIG. 2. Alignment of the sequences of mouse, rat, and human nociceptin precursors. The signal peptide is in italics and the nociceptin sequence is boxed. Asterisks denote amino acid identities and | emphasize conserved Cys residues. The putative proteolytic cleavage motifs are shown in white on black.

partially sequenced. This fragment contained 126 bp of the coding sequence overlaping with the rat cDNA clone, and 24 bp of the noncoding sequence separating the start codon from the putative intron–exon border.

Comparative Organization of the PPNOC and Opioid Peptides Precursor Genes. The nucleotide sequence of the murine and human PPNOC genes displays organizational and structural features that are very similar to those of the genes encoding the precursors to endogenous opioid peptides, enkephalins (PPENK), dynorphins/neo-endorphins (PPDYN), and β-endorphin (PPOMC) (Fig. 1*II*). In particular, the translated region of the murine and human PPNOC genes is interrupted by an intron located between codons for amino acids 42 and 43. An intron is also present at the equivalent site in the PPENK, PPDYN, and PPOMC genes (15–17). As it is the case in opioid peptide genes, another intron is also present in the 5′-UTR of the PPNOC gene. In addition to these two introns shared with the other precursor genes, the PPNOC gene also includes an intron in the 3′ untranslated region of the message. Taken together, the present data are compatible with the notion that the nociceptin and opioid peptides genes have evolved in parallel from a common ancestor.

The Murine and Human PPNOC Gene Products. Fig. 2 shows that the deduced amino acid sequence of PPNOC is highly conserved across murine and human species, especially the C-terminal end that hosts nociceptin itself. The N-terminal end of the precursor consists of a hydrophobic stretch of about 20 amino acids that may represent the signal peptide necessary for translocation into the rough endoplasmic reticulum (18), followed by a cystein-rich portion that is also found in other hormone precursors, especially those to endogenous opioid peptides (18). Indeed, the pattern of cystein residues in pronociceptin is exactly the same as in proenkephalin and prodynorphin (Fig. 1), suggesting a common mode of folding and/or processing of these precursor proteins. The lowest homology across murine and human PPNOCs is observed in the core of the molecule with insertion of a variable number of repeated acidic motifs. The unique nociceptin sequence is located in the C-terminal fourth of the precursor where it is flanked by canonic Lys-Arg proteolytic excision motifs (18). Interestingly, pronociceptin contains other potential cleavage sites: one, Lys-Arg in the murine or Arg-Arg in the human sequence, is located upstream of nociceptin, and the other, Arg-Arg-Arg, is located downstream of nociceptin. Pronociceptin may therefore serve as the precursor not only to nociceptin but also to other physiologically important neuropeptides (see *Discussion*).

Tissue Distribution of Rat PPNOC mRNAs. Northern blot analysis (Fig. 3) revealed that PPNOC mRNAs are present as a single species about 1.3 kb long in rat nerve tissue (brain and spinal cord) as well as in ovary. No signal could be detected in RNA extracts from peripheral tissues, including liver, intestine, stomach, lung, spleen, adrenal gland, and testis. In brain, the PPNOC mRNA was particularly abondant in hypothalamus and striatum and appeared as a weak signal in hippocampus and cortex. It was absent in pituitary gland and olfactory

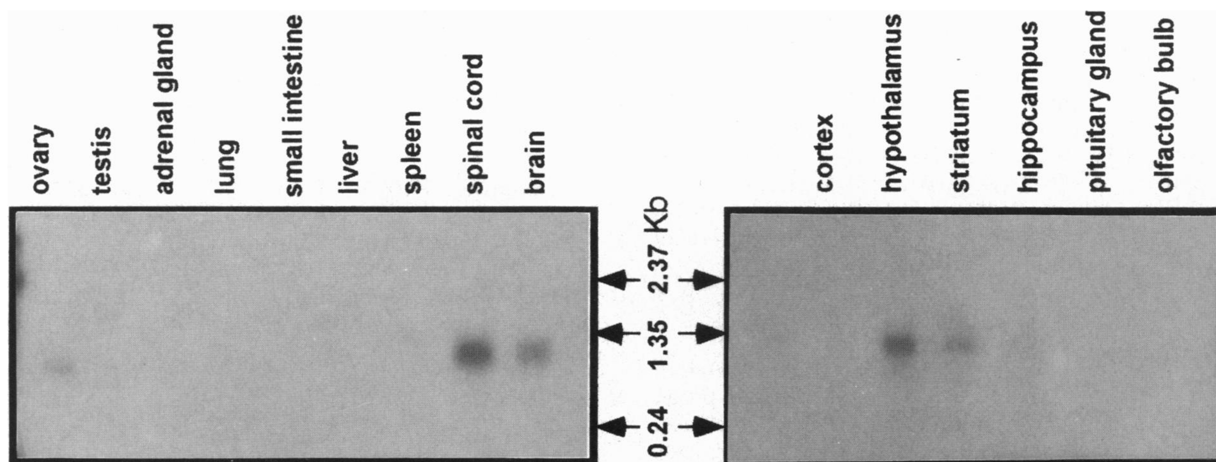

FIG. 3. Tissue-specific expression of rat PPNOC mRNA. Northern blotting analyses were performed with 5 μg poly(A)$^+$ mRNAs from rat tissues (*Left*) or 3 μg poly(A)$^+$ mRNAs from brain regions (*Right*). Hybridization was with the ^{32}P-labeled rat pronociceptin cDNA (926 bp; ref. 1) and the blots were exposed for 24 h. Calibration was with the RNA ladder from GIBCO/BRL.

bulb. Since integrity of individual RNA preparations was routinely checked with a β-actin probe (not shown), the absence of PPNOC mRNA was unlikely to be artifactual—i.e., accounted for by degradation. Overall, these data indicated that the PPNOC gene is expressed predominantly in the central nervous system. Expression of the PPNOC gene correlates well with expression of the ORL_1 (nociceptin) receptor gene (3–7, 9), with the possible exception of immune cells in which a few authors have reported the presence of ORL_1 transcripts or splice variants (10, 19).

Mapping of the Human PPNOC Gene. The human PPNOC gene was mapped by performing PCRs on the radiation hybrid cell lines of the GeneBridge 4 panel (data not shown; available on request). The RHMAPPER program allowed to assign unambiguously the gene to human chromosome 8, between STS markers WI-5833 and WI-1172 (Fig. 4). The PPNOC gene appears to be located closer to the WI-1172 marker [1.9 centiRays (cR)] than to the WI-5833 marker (6.9 cR). Given the relation between cR and megabase (Mb) reported for chromosome 8 (4.9 cR/Mb; ref. 14), this places the PPNOC gene about 0.4 Mb distally to WI-1172. This localization falls within the WC8.4 YAC contig, and in the 8p21 region of the genome, in close proximity of the NEFL locus encoding the neurofilament light polypeptide (also mapped between the WI-5833 and WI-1172 markers within the WC8.4 contig; ref. 14). The presence of the PPNOC gene was further investigated by PCR in 29 individual YAC clones belonging to the WC8.4 contig and 7 clones of the flanking WC8.1, WC8.2 and WC8.3 contigs (see *Materials and Methods*). None of these 35 YAC clones appeared to include the PPNOC gene, although the control PCR performed simultaneously on human genomic DNA was positive. This suggests that the PPNOC gene falls into a gap of the YAC coverage, within the WC8.4 contig. A chimeric YAC (686G9) reported to cover NEFL and loci proximal to WI-1172 also turned out to be negative.

DISCUSSION

Here, we have characterized PPNOC, the gene encoding the precursor protein of nociceptin/orphanin FQ (1, 2), the endogenous agonist of ORL_1 (3–10). The PPNOC gene closely resembles those of the PPENK, PPDYN, and PPOMC precursors to opioid peptides in that the four genes contain an intron splitting the coding sequence at a similar site and an intron in the 5′-UTR (Fig. 1). The PPNOC gene contains an additional intron in the 3′-UTR. This additional intron does not allow to encode alternate peptides or proteins by alternative splicing, since all frames are rapidly interrupted by stop codons. In addition, Northern blotting performed with the rat cDNA including the fourth exon detected a single transcript, with a size of around 1300 bases (Fig. 3). Interestingly, the genes encoding the nociceptin, μ-, δ- and κ-opioid receptors also bear organizational similitudes with two introns in their coding sequences, one in the first cytoplasmic and the other in the second exofacial loops (3, 8). These homologies suggest that the four precursor genes on the one hand, and the four receptor genes on the other hand, have evolved in parallel, each set from a common ancestor.

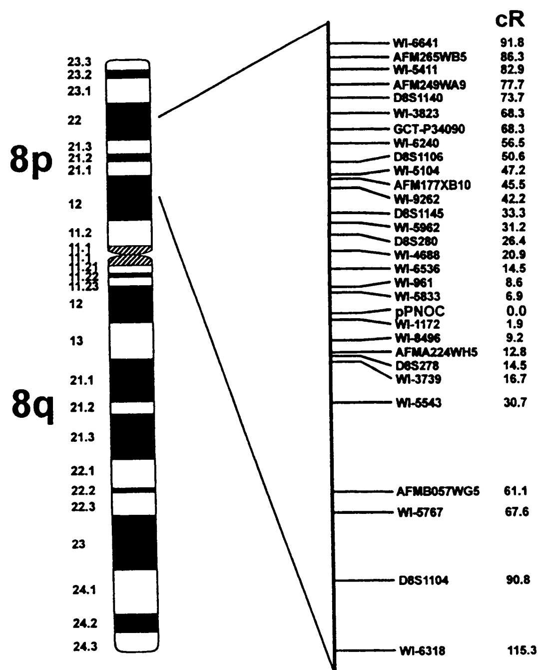

FIG. 4. Representation of human chromosome 8 and of the physical map of its short arm, in the neighborhood of the PPNOC gene. Distances are represented in cR, starting from the PPNOC locus on both sides.

The PPNOC gene encodes a single copy of nociceptin, differing in this respect from the PPENK and PPDYN genes which encode each several copies of Met- and/or Leu-enkephalin (15, 16). In fact, PPNOC is more like the gene of PPOMC (17) which encodes a single copy of enkephalin as well as of other hormones (γ-melanocyte stimulating hormone, adrenocorticotropic hormone, β-lipotropic hormone, etc.). The fact that PPNOC contains other potential cleavage recognition sites than those framing nociceptin, strongly suggest that PPNOC serves as the precursor not only to nociceptin but also to other biologically significant neuropeptides. There are several candidates including the obvious C-terminal peptide of 28 amino acids whose sequence is strictly conserved across murine and human species and which would be necessarily generated along with nociceptin. Yet further processing of this peptide at an arginyl triplet might also yield two additional neuropeptides, the heptadecapeptide FSEFMRQYLV-LSMQSSQ and the octapeptide TLHQNGNV. The sequence of PPNOC contains a third cleavage site (Lys-96–Arg or Arg96–Arg) which, together with the one at the N-terminal end of nociceptin, delimitate amino acid sequences of variable length and of low conservation across species. The variation in length between the human, rat, and mouse precursors is essentially due to the repetition of a six amino acid motif (DAEPGA), present a single time (and divergent from the above consensus) in human, twice in rat, and three times in mouse. In this latter species, the repeats are perfectly conserved, both at the DNA and protein level, and are therefore reminiscent of minisatellites that generate polymorphism among individuals by unequal crossing over. Such phenomenon is however not common in coding sequences, and it is not known whether such individual polymorphism of the PPNOC gene does occur in mouse. The acidic character of this portion is reminiscent to the CLIP (corticotropin-like intermediate lobe peptide), a peptide of yet-unknown function derived from the adrenocorticotropin hormone. These peptides may therefore not be of utmost physiological importance. It is worthy of note however that the Lys/Arg-96–Arg cleavage site is surrounded by strictly conserved amino acid sequences, suggesting that it might be normally used to produce, for instance, a PPNOC maturation intermediate.

The PPNOC gene is essentially expressed in nerve tissues where its distribution correlates with the localization of ORL_1 transcripts. The presence in ovary and hypothalamus suggests that the nociceptin system may also play a role in endocrine regulation. However, assuming that the precursor is potentially able to generate other peptides in addition to nociceptin, the actual release of each of the peptides in these tissue will require confirmation. Some authors have described the expression of the ORL_1 gene in spleen and lymphocytes, but only when the immune system is stimulated (10, 19). We could not detect PPNOC mRNAs in normal spleen (this study). Nevertheless,

it is not excluded that the nociceptin system could be activated under inflammatory conditions and could therefore modulate the immune response.

The PPNOC gene was localized to the 8p21 region of the human genome by using a radiation hybrid panel. The localization on chromosome 8 was independently confirmed by using a somatic hybrid cell panel (results not shown). No YAC clones containing the gene could be found, despite the fact that the interval between markers WI-5833 and WI-1172 is reported to be included within the WC8.4 YAC contig. This indicates that the PPNOC gene falls within a gap of the WC8.4 contig. The presence of a gap within this contig is also suggested by data available from the genome data base: the WI-12748 marker, also located between WI-5833 and WI-1172 by analysis of radiation hybrids, has no reported YAC hits. At present, the location of the PPNOC gene does not allow to suggest its implication in the pathogenesis of human diseases.

We thank M. Samson, D. Duchemin and N. Gomes for their help with the analysis of genomic clones and chromosomal mapping. This work was supported by the Belgian Programme on Interuniversity Poles of Attraction (Prime Minister's Office, Science Policy Programming), the Fonds Médical Reine Elisabeth, the Belgian Fonds de la Recherche Scientifique Médicale, the French Association pour la Recherche sur le Cancer, and Boehringer Ingelheim.

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