Journal of Neurochemistry, 2001, **77**, 720–729

Differential splicing of transcripts encoding the orphanin FQ/nociceptin precursor

Jamshid Arjomand* and Chris J. Evans†

**Neuroscience Interdepartmental Program, †Department of Biobehavioral Sciences and Psychiatry, UCLA, Los Angeles, USA*

Abstract

Orphanin FQ or nociceptin (OFQ/N), the heptadecapeptide agonist for the NOP receptor, is derived by proteolytic processing from a precursor protein, preproOFQ/N. Previous studies have reported alternative splicing between exons 3 and 4 of the mouse OFQ/N transcript, which, upon translation, would yield precursor proteins with different C-termini. Using RT-PCR, we identified similar alternative splicing of preproOFQ/N transcripts in humans and rats. In addition, we identified two novel human preproOFQ/N splice variants from which exon 2 has been excised and which also undergo alternative splicing between exons 3 and 4. Exon 2 contains the translational start site for preproOFQ/N and encodes the signal peptide sequence. *In vitro* translation of cRNAs lacking exon 2 yields shorter translation products which arise from an alternative initiator methionine located within exon 3. The resulting proteins would lack a signal peptide sequence, which would likely alter their cellular trafficking and processing.

Keywords: alternative splicing, nociceptin, NOP, ORL1, orphanin FQ, RT-PCR.

J. Neurochem. (2001) **77**, 720–729.

Orphanin FQ (OFQ) or nociceptin (N), a 17 amino acid peptide, has been isolated independently by two groups and shown to bind and activate the NOP receptor, also known as the ORL1 receptor (Meunier *et al*. 1995; Reinscheid *et al*. 1995). The NOP receptor is a seven transmembrane G protein-coupled receptor which has ≈60% amino acid identity to the classical opioid receptors (mu, delta and kappa) (Mollereau *et al*. 1994). Similar to the opiate system, OFQ/N binding to the NOP receptor activates pertussis toxin-sensitive Gi/o proteins, which can inhibit adenyl cyclase (Meunier *et al*. 1995; Reinscheid *et al*. 1995), inhibit voltage-gated calcium currents (Connor *et al*. 1996), induce potassium currents (Matthes *et al*. 1996) and activate the MAP kinase pathway (Hawes *et al*. 1998). However, unlike the opiate system, pharmacological studies have revealed a complex role for OFQ/N in the regulation of analgesia. Factors such as the route of peptide administration, dosage, strain of animals, behavioral state of the animals and the types of behavioral tests used have been shown to affect whether OFQ/N elicits analgesia or hyperalgesia (reviewed by Zaki 1998; Harrison and Grandy 2000). In addition to effects on pain perception, treatment with OFQ/N has also been shown to modulate locomotion, autonomic output, memory and states of anxiety in response to acute stressors (reviewed by Harrison and Grandy, 2000; Zaki 1998).

OFQ/N is derived from a longer precursor protein, preproOFQ/N (ppOFQ/N), which encodes other putative bioactive peptides (Meunier *et al*. 1995; Nothacker *et al*. 1996; Pan *et al*. 1996; Okuda-Ashitaka *et al*. 1998; Rossi *et al*. 1998). Immediately preceding OFQ/N, and flanked by dibasic residues, a peptide named nocistatin (Nct) has been isolated from rat, mouse and human CNS (Lee *et al*. 1999). When administered intrathecally, Nct is effective at inhibiting OFQ/N-induced hyperalgesia (Okuda-Ashitaka *et al*. 1998; Yamamoto and Sakashita 1999), inhibiting OFQ/N-induced glutamate release from rat cerebrocortical slices (Nicol *et al*. 1998), and blocking OFQ/N-precipitated memory impairment (Hiramatsu and Inoue 1999a, b). The effects of Nct are mediated by, as yet uncharacterized, receptor(s) that appear distinct from the NOP receptor (Okuda-Ashitaka *et al*. 1998). C-Terminal to the OFQ/N

Received September 21, 2000; revised manuscript received December 19, 2000; accepted December 20, 2000.

Address correspondence and reprints requests to Chris J. Evans, Department of Biobehavioral Sciences and Psychiatry, UCLA-NPI, 760 Westwood Plaza, Los Angeles, CA 90024-1759, USA.
E-mail: cevans@ucla.edu

Abbreviations used: Met, methionine; Nct, nocistatin; OFQ2, orphanin FQ2; ORL1, opioid receptor like 1; ppOFQ/N, preproorphanin FQ/nociceptin; RPA, ribonuclease protection assay; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1 PCR primers

No.	Species	Sense	Exon	Sequence (5′−3′)
P1	Rat	+	II	CTCTCCAGCGTGTTCAGC
P2	Rat	–	IV	GGGTGTTCAGTGCCTCAG
P3	Human	+	II	GGATCCGCACCATGAAAGTCCTGCTTTG
P3b	Human	–	III	GAATTCACTGAACCGCTTCTGATTGG
P4	Human	–	IV	AGCAACAGGGTTGTGGTGAAAC
P5	Human/Mouse	+	I	GATATATATGCTGGTGTGGCTG
P6	Human/Mouse	–	III/IV	TCACCTGGACGCTCATGGGTT
P7	Rat	+	β-Actin	TACAACCTCCTTGCAGCTCC
P8	Rat	–	β-Actin	TCTTCATGAGGTAGTCTGTC

Underlined bases correspond to a *Bam*HI or *Eco*RI restriction site added to primers P3 and P3b, respectively, for purposes of directional subcloning.

peptide in ppOFQ/N, and also flanked by dibasic amino acids, is a putative heptadecapeptide that is 100% conserved among mice, rats and humans. Named OFQ2, intracerebroventricular injections of this peptide in mice induce dopamine-mediated locomotor activity (Florin *et al*. 1997). In addition, either intracerebroventricular or intrathecal administration of OFQ2 in mice has been shown to have antinociceptive properties (Rossi *et al*. 1998). The mechanisms mediating these behavioral actions remain to be elucidated.

ppOFQ/N is encoded by an ≈1.3-kb transcript derived from four exons (Mollereau *et al*. 1996). Exon 1 constitutes the majority of the 5′-UTR. Exon 2 contains the translational start site and the signal peptide, while exon 3 contains the coding region for the multiple bioactive peptides. Exon 4 encodes the 3′-UTR and polyadenylation signal (Mollereau *et al*. 1996). Very recently the promoter region upstream of exon 1 has been characterized and shown to contain a number of regulatory sites including cAMP response elements and glucocorticoid receptor binding sites (Xie *et al*. 1999).

Coinciding with the discovery of OFQ/N, Saito and colleagues independently isolated both ppOFQ/N (N23K), as well as an alternative splice variant (N27K), from dibuyryl cAMP-treated mouse neuroblastoma cell line NS20Y (Saito *et al*. 1995, 1996). The alternatively spliced ppOFQ/N transcript, N27K, results from the excision of 57 bases from the 3′-end of exon 3. This shorter transcript removes a stop codon in exon 3 and thereby extends the C-terminus of ppOFQ/N by 25 amino acids and into exon 4 (Saito *et al*. 1996). This group demonstrated that transfection of NS20Y neuroblastoma cells with either of the alternative splice constructs promotes neurite outgrowth and that cotransfection with both splice products is more efficacious than transfection with either alone (Saito *et al*. 1997). This suggests that the two proteins may independently affect neuronal differentiation. In addition, processed OFQ/N peptide was shown to be ineffective at promoting this neuronal differentiation, suggesting that the NOP receptor alone does not mediate these effects (Saito *et al*. 1997).

Because alternative splicing of the mouse ppOFQ/N transcripts can yield two different precursor proteins, it was of interest to determine whether this splicing pattern is conserved in other species. Here, we used RT-PCR to analyze the spliced products of the rat and human ppOFQ/N transcripts and describe identical alternative use of donor sites in exon 3 of both species. Furthermore, from the human cortex, we identified two novel splice variants of ppOFQ/N that eliminate exon 2 from their transcripts. *In vitro* translation experiments of these novel transcripts reveal that translation can be initiated from an alternative translational start site located within exon 3, resulting in truncated forms of the precursor proteins devoid of signal peptide sequences.

Materials and methods

RNA preparation

Unless noted otherwise, adult male Sprague−Dawley rats and BALB/c mice were used and tissues of interest dissected and snap frozen on dry ice. Human surgical cortex was similarly frozen and kindly provided by Dr Harry Vinters. Total RNA was extracted using Trizol reagent (Life Technologies, Rockville, MD, USA) according to manufacturer's recommendations. mRNA from adult rat brain was isolated using the Poly(A)Tract kit (Promega, Madison, WI, USA). RNA integrity was assessed by agarose gel electrophoresis and concentration assessed spectrophotomentrically. Developmental Sprague−Dawley rat brain mRNA samples and human fetal brainstem total RNA samples were generously provided by Drs Jim Boulter and Thomas Prybil, respectively.

Reverse transcription PCR

First-strand cDNA synthesis was performed using either 2 μg of total RNA or 300 ng of poly(A^+) RNA with Superscript II (Life

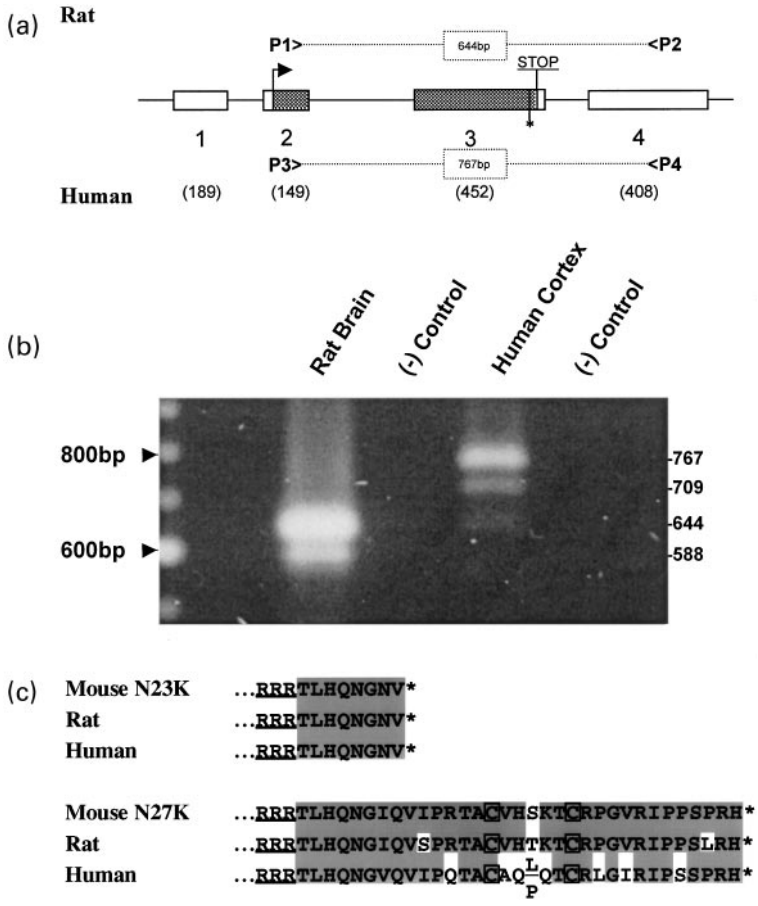


Fig. 1 Conservation of OFQ/N exon 3 splicing in the rat and human. (a) Gene structure of preproOFQ/N. Exons are boxed, numbered sequentially and the previously established exon sizes (in bp) are indicated for the human in parentheses (Mollereau *et al.* 1996). The translational start site for ppOFQ/N is marked with an arrow, the coding region is denoted with cross-hatching and the putative alternative splice junction is indicated by an asterisk. The PCR primers used to span the putative splice site at the 3′-end of exon 3 for each species are listed in Table 1 and are marked in bold. The expected lengths of the amplified products are in the dotted boxes. (b) RT-PCR of rat and human brain spanning exons 2–4. Two micrograms of total RNA was reversed transcribed and subjected to PCR using the primer pairs indicated in (a) and the parameters described in Materials and methods. The (–) control represent PCR reactions for rat and human RNA from which the reverse transcriptase was omitted. DNA molecular mass size markers are on the left, whereas the numbers on the right correspond to the size (bp) of the individual PCR products. (c) Comparison of the translated C-termini of the differentially spliced ppOFQ/N transcripts across species. Potential basic processing sites have been underlined, conserved amino acids shaded, and conserved cystein residues boxed.

Technologies) in the recommended buffers and either 150 ng of random hexamers or 50 ng of oligo(dT), respectively. One-tenth of the RT reactions were used in each of the subsequent amplification reactions using the primer pairs listed in Table 1. All primers were synthesized by Gibco-BRL (Life Technologies). ppOFQ/N primers were based on the rat and human published sequences (Accession nos U48262and U48263, respectively), whereas the β-actin primers were selected according to Raff *et al*. (1997). The final amplification conditions were as follows: 20–25 pmoles of each primer, 2.5 mM $MgCl_2$, 200 μM dNTP and 2.5 units of Hot Start Taq Polymerase (Qiagen, Valencia, CA, USA). Amplification for all tissues was carried out in a GeneAmp 2400 cycler (Perkin-Elmer, Foster City, CA, USA) and performed following an initial denaturation step of 15 min at 94°C. For rat tissues, 35 PCR cycles were performed with denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. Amplification from human cortex was carried out using a Touchdown-Hot Start PCR protocol. Briefly, denaturation and extension conditions were as described above, but the annealing temperature was reduced from 60 to 50°C, with three cycles at 60, 57, 54 and 52°C and 25 cycles at 50°C.

PCR products were separated by electrophoresis on 1.5% agarose gels in 1× TAE or 1× TBE, stained with ethidium bromide and visualized by UV illumination. Polaroid photographs were taken, scanned and imported into PHOTOSHOP 5.0 (Adobe, San Jose, CA, USA).

Subcloning and sequencing

PCR products were subcloned using PCR-Script Amp cloning kit (Stratagene, La Jolla, CA, USA) and individual clones sequenced using [^{35}S]dATP chain termination reaction of Sequenase 2.0 (Amersham, Piscataway, NJ, USA) or sent to the Davis sequencing facility for automated sequencing. Sequence comparison and post-translational protein analyses were performed using MACVECTOR or web browser-based software (http://www.cbs.tdu.dk).

Ribonuclease protection assay

A cloned fragment of the human OFQ/N transcript was further amplified for 15 cycles using primer pairs P3 and P3b. The resulting 456 bp fragment, corresponding to a segment spanning exons 2 and 3 of the human OFQ/N message, was subcloned into pBS SK(–) cloning vector (Stratagene), sequenced, linearized with *Not*I (Life Technologies), and used as a template for cRNA synthesis using T7 RNA polymerase (Promega) in the presence of [^{32}P]CTP nucleotide (NEN, Boston, MA, USA). Unlabeled sense cRNA was synthesized with T3 RNA polymerase (Promega), using *Eco*RV-digested clones representing human N23K and N23K

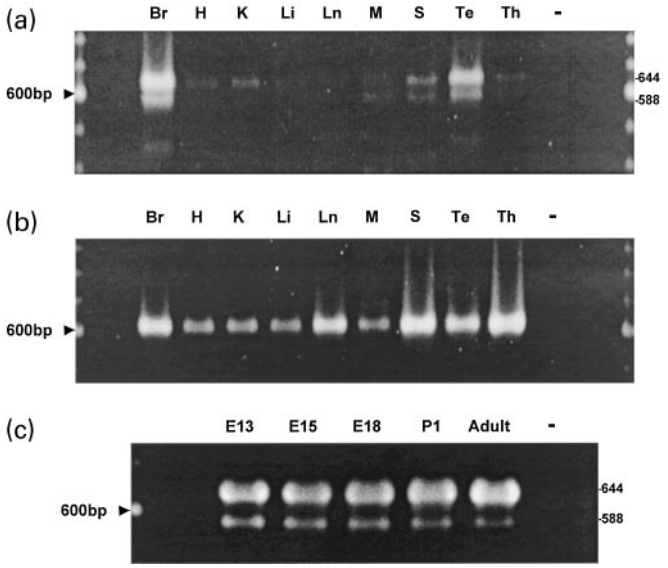


Fig. 2 Rat N23K and N27K ppOFQ/N transcript distribution. (a) Distribution of the rat N23K and N27K ppOFQ/N mRNAs in peripheral tissues and brain. Two micrograms of total RNA from various tissues were reversed transcribed and subjected to PCR using primers spanning exons 2–4. The different tissues analyzed were: Br, brain; H, heart; K, kidney; Li, liver; Ln, lung; M, muscle; S, spleen; Te, testis; Th, thymus; (–), brain RNA without RT. (b) Rat β-Actin control RT-PCR of various tissues. Tissue samples were amplified using actin primers P7 and P8 (Table 1) using conditions specified in Materials and methods. (c) Developmental expression of rat brain N23K and N27K ppOFQ/N transcripts. A sample of 300 ng poly(A^+) RNA from the CNS of embryonic to adult stages was reverse transcribed and amplified as described in Materials and methods. E13 to E18, embryonic day 13–18; P1, postnatal day 1; (–), negative control using water in lieu of cDNA.

minus exon 2 ppOFQ/N transcripts. The sense cRNA samples were purified through a Sephadex G50 column and their concentrations assessed spectrophotometrically. Protection assays were conducted using HybSpeed RPA kit (Ambion, Austin, TX, USA) according to the manufacturer's suggested protocols. Samples were electrophoresed in 5% acrylamide gels and exposed overnight to X-OMAT film (Eastman Kodak, Rochester, NY, USA) at −70°C with an intensifying screen. Quantitation of the protected fragments was performed by densitometry analyses (Molecular Dynamics, Sunnyvale, CA, USA).

In vitro transcription and translation

Depending on the orientation of the clones, vectors were linearized using either *Eco*RV or *Sac*II restriction enzymes (New England Biolabs, Beverly, MA, USA) that cut at unique sites within the multipurpose cloning site. Sense RNA was synthesized from each clone using the appropriate T3 or T7 polymerase (Promega). Following phenol/chloroform extraction and ethanol precipitation, the RNA was translated *in vitro* with [^{35}S]methionine using Retic Lysate IVT (Ambion). The resulting reactions were electrophoresed along with rainbow molecular mass markers (Amersham) on a 15% polyacrylamide gel, fixed with 10% acetic acid and 45% methanol, dried and exposed using a phosphoimager screen (Kodak/Molecular Dynamics).

Results

Alternative splicing of ppOFQ/N transcripts within exon 3 in rat and human brain

The gene structures of the rat and human ppOFQ/N and the location of the PCR primers used in this study are depicted in Fig. 1 (a). RT-PCR analysis of rat and human brain RNA using primers spanning exons 2–4 yielded the expected length fragment of 644 and 767 bp, respectively. In addition, a shorter band from both species was also amplified (Fig. 1b). Sequence analysis of all the amplified fragments revealed that the shorter bands corresponded to a ppOFQ/N message from which the 3′-terminal region of exon 3 is omitted. The use of such an alternative donor splice site corresponds exactly to the previously described murine N27K splice variant (Saito *et al*. 1996). The predicted translated C-terminus for both ppOFQ/N splice variants of the rat and human have been aligned with the mouse sequence in Fig. 1(c) (Saito *et al*. 1996). The C-terminal octapeptide following the presumptive processing site (Arg-Arg-Arg) of N23K is identical between rat, mouse and human. In the case of N27K, the 33 amino acid peptide that follows the same potential processing site has ≈66% identity between the species examined. Of note, is the conservation of two cysteine residues in all of the extended C-termini examined. In addition, a polymorphism at position 825 of the human sequence (adenine or cytosine) noted previously in the published sequence (Accession no. U48263; Nothacker *et al*. 1996) was also observed from the sequencing of multiple independent tissue samples.

Tissue distribution of the rat ppOFQ/N N23K and N27K messages

RT-PCR analysis was performed in order to analyze the tissue distribution of ppOFQ/N transcripts in the rat. Fragments corresponding in size to both N23K and N27K transcripts were observed following amplification of RNA isolated from brain and testis (Fig. 2a). Less intense bands

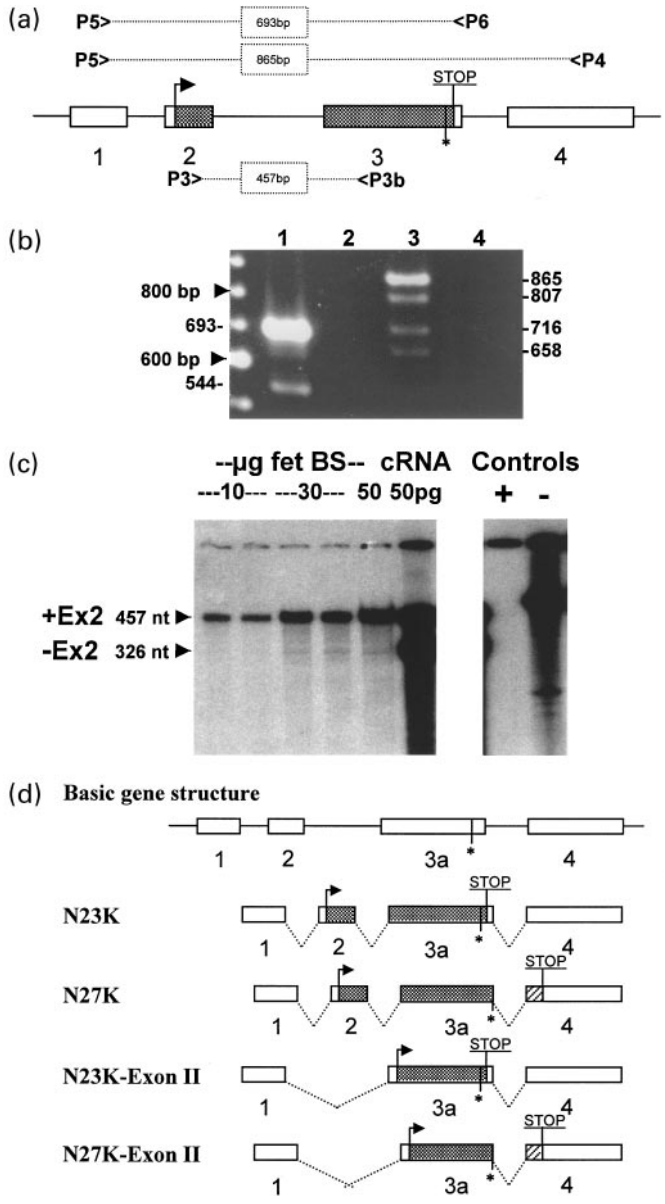


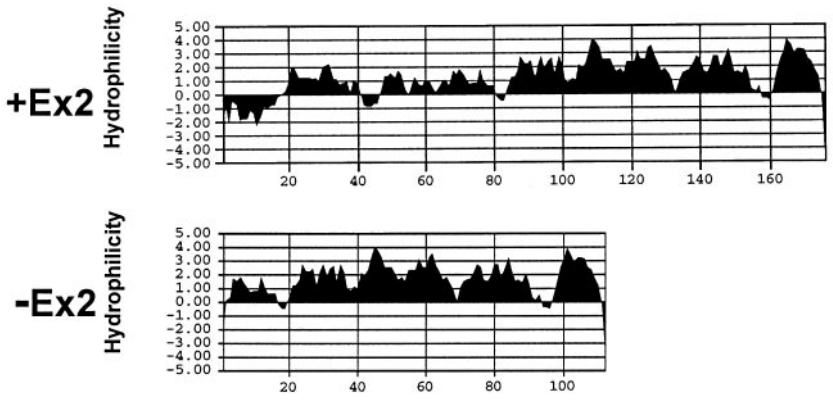
Fig. 3 Identification of human ppOFQ/N splice variants lacking exon 2. (a) Schematic of the human ppOFQ/N gene and location of PCR primers. Gene features are marked as in Fig. 1(a). The PCR primers used to span exon 2 and/or the 3′-end of exon 3 are marked in bold on top of the gene and are listed in Table 1. The PCR primers used for the preparation of the RPA probe are noted below the gene. The expected lengths of the amplified products are in the dotted boxes. (b) Human cortex cDNA was amplified either with primers spanning exons 1–3 or primers spanning exons 1–4. Lane 1, cDNA amplified with primers spanning exons 1–3; lane 2, no RT control for lane 1; lane 3, cDNA amplified with primers spanning exons 1–4; lane 4, no RT control for lane 3. (c) The abundance of ppOFQ/N transcripts with or without exon 2 was compared using an RPA probe spanning exons 2 and 3 of the message. The signals generated from increasing amounts of human brain stem RNA (10 and 30 µg, each assayed in duplicates, or 50 µg) were compared with the signal generated by incubating 50 pg of each RNA synthesized *in vitro*. The 457 nucleotide protected fragment represents transcripts containing exon 2, whereas the 326 nucleotide fragment represents transcripts lacking exon 2. (+) Control digest containing 50 µg yeast RNA, probe, and RNAse; (–) control reaction containing 50 µg yeast RNA and probe only. (d) Schematic of alternative splicing of human ppOFQ/N transcripts. N23K corresponds to the longest transcript detected. N27K depicts the splice product missing the 3′-end of exon 3, thus extending the ORF into exon 4. Other transcripts depicted are N23K and N27K lacking exon 2. Potential translation start sites of the different transcripts are depicted by arrows.

Fig. 4 Sequence and translation of human ppOFQ/N. (a) Translation of ppOFQ/N transcripts. Predicted translation start sites are in bold. Exon 2, which is spliced in some transcripts, is underlined. Paired basic amino acids delineating potential protease cleavage sites are boxed. The amino acid sequence denoting nocistatin is underlined, the OFQ/N sequence is in bold and the presumed OFQ 2 sequence is indicated by a dotted underline. The alternatively spliced 3′-region of exon 3 and the encoding amino acids are in bold and italicized and the resulting extended translation product is continued into exon 4. Stop codons are marked with asterisks. M# in the nucleotide sequence denotes the observed adenine or cytosine polymorphism. (b) Hydrophilicity plot for human N23K preproOFQ/N (upper), or N23K ppOFQ/N lacking exon 2 and translated from an in-frame methionine located in exon 3 (lower), as determined by Kyte-Doolittle with a window of 7. (c) *In vitro* translation of various cloned human ppOFQ/N cRNAs. Plasmids were linearized, *in vitro* transcribed with T3 or T7 RNA polymerase, and translated with reticulocyte lysate in the presence of [^{35}S]methionine. Lane 1, human N23K; lane 2, human N27K; lane 3, human N23K minus exon 2; lane 4, human N27K minus exon 2; (–), negative control devoid of RNA; (+), positive control *Xenopus* elongation factor-1; (L), ^{14}C labeled molecular size marker.

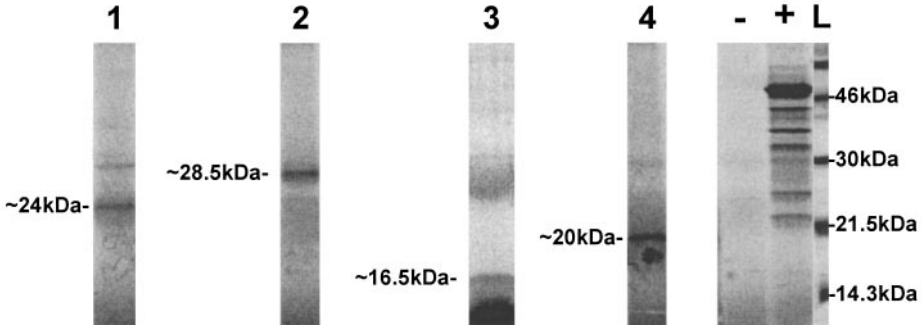
(a)

```
1     GCCAGGAAGGCTTGCAGGTTCTGCTGTTTGGTTGCTGAAGGGGGTCAGTGTGTGTATGTGTCATGGAGGTGGGCA
76    GGGAAGGGGAGGGCTGTGCGTGGGGGAGATGAGGATATATATGCTGGTGTGGCTGAGAAAGCGGAACCGAGCCTC
151   GCATCCATCGGAGGGAGCCGGGGACTGACAGCTCTCAGCACCTGCTTCCTGCTCCTGCACCATGAAAGTCCTGCT
                                                                M  K  V  L  L
226   TTGTGACCTGCTGCTGCTCAGTCTCTTCTCCAGTGTGTTCAGCAGTTGTCAGAGGGACTGTCTCACATGCCAGGA
        C  D  L  L  L  L  S  L  F  S  S  V  F  S  S  C  Q  R  D  C  L  T  C  Q  E
301   GAAGCTCCACCCAGCCCTGGACAGCTTCGACCTGGAGGTGTGCATCCTCGAGTGCGAAGAGAAGGTCTTCCCCAG
        K  L  H  P  A  L  D  S  F  D  L  E  V  C  I  L  E  C  E  E  K  V  F  P  S
376   CCCCCTCTGGACTCCATGCACCAAGGTCATGGCCAGGAGCTCTTGGCAGCTCAGCCCTGCCGCCCCAGAGCATGT
        P  L  W  T  P  C  T  K  V  M  A  R  S  S  W  Q  L  S  P  A  A  P  E  H  V
471   GGCGGCTGCTCTCTACCAGCCGAGAGCTTCGGAGATGCAGCATCTGCGGCGAATGCCCCGAGTCCGGAGCTTGTT
        A  A  A  L  Y  Q  P  R  A  S  E  M  Q  H  L  R  R  M  P  R  V  R  S  L  F
526   CCAGGAGCAGGAAGAGCCCGAGCCTGGCATGGAGGAGGCTGGTGAGATGGAGCAGAAGCAGCTGCAGAAGAGATT
        Q  E  Q  E  E  P  E  P  G  M  E  E  A  G  E  M  E  Q  K  Q  L  Q  K  R  F
601   TGGGGGCTTCACCGGGGCCCGGAAGTCGGCCAGGAAGTTGGCCAATCAGAAGCGGTTCAGTGAGTTTATGAGGCA
        G  G  F  T  G  A  R  K  S  A  R  K  L  A  N  Q  K  R  F  S  E  F  M  R  Q
676   ATACTTGGTCCTGAGCATGCAGTCCAGCCAGCGCCGGCGCACCCTGCACCAGAATGGTAATGTGTAGCCGGAAGG
        Y  L  V  L  S  M  Q  S  S  Q  R  R  R  T  L  H  Q  N  G  N  V  *
751   GGCGCTCCTCCCAGCTGTACCGGCCACTGCAACCCATGAGCGTCCAGGTGATCCCCCAAACAGCATGTGCTCAGM#
                                                          V  Q  V  I  P  Q  T  A  C  A  Q
826   CCCAGACCTGCCGCCTGGGAATCAGGATTCCTTCTTCCCCAAGGCACTGAGCGCCTGCAGATCCCGCAGGCTTCG
    L/P Q  T  C  R  L  G  I  R  I  P  S  S  P  R  H  *
901   TTTGCCTCCAGAACCTTCCCGTCTGATTGTTCCTCCCCAGCCCCCTGGCATGTTTCACCACAACCCTGTTGCTAC
976   ATCAGAGTGTATTTTTGTAATTCCTCTAGCTACCATTTCAATAGCCCCATCTCTCCTGCTCACCCGCCTCTTGCC
1051  CCTTCTAGGGGCAGGTGAAAGGAATAGGAAATTGAACCTGGGGTTTTGACTTGCCACTGCCATAACTTGTTTGTA
1126  AAAGAGCTGTTCTTTTTGACTGATTGTTTTAAACAACGATTTCTCCATTAAACTTCTACTGAGCAAATGGTTA
```

(b)



(c)



were observed in the spleen, whereas amplification of OFQ/N transcripts was either undetectable or negligible in the heart, liver, lymph nodes, muscle and thymus. Concurrent PCR reactions using actin primers of the same tissue samples yielded a 630-bp product corresponding to the rat β-actin and served as a positive control for the reverse transcription reaction (Fig. 2b).

Developmental expression of the rat ppOFQ/N N23K and N27K analogs

We examined the developmental expression of the rat ppOFQ/N transcripts by RT-PCR. Brain poly(A^+)-selected mRNA samples representing various time points during development were analyzed using RT-PCR for the presence of transcripts encoding N23K or N27K. Our analysis revealed that both messages were expressed from the earliest time point examined (embryonic day 13) into adulthood (Fig. 2c). Although the assay was not designed to be quantitative, the ratio of the full-length (N23K) transcript to the splice variant (N27K) appeared relatively consistent at all developmental time points examined, with the N23K band being more prominent.

Additional ppOFQ/N splice variants in the human cortex

Using primers P5 and P6, which span exons 1 and 3 of the human ppOFQ message, RT-PCR analysis of human cortex resulted in the amplification of the expected (693 bp) and a shorter (544 bp) fragment (Fig. 3b, lanes 1 and 2). Sequence analysis of the longer fragment corresponded to the expected ppOFQ/N transcript, whereas analysis of the shorter band revealed the complete excision of exon 2. Further PCR analysis using primer pairs spanning exons 1–4 revealed a total of four fragments (Fig. 3b, lane 3). Analogous RT-PCR experiments using human fetal brain stem RNA revealed a similar amplification pattern to cortex (data not shown). Sequence analysis revealed that the two longest products, 865 and 807 bp, correspond to the previously described N23K and N27K ppOFQ/N transcripts, whereas the shorter fragments represent the same transcripts lacking exon 2. In order to assess the abundance of these additional transcripts, we conducted ribonuclease protection assays (RPA). Increasing concentrations of human fetal brainstem total RNA or *in vitro* transcribed sense RNA corresponding to the human N23K and N23K minus exon 2 were hybridized to a cRNA probe partially spanning exons 2–3 (Fig. 3c). Protected fragments of 457 nucleotides represent transcripts containing exon 2, whereas the shorter protected fragments of 326 nucleotides depict transcripts lacking exon 2. Calculations based on densitometric analyses of the protected fragments in four independent experiments revealed that transcripts lacking exon 2 constituted only 7.1 ± 3% of the signal generated by exon 2-containing bands. The diagram in Fig. 3(d) illustrates the various ppOFQ/N splice variants present in human cortex. Transcripts lacking exon 2 were not detected in mouse CNS in PCR experiments using primers spanning exon 1 to exon 3 (data not shown).

***In vitro* translation of various human ppOFQ/N transcripts**

Examination of the human splice variants missing exon 2 indicates that Met65, located in exon 3, is in-frame with the OFQ/N peptide and encoded within a favorable Kozac consensus sequence (Fig. 4a). Computer-based analysis of the human N23K ppOFQ/N reveals that the first 19 amino acid residues are highly hydrophobic and represent a candidate signal peptide sequence, whereas the N23K minus exon 2 ppOFQ/N results in a hydrophilic N-terminal, characteristic of cytosolic proteins (Fig. 4b). Furthermore, the minus exon 2 translation products do not have any stretches of hydrophobic residues that could serve as potential signal peptides. *In vitro* translation of the human N23K and N27K cRNA transcripts resulted in the translation of proteins migrating at an estimated molecular mass of 24 and 28.5 kDa, respectively (Fig. 4d, lanes 1 and 2). Translation of the N23K and N27K splice variants lacking exon 2 clones resulted in proteins of ≈ 16.5 and 20 kDa, respectively (Fig. 4d, lanes 3 and 4).

Discussion

Previous studies of the mouse ppOFQ/N transcripts revealed alternative splicing at the 3′-end of exon 3 yielding two precursor proteins with different C-terminal sequences (Saito *et al*. 1996). The longer transcript, named N23K, corresponds to the cDNA clones isolated during the search for the endogenous NOP receptor ligand (Meunier *et al*. 1995; Reinscheid *et al*. 1995), and gives rise to a predicted precursor protein of 23 kDa containing a short C-terminus following OFQ2 (Fig. 1c). In the shorter transcript, named N27K, the stop codon in the ORF of exon 3 is removed, resulting in a longer predicted precursor protein with an extended C-terminal sequence.

Here, we report that the pattern of ppOFQ/N transcript splicing originally observed in the mouse is conserved in rat and human tissues (Fig. 1b) and that the predicted translation products of the shorter messages analogous to mouse N27K, also result in extended C-termini. Furthermore, there is a high degree of conservation at the C-terminus of the mouse, rat and human precursors in the predicted protein sequence from both transcripts (Fig. 1c). Following the dibasic amino acids of OFQ/N, the C-terminal of ppOFQ/N encodes the presumed peptide OFQ2, which is followed by three arginine residues and a varying carboxy tail depending on the splicing pattern of the ppOFQ/N message. Whether OFQ2 is cleaved *in vivo* at the triple arginines is not known, because the 17 amino acid peptide corresponding to the isolation of the predicted OFQ2 peptide have not yet been reported. If cleavage at this site does not occur, OFQ2 would

exist *in vivo* as a 28 amino acid peptide from N23K and a 53 amino acid peptide from N27K. Alternatively, if there is cleavage at the triple arginine residues, a C-terminal octapeptide would be released from N23K and a 33 amino acid peptide from N27K. The C-terminal octapeptide sequence of N23K is 100% conserved, whereas the 33 amino acids at the C-terminus of N27K are ≈66% identical across the species examined (Fig. 1c). As reported previously, the human sequence is polymorphic at residue 825 and was assumed to encode the 3′-UTR of the ppOFQ/N transcript (Fig. 4a) (Nothacker *et al*. 1996). In our study, polymorphism was confirmed by sequencing clones form several distinct donors and was mapped to the coding region of the extended C-terminal of the N27K splice variant. Polymorphism (adenine or cytosine) would alter the translation of the extended precursor protein to either a leucine or proline. Interestingly, this amino acid is the only highly divergent region of the extended N27K C-termini in the species examined and is located between two conserved cysteine residues (Fig. 1c). These conserved cysteine residues could potentially form disulfide bridges. Further analyses of the endogenous ppOFQ/N derived proteins are required to identify the exact nature of the peptides excised from the C-terminus of both the N23K and N27K.

Studies conducted by Saito *et al*. (1997) reported that murine ppOFQ/N splice variants were expressed during neuronal differentiation and that both transcript and protein levels diminished with maturation. Figure 2(c) demonstrates that both ppOFQ/N splice variants were present as early as embryonic day 13 in the rat brain and that the expression persisted into adulthood. In addition, it appears that the shorter transcript, corresponding to N27K, was consistently less abundant than the longer N23K transcript, at each time point examined. The differences observed may be attributed to the more sensitive RT-PCR method that we employed for the detection of OFQ/N gene products, as opposed to northern and western blots used by Saito *et al*. (1997). Given that the RT-PCR technique used here was not quantitative, it is not possible to assess whether the transcript levels varied over the course of rat brain development; however, the observed relative ratios of the two transcripts at each time point persisted even with fewer amplification cycles (data not shown).

Transcripts encoding OFQ/N have been identified in certain peripheral tissues using northern blots (Mollereau *et al*. 1996; Nothacker *et al*. 1996). Because this technique can not adequately resolve the differences in size between the alternative splice variants, we examined peripheral distribution of the transcripts using RT-PCR. In the rat tissues examined, we noted expression of both transcripts in the testis and minor expression in the spleen and kidney (Fig. 2a). The low expression of ppOFQ/N transcripts in the spleen and kidney tissues may be due to sympathetic innervation of these tissues, as it has been demonstrated that peripheral nerves express ppOFQ/N transcripts (Kummer and Fischer 1997). It is noteworthy that OFQ/N RNA expression in rat or mouse testes was not detected in previous studies (Saito *et al*. 1995; Mollereau *et al*. 1996; Nothacker *et al*. 1996), although faint expression was detected in rat ovaries (Mollereau *et al*. 1996). We attribute these differences to the sensitivity of the assays, as the aforementioned studies were based on northern analyses whereas our approach was PCR based. Interestingly, all classical opioid peptide precursor transcripts (pro-opiomelanocortin, proenkephalin, prodynorphin) have been isolated from testis (Pintar *et al*. 1984; Kilpatrick and Rosenthal 1986; Douglass *et al*. 1987). Because OFQ/N inhibits electrically evoked vas deferens constriction, it is highly probable that some of the ppOFQ/N-derived peptides may mediate reflexes involved in male sexual behavior (Berzetei-Gurske *et al*. 1996; Calo *et al*. 1996; Champion *et al*. 1997; Zhang *et al*. 1997; Nicholson *et al*. 1998). Interestingly, OFQ may also modulate sexual behavior at the central level, given that intracerebroventricular OFQ/N administration to female rats enhances the lordosis quotient in a dose-dependent manner (Sinchak *et al*. 1997).

In addition to the conserved alternative splicing at the 3′-end of exon 3, we isolated two novel human OFQ/N splice variants. RT-PCR experiments demonstrate that all four transcripts are detectable in human cortex (Fig. 3b, lane 3). Sequence analyses of the amplified fragments revealed that the most prominent and largest amplified fragments correspond to N23K and N27K, respectively, and are followed by two shorter fragments corresponding to transcripts devoid of exon 2.

In order to assess the abundance of the novel OFQ/N splice variants missing exon 2, we performed RPA experiments using human fetal brainstem RNA. Our findings suggest that the OFQ/N transcripts devoid of exon 2 are rare transcripts relative to those of N23K and N27K (Fig. 3c). Because of tissue scarcity, we did not examine the distribution or abundance of the OFQ/N splice variants in the adult human brain. Thus, we can not be certain whether the pattern of the protected fragments observed with the fetal brainstem RNA is representative of other brain regions and other developmental time points. When we examined the mouse for the occurrence similar OFQ/N splicing pattern, only transcripts containing exon 2 were amplified (data not shown). Because the rat OFQ/N exon 1 sequence has not yet been isolated, we were unable to perform similar analysis in this species. Further comparison with other species will be necessary to ascertain the species specificity of OFQ/N splicing pattern described in this study.

Splicing exon 2 from the primary ppOFQ/N transcript would remove the translational start site and the signal peptide sequence (a stretch of hydrophobic residues required for the targeting of proteins to the secretory pathway) from

the precursor. Sequence analyses of these splice variants reveal that the longest ORF resides in-frame with the OFQ/N peptide and begins at Met65 of the human ppOFQ/N (Fig. 4a). This methionine is encoded within a favorable Kozak consensus sequence containing a purine at position −3 and a guanine residue at position +4, relative to the ATG start site (Kozak 1996). Recently, it has been shown that functional signal peptides can exist in the middle of the sequence of precursor protein (Miyakawa *et al*. 1999). However, analysis of the predicted truncated ppOFQ/N precursor stemming from translation at Met65 indicates that these precursors would be devoid of any continuous stretch of hydrophobic amino acids that could serve as an internal signal peptide (Fig. 4c).

Previous studies have described multiple truncated POMC transcripts expressed in both rat and human extrapituitary tissues which, if translated, would lack a signal peptide (Jeannotte *et al*. 1987; Lacaze-Masmonteil *et al*. 1987). Due to the similarities between the truncated POMC and OFQ/N messages, we were interested in testing whether the OFQ/N Met65 could serve as a translational initiation site. The predicted translation products from N23K minus exon 2 and N27K minus exon 2 are calculated to be 13 and 15.8 kDa, respectively. All four human OFQ/N splice variants could be translated *in vitro*; however, the molecular masses obtained from the translation products were all several kDa larger than the calculated values. We attribute the curious migration of the translation products to the electrophoretic properties of the ppOFQ/N splice variants, because both murine N23K and N27K proteins were named based on their observed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) mobility, as opposed to their smaller calculated molecular masses of 20.9 and 23.7 kDa, respectively (Saito *et al*. 1995, 1996).

In summary, we have shown that the pattern of ppOFQ/N mRNA splicing described previously in the mouse is conserved in both rats and humans. The splicing of the 3′-end of exon 3 results in elongated and 'conserved' C-termini of the precursor protein. Furthermore, we have described two additional human OFQ/N splice variants which were devoid of exon 2, and whose expression levels in fetal brain were not abundant relative to the messages encoded by N23K and N27K. Finally, although exon 2 contains the predicted translational start site, these latter splice variants could be translated *in vitro* from an internal methionine, which is in frame with the OFQ/N peptide. The functional roles of the precursor proteins resulting from these novel splicing events, should they be expressed *in vivo*, remain to be elucidated.

Acknowledgements

This work was supported by the National Institute on Drug Abuse Grant DA-05010. JA is a Hatos scholar and supported by the Norman Cousins program in Psychoneuroimmunology, UCLA. The authors would like to thank Drs Jim Boulter, Brigitte Keiffer, Paulette Zaki and Duane Keith for their helpful suggestions in the assembly of this manuscript.

References

Berzetei-Gurske I. P., Schwartz R. W. and Toll L. (1996) Determination of activity for nociceptin in the mouse vas deferens. *Eur. J. Pharmacol.* **302**, R1–R2.

Calo G., Rizzi A., Bogoni G., Neugebauer V., Salvadori S., Guerrini R., Bianchi C. and Regoli D. (1996) The mouse vas deferens: a pharmacological preparation sensitive to nociceptin. *Eur. J. Pharmacol.* **311**, R3–R5.

Champion H. C., Wang R., Hellstrom W. J. and Kadowitz P. J. (1997) Nociceptin, a novel endogenous ligand for the ORL1 receptor, has potent erectile activity in the cat. *Am. J. Physiol.* **273**, E214–E219.

Connor M., Yeo A. and Henderson G. (1996) The effect of nociceptin on Ca^{2+} channel current and intracellular Ca^{2+} in the SH-SY5Y human neuroblastoma cell line. *Br. J. Pharmacol.* **118**, 205–207.

Douglass J., Cox B., Quinn B., Civelli O. and Herbert E. (1987) Expression of the prodynorphin gene in male and female mammalian reproductive tissues. *Endocrinology* **120**, 707–713.

Florin S., Suaudeau C., Meunier J. C. and Costentin J. (1997) Orphan neuropeptide NocII, a putative pronociceptin maturation product, stimulates locomotion in mice. *Neuroreport* **8**, 705–707.

Harrison L. M. and Grandy D. K. (2000) Opiate modulating properties of nociceptin/orphanin FQ. *Peptides* **21**, 151–172.

Hawes B. E., Fried S., Yao X., Weig B. and Graziano M. P. (1998) Nociceptin (ORL-1) and mu-opioid receptors mediate mitogen-activated protein kinase activation in CHO cells through a Gi-coupled signaling pathway: evidence for distinct mechanisms of agonist-mediated desensitization. *J. Neurochem.* **71**, 1024–1033.

Hiramatsu M. and Inoue K. (1999a) Effects of nocistatin on nociceptin-induced impairment of learning and memory in mice. *Eur. J. Pharmacol.* **367**, 151–155.

Hiramatsu M. and Inoue K. (1999b) Nociceptin/orphanin FQ and nocistatin on learning and memory impairment induced by scopolamine in mice. *Br. J. Pharmacol.* **127**, 655–660.

Jeannotte L., Burbach J. P. and Drouin J. (1987) Unusual proopiomelanocortin ribonucleic acids in extrapituitary tissues: intronless transcripts in testes and long poly(A) tails in hypothalamus. *Mol. Endocrinol.* **1**, 749–757.

Kilpatrick D. L. and Rosenthal J. L. (1986) The proenkephalin gene is widely expressed within the male and female reproductive systems of the rat and hamster. *Endocrinology* **119**, 370–374.

Kozak M. (1996) Interpreting cDNA sequences: some insights from studies on translation. *Mamm. Genome* **7**, 563–574.

Kummer W. and Fischer A. (1997) Nociceptin and its receptor in guinea-pig sympathetic ganglia. *Neurosci. Lett.* **234**, 35–38.

Lacaze-Masmonteil T., de Keyzer Y., Luton J. P., Kahn A. and Bertagna X. (1987) Characterization of proopiomelanocortin transcripts in human nonpituitary tissues. *Proc. Natl Acad. Sci. USA* **84**, 7261–7265.

Lee T. L., Fung F. M., Chen F. G., Chou N., Okuda-Ashitaka E., Ito S., Nishiuchi Y., Kimura T. and Tachibana S. (1999) Identification of human, rat and mouse nocistatin in brain and human nocistatin in brain and human cerebrospinal fluid [published errata appear in *Neuroreport* (1999) 10, 2879; (2000) 11, inside back cover, viii; and (2000) 11, inside back cover]. *Neuroreport* **10**, 1537–1541.

Matthes H., Seward E. P., Kieffer B. and North R. A. (1996) Functional selectivity of orphanin FQ for its receptor coexpressed with

potassium channel subunits in *Xenopus laevis* oocytes. *Mol. Pharmacol.* **50**, 447–450.

Meunier J. C., Mollereau C., Toll L. *et al.* (1995) Isolation and structure of the endogenous agonist of opioid receptor- like ORL1 receptor [see comments]. *Nature* **377**, 532–535.

Miyakawa K., Hatsuzawa K., Kurokawa T., Asada M., Kuroiwa T. and Imamura T. (1999) A hydrophobic region locating at the center of fibroblast growth factor-9 is crucial for its secretion. *J. Biol. Chem.* **274**, 29352–29357.

Mollereau C., Parmentier M., Mailleux P., Butour J. L., Moisand C., Chalon P., Caput D., Vassart G. and Meunier J. C. (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett.* **341**, 33–38.

Mollereau C., Simons M. J., Soularue P., Liners F., Vassart G., Meunier J. C. and Parmentier M. (1996) Structure, tissue distribution, and chromosomal localization of the prepronociceptin gene. *Proc. Natl Acad. Sci. USA* **93**, 8666–8670.

Nicholson J. R., Paterson S. J., Menzies J. R., Corbett A. D. and McKnight A. T. (1998) Pharmacological studies on the 'orphan' opioid receptor in central and peripheral sites. *Can. J. Physiol. Pharmacol.* **76**, 304–313.

Nicol B., Lambert D. G., Rowbotham D. J., Okuda-Ashitaka E., Ito S., Smart D. and McKnight A. T. (1998) Nocistatin reverses nociceptin inhibition of glutamate release from rat brain slices. *Eur. J. Pharmacol.* **356**, R1–R3.

Nothacker H. P., Reinscheid R. K., Mansour A., Henningsen R. A., Ardati A., Monsma F. J. Jr, Watson S. J. and Civelli O. (1996) Primary structure and tissue distribution of the orphanin FQ precursor. *Proc. Natl Acad. Sci. USA* **93**, 8677–8682.

Okuda-Ashitaka E., Minami T., Tachibana S., Yoshihara Y., Nishiuchi Y., Kimura T. and Ito S. (1998) Nocistatin, a peptide that blocks nociceptin action in pain transmission. *Nature* **392**, 286–289.

Pan Y. X., Xu J. and Pasternak G. W. (1996) Cloning and expression of a cDNA encoding a mouse brain orphanin FQ/nociceptin precursor. *Biochem. J.* **315**, 11–13.

Pintar J. E., Schachter B. S., Herman A. B., Durgerian S. and Krieger D. T. (1984) Characterization and localization of proopiomelanocortin messenger RNA in the adult rat testis. *Science* **225**, 632–634.

Raff T., van der Giet M., Endemann D., Wiederholt T. and Paul M. (1997) Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. *Biotechniques* **23**, 456–460.

Reinscheid R. K., Nothacker H. P., Bourson A., Ardati A., Henningsen R. A., Bunzow J. R., Grandy D. K., Langen H., Monsma F. J. Jr and Civelli O. (1995) Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor. *Science* **270**, 792–794.

Rossi G. C., Mathis J. P. and Pasternak G. W. (1998) Analgesic activity of Orphanin FQ2, murine prepro-orphanin FQ141-157 in mice [in process citation]. *Neuroreport* **9**, 1165–1168.

Saito Y., Maruyama K., Saido T. C. and Kawashima S. (1995) *N23K*, a gene transiently up-regulated during neural differentiation, encodes a precursor protein for a newly identified neuropeptide nociceptin. *Biochem. Biophys. Res. Commun.* **217**, 539–545.

Saito Y., Maruyama K., Kawano H., Hagino-Yamagishi K., Kawamura K., Saido T. C. and Kawashima S. (1996) Molecular cloning and characterization of a novel form of neuropeptide gene as a developmentally regulated molecule. *J. Biol. Chem.* **271**, 15615–15622.

Saito Y., Maruyama K., Saido T. C. and Kawashima S. (1997) Overexpression of a neuropeptide nociceptin/orphanin FQ precursor gene, *N23K/N27K*, induces neurite outgrowth in mouse NS20Y cells. *J. Neurosci. Res.* **48**, 397–406.

Sinchak K., Hendricks D. G., Baroudi R. and Micevych P. E. (1997) Orphanin FQ/nociceptin in the ventromedial nucleus facilitates lordosis in female rats. *Neuroreport* **8**, 3857–3860.

Xie G. X., Ito E., Maruyama K., Suzuki Y., Sugano S., Sharma M., Pietruck C. and Palmer P. P. (1999) The promoter region of human prepro-nociceptin gene and its regulation by cyclic AMP and steroid hormones. *Gene* **238**, 427–436.

Yamamoto T. and Sakashita Y. (1999) Effect of nocistatin and its interaction with nociceptin/orphanin FQ on the rat formalin test. *Neurosci. Lett.* **262**, 179–182.

Zaki P. A. and Evans C. J. (1998) ORL-1: an awkward child of the opioid receptor family. *Neuroscientist* **4**, 172–184.

Zhang G., Murray T. F. and Grandy D. K. (1997) Orphanin FQ has an inhibitory effect on the guinea pig ileum and the mouse vas deferens. *Brain Res.* **772**, 102–106.