Identification and differential regional expression of KOR-3/ORL-1 gene splice variants in mouse brain

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Abstract KOR-3, also known as ORL-1, is a member of the opioid receptor family, encoding the murine receptor for orphanin FQ/nociceptin. In the current studies we have identified five different splice variants of KOR-3 in mouse brain, three of which have not been previously reported. In addition to variants with a 15 bp deletion at the 3′-end of the first coding exon (KOR-3d) and an 81 bp insertion between the second and third coding exons (KOR-3e), three new variants with insertions of 34 (KOR-3a), 98 (KOR-3b), and 139 bp (KOR-3c) between the first and second coding exons have been obtained. The expression of the three variants in mouse brain varies markedly among brain regions with a distribution which is quite distinct from KOR-3 itself. Of greatest interest was the presence of high levels of KOR-3a in the striatum, a region with no demonstrable KOR-3, and in the cortex. KOR-3c was seen in the periaqueductal gray and hypothalamus, regions where KOR-3 predominated. The brainstem had similar levels of KOR-3, KOR-3a, and KOR-3d. In contrast, KOR-3d was most prominent in the cerebellum. KOR-3b levels were very low throughout.

Key words: Opioid receptor; KOR-3; Orphanin FQ; Nociceptin; Kappa$_3$ receptor; Splicing; ORL-1

1. Introduction

A novel member of the opioid receptor family has been recently cloned from human (ORL-1), mouse (KOR-3) and other species [1–8]. The murine KOR-3 gene has been cloned and sequenced and contains five exons separated by four introns [4]. Although structurally homologous with the cloned traditional opioid receptors, the receptor encoded by KOR-3 has low affinity for most opioids and opioid peptides. The recent discovery of the endogenous ligand for this receptor, the neuropeptide orphanin FQ [9]/nociceptin [10] (OFQ/N) has provided important insights into the functional significance of this receptor. OFQ/N is intimately involved with pain perception, but its actions are complex. Initially, it was reported to be hyperalgesic [9,10] and low doses reverse the actions of opioids [11–16]. Yet, OFQ/N is also an analgesic [14,17–20].

The complex pharmacology of OFQ/N and its analogs, along with other studies, has raised the possibility of multiple OFQ/N receptors. Radiolabeled OFQ/N binding to brain homogenates [21] is quite distinct from that to the cloned receptor [9,22] and is consistent with binding site heterogeneity in the brain. Antisense mapping the three coding exons of the receptor encoded by KOR-3 also raised the question of alternative splicing [1,2,16,17,23]. Antisense probes targeting the first coding exon blocked OFQ/N hyperalgesia, but not analgesia, while other probes targeting the second and third exons blocked analgesia and not hyperalgesia. The second and third coding exons, but not the first, also have been implicated in kappa$_3$ analgesia. These observations raised the possibility that the kappa$_3$ receptor and KOR-3 might result from alternative splicing of the same gene [4]. Two alternatively spliced variants have been reported, including a rat variant (XOR1L) which contains a 28 amino acid insertion between the second and third coding exons [24] and another with a 15 base deletion corresponding to Tyr^{71}–Arg^{75} in the first intracellular loop [25]. Here we report the identification and regional expression of additional KOR-3/ORL-1 variants isolated from mouse brain.

2. Materials and methods

2.1. Identification of alternative splicing variants by RT-PCR

A sense (5′-TGCC TTC CTG CCC CTT GGA C-3′; positions 419–438) and an antisense primer (5′-CCC AGA AGG ATG TCT GTG CCC-3′; position 610–630) based upon the nucleotide sequence of the mouse KOR-3 clone (GenBank accession number U09621) were used to amplify cDNA fragments using PCR. The template was first-strand cDNA reverse transcribed with random hexamers from C57BL/6 mouse brain total RNA prepared as described [26]. Multiple bands were obtained by PCR, isolated, subcloned and sequenced in both directions. Four KOR-3-related clones were identified.

2.2. cDNA library screening

We screened a C57BL/6 mouse brain λZAP cDNA library with a ^{32}P-labeled 1.1 kb fragment containing the full length KOR-3 coding region at high stringency, identifying 32 positives. Three contained a 34 bp insertion (KOR-3a), one had a 98 bp insertion (KOR-3b), one had a 139 bp insertion (KOR-3c) and another had an 81 bp insertion between coding exons 2 and 3 (KOR-3e). We sequenced KOR-3a and KOR-3c clones of approximately 2.9 kb and a 1.2 kb clone of KOR-3b. We did not observe any clones with the 15 bp deletion in the first exon in these screens.

2.3. Northern blot analysis

Polyadenylated RNAs were isolated from mouse total RNAs using oligo(dT) chromatography (Pharmacia, Piscataway, NJ) as described [2]. Northern blotting followed the protocol for GeneScreen Plus membranes (New England Nuclear, Boston, MA). Probes for KOR-3a (5′-GGT GTG CCT GCT GTC TCC AGT TCC CCT CAA TGC CCT CCC AGC TGA GGA-3′) and KOR-3b/KOR-3c (5′-CCT CAG TCT CTC TTA AGA CTC CTC TCA GAG GGT TTT CAG GGC ACT GCC-3′) were 5′-end ^{32}P-labeled by T4 polynucleotide kinase. A ^{32}P-labeled 1.1 kb fragment containing the full length of the KOR-3 coding region was generated by PCR with appropriate primers.

2.4. Analysis of KOR-3a, KOR-3b and KOR-3c expression in various brain regions

Total RNAs from various C57BL/6 mouse brain regions were extracted and reverse transcribed using random hexamers. Two primers were designed from the nucleotide sequence of mouse KOR-3/ORL-1

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receptor at positions 486–505 (sense primer, 5′-TCC TGG GGA ACT GCC TCG TC-3′) and 610–630 (antisense primer, 5′-CCC AGA AGG ATG TCT GTG CCC-3′) and used in sequential PCR reactions with the first-strand cDNAs as templates. The predicted sizes of the amplified cDNA fragments for KOR-3, KOR-3a, KOR-3b and KOR-3c are 145 bp, 179 bp, 232 bp and 284 bp, respectively. The PCR products were then separated by 1.5% agarose gel, transferred on GeneScreen Plus membranes and hybridized with a ^{32}P-labeled 107 bp fragment of the coding exon 2 generated by PCR (Fig. 1). The RNA loading in the RT-PCR reactions was estimated by parallel PCR with β_2-microglobulin primers (Clontech).

3. Results

3.1. cDNA cloning of alternative splicing variants of KOR-3 gene

To look for variants differing in the region between the first and second coding exons, we performed RT-PCR using an upstream primer in the first coding exon and a downstream primer in the second. This led to the identification of four splice variants. Three had insertions between the first and second coding exons (Fig. 1) while the fourth had a 15 bp deletion at the 3′-end of the first coding exon, corresponding to a variant cloned from lymphocytes [25]. We then isolated full length cDNAs containing the 34 bp (KOR-3a), the 98 bp (KOR-3b) and the 139 bp (KOR-3c) insertions by screening a mouse brain cDNA library with a full length KOR-3 probe. The sequences of the new cDNAs were identical to that of the KOR-3 cDNA except for the insertions. The insertion sequences of KOR-3a, KOR-3b and KOR-3c were aligned to the intron between the first and second coding exons of the KOR-3 gene [4] and the 81 bp insertion in KOR-3e to the intron between the second and third coding exons (Fig. 2). All of the splice sites were in agreement with the GT/AG rule.

3.2. Northern analysis of KOR-3 and its splice variants

We next used Northern blot analysis to investigate the full length transcripts of the variants obtained from whole brain mRNAs (Fig. 3). The KOR-3a probe hybridized a major transcript with a size of approximately 2.9 kb, with another smaller sized band of lower abundance. Since the 139 bp insertion in KOR-3c contains the 98 bp in KOR-3b, there is no specific probe for the KOR-3b. We therefore used a 48 bp probe derived from the 98 bp to detect both the KOR-3b and KOR-3c expression. This probe hybridized a major transcript with a size of approximately 3.4 kb, which was similar to that probed by the KOR-3 probe. The relative abundance of the transcripts revealed by the KOR-3a probe was much higher than that by the KOR-3b and KOR-3c probe,

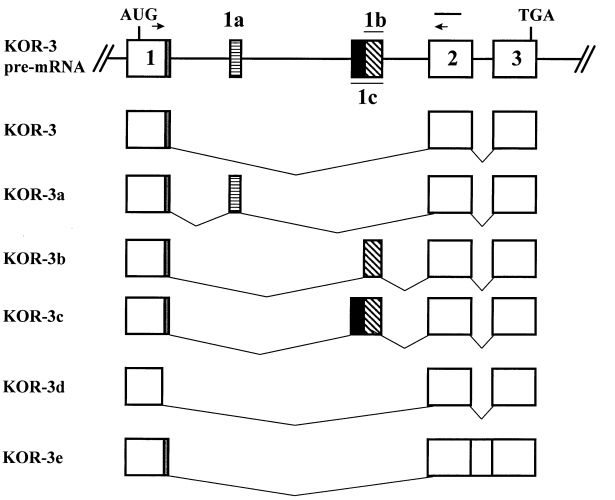
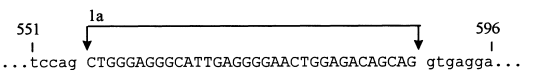
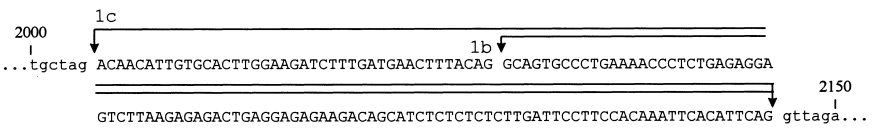


Fig. 1. Schematic of KOR-3 gene and alternative splicing. Exons and introns are indicated by open boxes and horizontal lines, respectively. Mini-exon 1a with 34 bp is shown by the solid box with horizontal lines, mini-exon 1b with 98 bp by the solid box with diagonal lines and mini-exon 1c with 139 bp (including mini-exon 98 bp) by a solid black box and box 1b. The shaded area of exon 1 represents the deletion originally reported in lymphocytes [25] which we also saw in brain. Arrows represent the primers used in RT-PCR reaction to clone the splicing variants. The short heavy line over exon 2 denotes the probe used in Southern blotting to detect expression of KOR-3 gene splicing variants.

a) Insertion sequence KOR-3a



b) Insertion sequence KOR-3b and KOR-3c



c) Insertion sequence KOR-3e

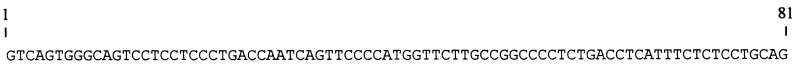


Fig. 2. Insertion sequences of KOR-3a,b,c and e. Insertion sequences of (a) KOR-3a, (b) KOR-3b and KOR-3c, (c) KOR-3e. Intronic sequences are in lower case and exons are given in upper case. The complete KOR-3a, KOR-3b and KOR-3c cDNA sequences have been submitted to GenBank under accession numbers AF043276, AF043277 and AF043278, respectively. Base numbers for genomic and cDNA sequences are based upon intron 2 of the KOR-3 gene (GenBank accession number U32939) and KOR-3 cDNA (accession number U09421) sequences. The splicing junctions are indicated by arrows. In (b) arrows indicate the splicing junctions for KOR-3c and KOR-3b.

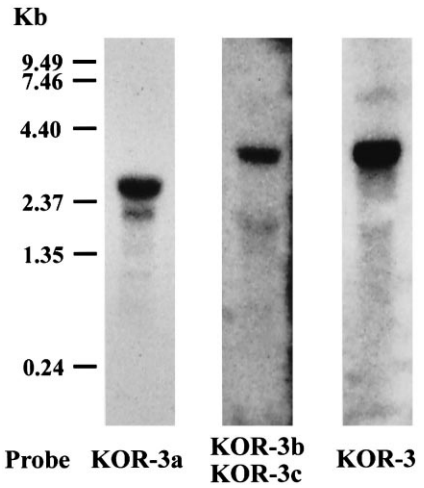


Fig. 3. Northern blotting of KOR-3 and its splice variants. Poly-$(A)^+$ RNA isolation and Northern blotting are described in Section 2. Lanes were loaded with 15 μg of poly$(A)^+$ RNA.

although it was less abundant than that by the KOR-3 probe. We saw no bands with the KOR-3e probe.

3.3. Regional expression of the splice variants

We next examined the regional distribution of the variants using RT-PCR and Southern blotting. Four major bands were obtained with different intensities among the various regions (Fig. 4). The sizes of the four bands from the lower to the higher matched those of the 15 bp deletion (clone D), KOR-3, 34 bp insertion (clone A) and 139 bp insertion (clone C), respectively. To confirm that the amplified bands correspond to the KOR-3 splice variants, each band was extracted from the agarose gel, subcloned into the Bluescript vector and sequenced. In all cases, the sequences of the bands were identical to those of the variant clones. The relative abundance in whole brain of KOR-3 and its variants was similar to that seen with Northern blotting: KOR-3 > KOR-3a > KOR-3c. Although KOR-3b expression was not seen in the initial PCR and the blotting, the KOR-3b fragment was amplified by a second round PCR using the gel extracts corresponding to the KOR-3b from the first PCR as templates (data not shown). This implies a very low level of expression of KOR-3b in brain.

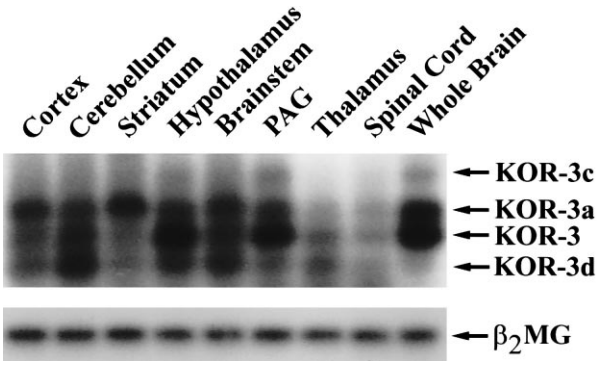


Fig. 4. Regional distribution of the alternatively spliced variants. Total RNAs were extracted from the indicated brain regions and used in the RT reactions with a sense primer targeted close to 3′ of the coding exon 1 and an antisense primer near to 5′ of the coding exon 2 (see arrows, Fig. 1). β_2-Microglobulin (β_2MG) served as an internal control for the regions. The PCR products were assayed by Southern blot.

Among the regions examined, the KOR-3 transcript was most abundant in the hypothalamus and periaqueductal gray (PAG), while the cortex, striatum and brainstem had higher levels of the KOR-3a. The KOR-3c was highly expressed in PAG and hypothalamus and the KOR-3d in cerebellum, hypothalamus and brainstem. Lower levels of KOR-3c were seen in the cortex, PAG and thalamus. Interestingly, the only major variant expressed in the striatum was KOR-3a. This differential expression of the variants among the regions implies region-specific splicing and argues strongly for their functional relevance.

4. Discussion

In addition to the variants reported earlier, the current studies have identified a number of additional splice variants of KOR-3/ORL-1. The insertions observed in the new variants correspond to the region between TM1 and the first intracellular loop, a splice site common among all the opioid receptors. Similar splicing variants with insertions between exons one and two have been identified in mouse delta receptor (DOR-1) gene [27] and the mu (MOR-1) gene (Y.-X. Pan, J. Xu and G.W. Pasternak, unpublished observations). The presence of such a large number of variants underscores the extensive alternative splicing at this location. The splicing becomes even more interesting in view of the differential regional expression of these variants. Perhaps the best example is the striatum, which has been reported to be devoid of ORL-1 message and OFQ/N receptors. We also saw little evidence for any appreciable levels of KOR-3/ORL-1 in this region, in contrast to the high expression of KOR-3a. This regional specific mRNA processing is quite interesting.

Identification of multiple KOR-3 variants with insertions between the first and second coding exons implies extensive alternative splicing at this location. G triplet repeats may play a role in exon-intron border selection and in alternative exon determination [28] and the 34 bp insertion sequence in KOR-3a contains three G triplets. Although the role of the G triplets within the 34 bp in KOR-3a splicing has not been established, it may be interesting to determine whether specific factors capable of binding the G triplets are differentially expressed among brain regions. It has also been reported that many alternatively spliced exons contain GAR repeats, where R is a purine, which have been referred to as exonic splicing enhancers or ESEs [29]. SRp55 is a specific ESE binding protein. Thus, the presence of multiple GAR repeats in the insertion sequences of both the KOR-3b and the KOR-3c might contribute to the regional expression of the KOR-3b and the KOR-3c.

The KOR-3e appears to be an intron-retention variant and is very similar to a rat variant [24]. Unlike the murine version, which contains a termination codon which would lead to a truncated receptor lacking the last three transmembrane regions, the published rat version does not appear to have the termination codon.

Translation of the cDNAs using the start codon AUG of KOR-3 results in early termination either at the mini-exon insertion (KOR-3c) or shortly after the insertion (KOR-3a and KOR-3b). Yet, in preliminary studies the expressed full length clones containing these insertions bind the kappa$_3$ lig-

and [^{3}H]naloxone benzoylhydrazone quite well (unpublished observations). Although the truncated protein may retain high affinity for the ligands, this seems unlikely. Alternative explanations include an alternative start codon which would be in frame with the second and third coding exons or possibly RNA editing.

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References

[1] Pan, Y.-X., Cheng, J., Xu, J. and Pasternak, G.W. (1994) Regul. Pept. 54, 217–218.
[2] Pan, Y.-X., Cheng, J., Xu, J., Rossi, G.C., Jacobson, E., Ryan-Moro, J., Brooks, A.I., Dean, G.E., Standifer, K.M. and Pasternak, G.W. (1995) Mol. Pharmacol. 47, 1180–1188.
[3] Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.L., Moisand, C., Chalon, P., Caput, D., Vassart, G. and Meunier, J.C. (1994) FEBS Lett. 341, 33–38.
[4] Pan, Y.-X., Xu, J. and Pasternak, G.W. (1996) Gene 171, 255–260.
[5] Wick, M.J., Minnerath, S.R., Lin, X., Elde, R., Law, P.-Y. and Loh, H.H. (1994) Mol. Brain Res. 27, 37–44.
[6] Fukuda, K., Kato, S., Mori, K., Nishi, M., Takeshima, H., Iwabe, N., Miyata, T., Houtani, T. and Siguimoti, T. (1994) FEBS Lett. 343, 42–46.
[7] Bunzow, J.R., Saez, C., Mortrud, M., Bouvier, C., Williams, J.T., Low, M. and Grandy, D.K. (1994) FEBS Lett. 347, 284–288.
[8] Keith Jr., D., Maung, T., Anton, B. and Evans, C. (1994) Regul. Pept. 54, 143–144.
[9] Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma, F.J. and Civelli, O. (1995) Science 270, 792–794.
[10] Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P., Monsarrat, B., Mazargull, H., Vassart, G., Parmentier, M. and Costentin, J. (1995) Nature 377, 532–535.
[11] Mogil, J.S., Grisel, J.E., Zhangs, G., Belknap, J.K. and Grandy, D.K. (1996) Neurosci. Lett. 214, 1–4.
[12] Mogil, J.S., Grisel, J.E., Reinscheid, K.K., Civelli, O., Belknap, J.K. and Grandy, D.K. (1996) Neuroscience 75, 333–337.
[13] Grisel, J.E., Mogil, J.S., Belknap, J.K. and Grandy, D.K. (1996) NeuroReport 7, 2125–2129.
[14] Tian, J.H., Xu, W., Fang, Y., Mogil, J.S., Grisel, J.E., Grandy, D.K. and Han, J.S. (1997) Br. J. Pharmacol. 120, 676–680.
[15] Zhu, C.B., Cao, X.D., Xu, S.F. and Wu, G.C. (1997) Neurosci. Lett. 235, 37–40.
[16] King, M.A., Chang, A. and Pasternak, G.W. (1998) Biochem. Pharmacol. 55, 1537–1540.
[17] Rossi, G., Leventhal, L., Boland, E. and Pasternak, G.W. (1997) J. Pharmacol. Exp. Ther. 282, 858–865.
[18] Rossi, G.C., Leventhal, L. and Pasternak, G.W. (1996) Eur. J. Pharmacol. 311, R7–R8.
[19] King, M.A., Rossi, G.C., Chang, A.H., Williams, L. and Pasternak, G.W. (1997) Neurosci. Lett. 223, 113–116.
[20] Yamamoto, T., Nozaki-Taguchi, N. and Kimura, S. (1997) Neuroscience 81, 249–254.
[21] Mathis, J.P., Ryan-Moro, J., Chang, A., Hom, J.S.H., Scheinberg, D.A. and Pasternak, G.W. (1997) Biochem. Biophys. Res. Commun. 230, 462–465.
[22] Pan, Y.-X., Xu, J., Ryan-Moro, J., Mathis, J., Hom, J.S.H., Mei, J.F. and Pasternak, G.W. (1996) FEBS Lett. 395, 207–210.
[23] Pasternak, G.W. and Standifer, K.M. (1995) Trends Pharmacol. Sci. 16, 344–350.
[24] Wang, J.B., Johnson, P.S., Imai, Y., Persico, A.M., Ozenberger, B.A., Eppler, C.M. and Uhl, G.R. (1994) FEBS Lett. 348, 75–79.
[25] Halford, W.P., Gebhardt, B.M. and Carr, D.J.J. (1995) J. Neuroimmunol. 59, 91–101.
[26] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[27] Gavériaux-Ruff, C., Peluso, J., Befort, K., Simonin, F., Zilliox, C. and Kieffer, B.L. (1997) Mol. Brain Res. 48, 298–304.
[28] McCullough, A.J. and Berget, S.M. (1997) Mol. Cell. Biol. 17, 4562–4571.
[29] Nagel, R.J., Lancaster, A.M. and Zahler, A.M. (1998) RNA 4, 11–23.