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cDNA cloning of an orphan opiate receptor gene family member and its splice variant

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Abstract

Radioligand binding and cDNA homology studies have suggested the existence of opiate receptors distinct from the recently-cloned μ, δ and κ receptors. XOR1S, a rat brain cDNA whose predicted translation product displays 67–72% homology with those encoded by μ1, δ1 and κ1 opiate receptor cDNAs, was constructed from two partial cDNAs identified through cDNA homology approaches. A longer XOR1L variant of this cDNA was also identified by polymerase chain reaction studies using genomic DNA and cDNA from brain and peripheral tissues. XOR1 mRNA is most highly expressed in hypothalamus. COS cell expression of both clones confers neither robust binding of opiate ligands nor reproducible opiate inhibition of forskolin-stimulated adenylate cyclase. These studies identify an orphan clone that helps to define features of the opiate receptor gene family, including apparent differential splicing and expression in peripheral tissues.

Key words: G-linked receptor; Morphine; Seven transmembrane domain; Hypothalamus; Alternative splicing

1. Introduction

Multiple receptor sites recognizing exogenous opiate drugs and endogenous opioid peptides have been defined based on radioligand binding and pharmacological experiments [1,2]. μ, δ and κ opioid receptors have been the focus of most attention, although an ε receptor has also been postulated (e.g. [1–6]). Recent cDNA cloning studies have identified members of the seven transmembrane domain, G-protein-linked neuropeptide receptor family that encode prototypic members of the μ, δ and κ opioid receptor families [7–15].

We now report evidence, obtained by screening cDNA libraries with hybridization probes derived from various opiate receptor sequences, that this opiate receptor gene subfamily contains members that differ from the initially-cloned μ, δ and κ_1 receptors [7–15]. Using polymerase chain reaction (PCR) and cDNA homology approaches, we have identified several overlapping cDNAs that encode a translation product termed XOR1S, due to its high homology with the initially-cloned μ, δ and κ_1 receptors [7–15]. The XOR1 mRNA appears to display splice variants altering amino acid sequences to form XOR1L and XOR1S. Long and short XOR1 forms are differentially expressed in brain regions and peripheral tissues, but neither manifests either robust binding of opiate ligands or opiate inhibition of forskolin-stimulated adenylate cyclase activity when expressed in COS cells. These receptors' sequences contribute to our understanding of the opiate receptor gene subfamily and its relation to the subfamily of neuropeptide neurotransmitter receptors.

2. Materials and methods

2.1. Cloning candidate rat brain opioid receptor subfamily cDNAs

Candidate rat opioid receptor cDNAs and genomic clones were obtained using several mRNA and cDNA sources and several oligonucleotide primers for PCR amplification, as described [7]. pPCR4A was a 700 base pair (bp) pPCRII (InVitrogen) subclone of a partial μ opiate receptor cDNA amplified from single stranded rat brain cDNA, as described [7]. The 700 bp pPCR4A insert was excised with *Eco*RI, radiolabelled by random priming, and used to isolate cDNAs from a size-selected rat cerebral cortex lambda ZAP cDNA library, as described [7]. Sequence analyses of the inserts from autoexcised plasmids revealed apparent partial sequences with substantial homology to other cloned opiate receptors, including a 2.8 kb cDNA termed RC9-1-5. A 5′ 500 bp fragment of RC9-1-5 was isolated using *Hin*dIII, the fragment was radiolabeled by random priming, and used to isolate other more 5′ cDNAs including a 3 kb cDNA, RC13-1, as described [7]. The 5′ end from RC13-1 and the 3′ end from RC9-1-5 were cut and ligated to form a fused clone termed XOR1S.

Convergent data was also obtained from PCR-based studies of rat genomic DNA. pOpiorph1 was a 636 bp pPCRII cDNA subclone amplified from rat genomic DNA using two rounds of polymerase chain reaction and primers 5′-ACGATGAA(GC)AC(TGA)GCCAC-CACCA-3′ [16] and 5′-CTTCAA(TC)CTGGC(TC)TTGGCTGAT-3′ [12], derived from μ and δ receptor cDNA sequences, respectively.

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Abbreviations: DAMGO, [D-Ala2,*N*-Methyl-Phe4,Glyol5]enkephalin; DPDPE, [D-Pen2,Phe4,D-Pen5]enkephalin; DADLE, [D-Ala2,D-Leu5]enkephalin; U-50,488 *trans* ± 3,4-dichloro-*N*-methyl-*N*[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; U 69,593 (5α,7α,8β)−(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspirol[4,5]dec-8-yl)benzacetamide.

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pXOR1S and pOPIORPH1 were subjected to complete sequencing of both strands using automated and manual methods as described [7]. The long form splice variant cDNA, pXOR1L, was generated by fusing pOpiorph1 and the pXOR1S. Both pXOR1S and pXOR1L were also subcloned into pcDNA1 (InVitrogen) to yield pcDNA1XOR1S and pcDNAXOR1L. DNA sequences were analyzed using GCG software [17].

2.2. Expression in COS cells and assessment of opiate pharmacologic properties

COS cells were transfected by electroporation with 20 μg/10^7 cells of pcDNA1XOR1L, pcDNA1XOR1S, or pcDNA1 vector, as described [18,19]. Transfected cells were plated in Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS), cultured for 2–3 days, and tested for opiate receptor expression by radioligand binding and adenylate cyclase assays as described [7]. Radioligands included: [^{3}H]bremazocine (29.2 Ci/mmol, NEN), [^{3}H]naloxone (47.2 Ci/mmol, NEN), [^{3}H]diprenorphine (29 Ci/mmol, NEN), [^{3}H]DAMGO ([D-Ala2,*N*-Methyl-Phe4,Glyol5]enkephalin; 60 Ci/mmol, Amersham), [^{3}H]DPDPEpCl ([D-Pen2,4'-Cl-Phe4,D-Pen5]enkephalin; 51 Ci/mmol, NEN), [^{3}H]DADLE (D-Ala2,D-Leu5 enkephalin; 37 Ci/mmol, NEN), [^{3}H]ethylketocyclazocine (28.5 Ci/mmol, NEN), [^{3}H]etorphine (38.7 Ci/mmol, NEN), [^{3}H]buprenorphine (13.4 Ci/mmol, RBI), [^{125}I]β-endorphin (2,000 Ci/mmol, Amersham) and [^{3}H]U-69,593 (57 Ci/mmol, Amersham). cAMP accumulation stimulated by 10 μM forskolin was assessed in 24-well plate COS cell cultures preincubated for 15 min with 1 mM IBMX (3-isobutyl-1-methylxanthine) as described [7] (kit No. KAPH2, Diagnostic Products, Los Angeles).

2.3. XOR expression

RNA was prepared from rat tissues that were rapidly dissected and frozen at −70°C. 20 μg of total RNA was prepared and electrophoresed along with molecular weight standards (BRL) and transferred to nylon membranes as described [20]. Blots were hybridized with the 2.8 kb RC9-1-5 cDNA radiolabelled with ^{32}P by random priming in 50% formamide, 5 × SSC, 50 mM $NaPO_4$, 1% SDS, 2.5 × Denhardt's solution, 200 μg/ml salmon sperm DNA at 42°C overnight, washed twice in 0.1 × SSC/0.1% SDS for 30 min at 65°C, and radioactive patterns identified using a phosphorimaging device (Molecular Dynamics) following overnight exposures.

Reverse transcription-PCR was performed using 5 μg of total RNA extracted from different tissues and oligonucleotide primers 5'-ACCCTGGTCTTGCTAACA-3' and 5'-CAGCACCAGTCGAGTGAT-3', as described (Perkin-Elmer RT-PCR Kit). Single-stranded cDNA was amplified by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 92°C for 1 min), with separation of PCR products by 2% agarose gel electrophoresis, transfer to nylon membranes, hybridization overnight with a ^{32}P-labeled 9-1-5 cDNA probe at 42°C as described [7], and phosphorimaging.

3. Results

Analyses of the rat cerebral cortex cDNAs RC9-1-5 and RC13-1 revealed an open reading frame of 367 amino acids with 52% identity to the rat δ-opioid receptor cDNA [9], 54% to the rat μ-opiate receptor [7–9], and 52% to the rat κ-opiate receptor sequence [13–15] (Figs. 1 and 2). This sequence was confirmed by partial sequencing of more than four additional distinct cDNAs (data not shown). The composite cDNA fused from RC9-1-5 and RC13-1 yielded a 3.2 kb opiate receptor subfamily fusion cDNA with 120 bp of apparent 5' untranslated region, 2.0 kb of 3' untranslated region including a polyadenylation sequence, and 1,101 bp encoding the open reading frame. Hydrophobicity analyses revealed that the seven hydrophobic candidate transmembrane regions of 20–24 amino acids contained sequences especially conserved with other G-linked receptors. Three consensus sequences for N-linked glycosylation in the putative extracellular N-terminal domain, and serine and threonine residues found in possible intracellular domains in sequence contexts favorable for protein kinase A and C phosphorylation were also notable [21,22]; (Fig. 1).

Comparison of these sequences with those of pOPIORPH1 revealed 84 additional base pairs in pOPIORPH1 that are flanked by consensus sequences for hnRNA splicing and encode additional open reading frame segments (Fig. 1). The spliced segment contains an additional site for potential N-linked glycosylation (Fig. 1).

Northern analysis of the distribution of XOR1 mRNA reveals highest levels of expression in the hypothalamus (Fig. 3A). At least three hybridizing mRNA species are observed in this brain region and in brainstem, midbrain, cerebral cortex, thalamus and hippocampus but not in striatum or cerebellum (Fig. 3A and data not shown). RT-PCR approaches revealed two splice variant products in tested brain regions as well as in several peripheral tissues such as intestine, skeletal muscle, vas deferens and spleen (Fig. 3B). The ratio between XOR1S and XOR1L expression also varies among the brain regions and peripheral tissues examined (Fig. 3B,C).

Transient COS cell expression of neither the XOR1S nor XOR1L pcDNA1 subclone yielded consistent binding of any tested ligand. In 14 experiments in which robust binding to rat or human μOR1 cDNAs provided positive control data, eight of ten displayed no specific radioligand binding above background levels to cells expressing XOR1S and four of four displayed no specific binding to cells expressing XOR1L. In two experiments, modest naloxone-displacable diprenorphine, bremazocine, and β-endorphin binding above background values was noted in XOR1S-transfected cells (data not shown). However, intermittent naloxone-displacable binding of naloxone and β-endorphin was also observed in mock-transfected COS cells in several negative control experiments. Neither radiolabeled diprenorphine, bremazocine, nor β-endorphin displayed specific binding in eight additional experiments; neither ethylketocyclazocine, naloxone, DAMGO, DPDPE, U,69,593, etorphine, buprenorphine, nor DADLE revealed specific binding in any experiment (more than two experiments each; data not shown).

COS cells transfected with pXOR1S or pXOR1L failed to display consistent opiate-induced alterations in forskolin-stimulated adenylate cyclase activity. In 14 experiments in which morphine-inhibited adenylate cyclase activity in COS cells expressing rat or human μOR1 cDNAs served as positive controls, eight of 10 experiments displayed no opiate-mediated inhibition of forskolin-stimulated cyclase activity in cells expressing XOR1S and four of four experiments displayed no opi-

ate-mediated inhibition of forskolin-stimulated cyclase activity in cells expressing XOR1L. In two experiments, bremazocine, buprenorphine, etorphine and β-endorphin did display modest naloxone-reversible inhibition of forskolin-stimulated cyclase activity in cells expressing XOR1S. However, intermittent naloxone-reversible β-endorphin effects were also noted in some experiments in mock-transfected cells (data not shown). Neither bremazocine, buprenorphine, etorphine nor β-endorphin altered forskolin-stimulated cAMP levels in eight additional experiments; neither DADLE, Dynophin A, morphine nor U50,488 altered cAMP levels in any experiment (more than two experiments each; data not shown).

4. Discussion

The sequences of the XOR1S and XOR1L cDNAs described here clearly place them within the family of G-protein linked, seven transmembrane domain receptors, and within the opiate receptor subfamily of receptor genes (Fig. 2). The distributions of the mRNAs ex-

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           1                                                      #  50
rXor1      ..........  ..........  .....MESLF PAPYWEVLYG SHFQGNLSLL
rMor1      MDSSTGPGNT SDCSDPLAQA SCSPAPGSWL NLSHVDGNQS DPCGLNRTGL
rDor1      ..........  ......MEPV PSARAE...L QFSLL.ANVS DTFPSAFPSA
rKor1      ..........  ..MESPIQIF RGEPGPTCAP SACLLPN..S SSWFPNWAES
Consensus  ----------  ----------  ----------  ---------S ----------

           #51         #                                          100
rXor1      NETVPHHLLL NASHSAFLPL GLKVTIVGLY LAVCIGGLLG NCLVMYVILR
rMor1      GGNDSLCPQ. ...TGSP.SM VTAITIMALY SIVCVVGLFG NFLVMYVIVR
rDor1      SANASGSPG. ...ARSASSL ALAIAITALY SAVCAVGLLG NVLVMFGIVR
rKor1      DSNGSVGSED QQLEPAHISP AIPVIITAVY SVVFVVGLVG NSLVMFVIIR
Consensus  --N-S-----  --------S- ---I-I-A-Y S-V--VGL-G N-LVMF-IVR

           101                                                    150
rXor1      HTKMKTATNI YIFNLALADT LVLLTLPFQG TDILLGFWPF GNALCKIVIA
rMor1      YTKMKTATNI YIFNLALADA LATSTLPFQS VNYLMGTWPF GTILCKIVIS
rDor1      YTKLKTATNI YIFNLALADA LATSTLPFQS AKYLMETWPF GELLCKAVLS
rKor1      YTKMKTATNI YIFNLALADA LVTTTMPFQS AVYLMNSWPF GDVLCKIVIS
Consensus  YTKMKTATNI YIFNLALADA L-T-TLPFQS --YLM--WPF G--LCK-V-S

           151                                                    200
rXor1      IDYYNMFTST FTLTAMSVDR YVAICHPIRA LDVRTSSKAQ AVNVAIWALA
rMor1      IDYYNMFTSI FTLCTMSVDR YIAVCHPVKA LDFRTPRNAK IVNVCNWILS
rDor1      IDYYNMFTSI FTLTMMSVDR YIAVCHPVKA LDFRTPAKAK LINICIWVLA
rKor1      IDYYNMFTSI FTLTMMSVDR YIAVCHPVKA LDFRTPLKAK IINICIWLLA
Consensus  IDYYNMFTSI FTL--MSVDR YIAVCHPVKA LDFRTP--AK -INIC-W-L-

                                          #
                    GQNVVLLPDSLVSHGFLLVPLPPNPSPA
           201                                                    250
rXor1      SVVGVPVAIM GSAQVED..E EIECLVEIPA PQ.DYWGPVF AICIFLFSFI
rMor1      SAIGLPVMFM ATTKYRQ..G SIDCTLTFSH PTW.YWENLL KICVFIFAFI
rDor1      SGVGVPIMVM AVTQPRD..G AVVCTLQFPS PSW.YWDTVT KICVFLFAFV
rKor1      SSVGISAIVL GGTKVREDVD VIECSLQFPD DEYSWWDLFM KICVFVFAFV
Consensus  S-VG-----M --T--R---- -I-C-L-F-- --W-YWD--- KICVF-FAFV

           251                                                    300
rXor1      IPVLIISVCY SLMIRRLRGV RLLSGSREKD RNLRRITRLV LVVVAVFVGC
rMor1      MPVLIITVCY GLMILRLKSV RMLSGSKEKD RNLRRITRMV LVVVAVFIVC
rDor1      VPILIITVCY GLMLLRLRSV RLLSGSKEKD RSLRRITRMV LVVVGAFVVC
rKor1      IPVLIIIVCY TLMILRLKSV RLLSGSREKD RNLRRITKLV LVVVAVFIIC
Consensus  -PVLII-VCY -LM-LRL-SV RLLSGS-EKD R-LRRIT-MV LVVV--FIVC

           301                                                    350
rXor1      WTPVQVFVLV QGL.GVQPGS ETAVAILRFC TALGYVNSCL NPILYAFLDE
rMor1      WTPIHIYVII KALITI.PET TFQTVSWHFC IALGYTNSCL NPVLYAFLDE
rDor1      WAPIHIFVIV WTLVDINRRD PLVVAALHLC IALGYANSSL NPVLYAFLDE
rKor1      WTPIHIFILV EALGSTSHST A.VLSSYYFC IALGYTNSSL NPVLYAFLDE
Consensus  W-PIHIFV-V --L-------  --------FC IALGY-NS-L NPVLYAFLDE

           351                                                    400
rXor1      NFKACFRKFC LLSSLHREMQ VSDRVRSIAK DVGLGCKTSE TVPRPA....
rMor1      NFKRCFREFC IPTSSTIEQQ NSTRVRQNTR EHPSTANTVD RTNHQLENLE
rDor1      NFKRCFRQLC RAPCGGQEPG SLRRPRQATA RERVTACTPS ......DGPG
rKor1      NFKRCFRDFC FPIKMRMERQ STNRVR.NTV QDPASMRDVG GMNKPV....
Consensus  NFKRCFR-FC -------E-- ---R-R--T- ---------- ----------
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Fig. 1. Amino acid sequence of the rat brain orphan opiate receptor family member predicted from the sequences of the cDNA for XOR1S and XOR1L, and comparison of the sequence to other opiate receptor family members. XOR1S and XOR1L were subjected to double stranded sequencing by automated and manual means, and the translation product open reading frame aligned with those of the rat δ-opioid receptor (rDor1), rat μ-opiate receptor (rMor1) and the rat κ-opiate receptor (rKor1) using the program PILEUP. Putative transmembrane domains are indicated by boldface type and shading, putative sequences for N-linked glycosylation by #, the location of the 28 additional amino acids encoded by the splice variant is shown by the lines. The sequences have been truncated at 400 amino acids. The consensus sequence was derived by setting the minimum number of similar residues equal to three and giving the μ, δ, and κ sequences greater weight in the PILEUP program. The XOR1 nucleotide sequence has been deposited with Genbank (Accession No. L33916).

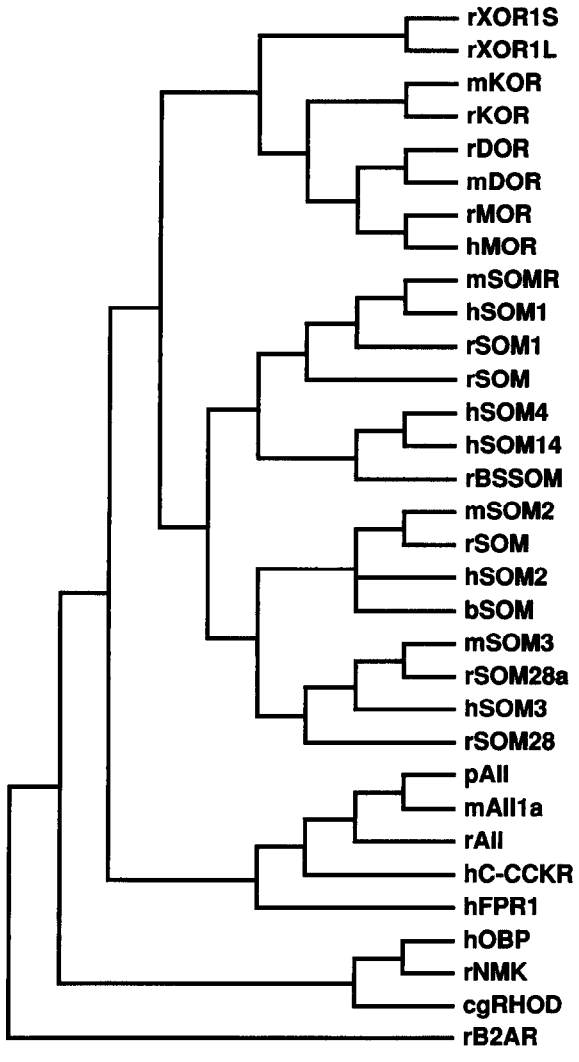

Fig. 2. Dendrogram depicting evolutionary relatedness of the proteins predicted from XOR1 and other G-protein-linked receptor cDNAs. The dendrogram was generated from the top 40 most related protein sequences identified by searching all available data bases using the computer programs Tfasta and PILEUP. Other G-protein linked receptors are also included to show family relatedness. The sequence accession numbers are as follows: rXOR1S, rXOR1L, mKOR (L11065), rKOR (U00442), rDOR (D16348), mDOR (L16064), rMOR (L20684), hMOR (L25119), mSOMR (M81831), hSOM1 (L07833), rSOM1 (X62314), rSOM (M93273), hSOM4 (L07061), hSOM14 (S59504), rBSSOM (M96544), mSOM2 (M81832), rSOM (L04535), hSOM2 (M818130), bSOM (L06613), mSOM3 (M91000), rSOM28a (X63574), hSOM3 (L07062), rSOM28 (S53287), pAII (D11340), mAII1a (S37484), rAII (M86912), hC-CCKR (L09230), hFPR1 (L10820), hOBP (M84605), rNMK (J05189), cgRHOD (X61084), rB2AR (X17607).

pressed from this gene are consistent with the possibility that it could encode a neurotransmitter receptor protein.

The elucidation of the present cDNAs appear to add substantially to evidence for the diversity of the gene subfamily that contains opiate receptors. The splice variant documented for this receptor represents the first example of differential splicing in this receptor gene sub-

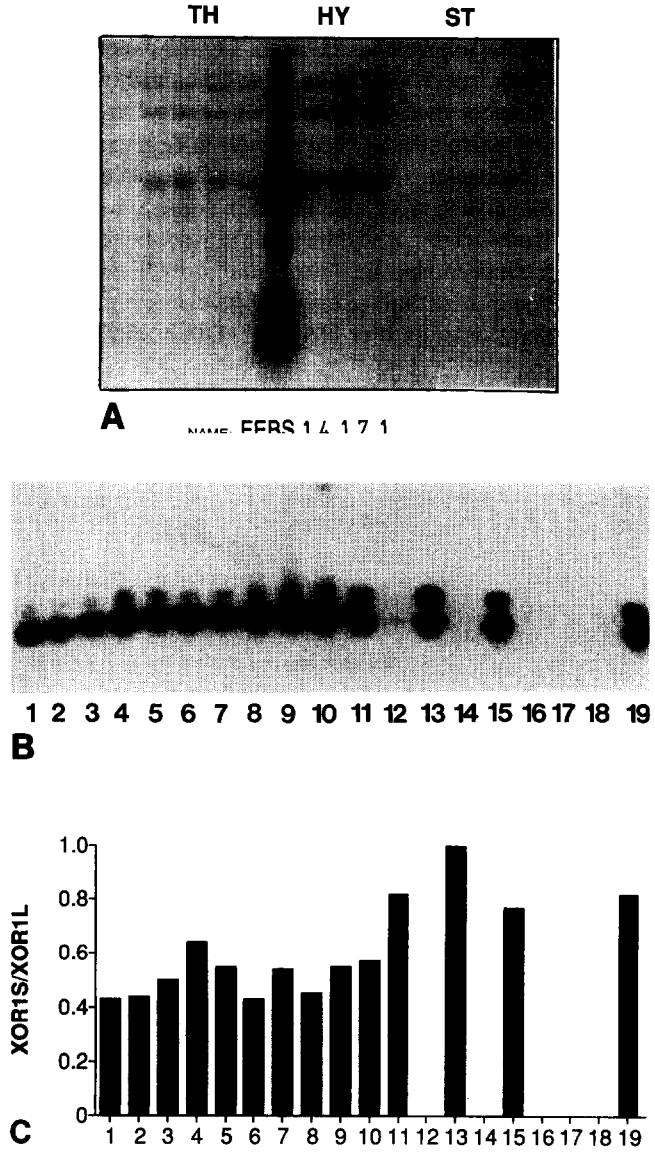

Fig. 3. Expression of XOR1 mRNA. (A) Northern analyses from a representative phosphorimager autoradiogram of XOR1 mRNA hybridization to radiolabeled RC9-1-5 cDNA in 20 μg total RNA extracted from rat thalamus (lanes 1–4), hypothalamus (lanes 6–8) and striatum (lanes 9–12). Size markers (lane 5) suggest 3.3 kb, 13.0 kb, 23.0 kb sizes for the three hybridizing mRNA species. (B) Reverse transcription/polymerase chain reaction amplification products of mRNAs extracted from various brain and peripheral tissues with XOR1 gene products detected by hybridization with a radiolabeled RC9-1-5 cDNA. Lane 1, cerebellum; lane 2, cerebral cortex; lane 3, striatum; lane 4, midbrain; lane 5, hippocampus; lane 6, brainstem; lane 7, olfactory bulb; lane 8, spinal cord; lane 9, thalamus; lane 10, hypothalamus; lane 11, intestine; lane 12, skeletal muscle; lane 13, vas deferens; lane 14, esophagus; lane 15, liver; lane 16, kidney; lane 17, testis; lane 18, adrenal gland; lane 19, spleen. The top band (long form) is 591 bp and the bottom band (short form) is 507 bp, molecular size markers not shown. (C) Ratio of hybridization to XOR1S to XOR1L reverse transcription/polymerase chain reaction products derived from mRNAs in each tissue is expressed as the ratio of radiolabeled hybridization probe recognizing the short form to that recognizing the long form, with hybridization, washing, and hybridization quantitation by phorphorimaging performed as described in the text. Similar results were noted in three replicate experiments.

family, and suggests an intron-exon border likely to be conserved in several opiate receptor subfamily genes [23]. Although this variant's presence is well-documented in reverse transcriptase/polymerase chain reaction approaches, none of the hybridizing bands noted in Northern analyses differ in gel motility by the 84 bp predicted from the splice variant documented in the coding sequence. Conceivably, two of these three mRNAs could represent products of different genes closely related to XOR1 in sequence. Alternatively, mRNA splicing and/or polyadenylation site usage events in the XOR1 gene's untranslated regions could yield the significant differences in transcript molecular mass noted in Northern analyses.

Several features of the XOR1's structure are consistent with specific functional implications. The size of the third putative intracellular loop predicted by the XOR1 cDNA is modest, consistent with sizes of the homologous segments in the seven transmembrane domain receptors that do not couple to adenylate cyclase stimulating G_s proteins [24]. Although many residues lying in transmembrane regions are conserved, the XOR1 sequence contain a glutamine at position 305 (Fig. 1) instead of the histidine that lies in comparable positions in the μ, κ and δ opiate receptor sequences. The XOR1L longer splice variant encodes 28 additional amino acids that separate a number of negatively charged residues in the putative third extracellular segment from each other (Fig. 1).

The failure of the binding site conferred on COS cells expressing this cDNA to consistently recognize radiolabeled peptides or drugs that bind opiate receptors, and its inconsistent actions on adenylate cyclase suggests that this receptor's natural ligands may not be opioid peptides and that it may contribute little to the brain actions of opiate drugs. Gene relatedness does not unfailingly predict agonist identity. The current data do suggest that XOR1 receptors are derived from the same evolutionary precursors that contributed to the opiate receptor subfamily of G-linked receptor genes.

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Note added in proof

During the handling of this paper, another editor accepted a paper with a similar content (Fukuda et al., FEBS Lett. 343 (1994) 42–46).