

Cloning and expression of a cDNA for the rat κ -opioid receptor

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We cloned a cDNA for the rat κ -opioid receptor from a rat thalamus cDNA library. The deduced amino acid sequence consists of 380 residues with features shared by members of the G protein-coupled receptor family. The specific binding of [³H]bremazocine to the membrane of COS-7 cells transfected with the cDNA was displaced by κ -specific opioid ligands, but not by μ - and δ -specific ligands. *Xenopus* oocytes injected with the in vitro transcribed mRNA responded to opioid ligands with the same subtype specificity. Northern blot analysis demonstrated that κ -opioid receptor mRNA is expressed in a regionally specific manner in rat brain.

Opioid receptor; κ -Type; Dynorphin A; U50488H

1. INTRODUCTION

Opiates produce various pharmacological effects, such as analgesia, respiratory depression, catalepsy and inhibition of diarrhea, through their specific receptors. Dozens of endogenous ligands of these receptors have been identified as an opioid peptide family. Pharmacological studies have defined three types of opioid receptors, i.e., μ -, δ - and κ -opioid receptors, which preferentially bind to morphine, enkephalins and dynorphins, respectively [1].

Recently, the mouse δ -opioid receptor was cloned by expression cloning and shown to belong to the G protein-coupled receptor family [2]. To understand the physiological roles of endogenous opioid peptides and mechanisms of action of opiates at a molecular level, cloning of other types of opioid receptors is essential. Since there is evidence that μ - and κ -opioid receptors also belong to the G protein-coupled receptor family [3], we tried to clone cDNAs for these receptors using the PCR technique. RT-PCR was carried out with total RNA from rat dorsal root ganglia. This tissue contains cell bodies of primary afferent neurons and the release of substance P from their terminals was shown to be inhibited by morphine [4]. RT-PCR led to a clone which had a nucleotide sequence 70.8% homologous to mouse δ -opioid receptor in the corresponding region. A rat

cDNA library was screened using the clone as a probe and we obtained two types of clones homologous to the mouse δ -opioid receptor. Here, we show that one of these clones encodes rat κ -opioid receptor.

2. MATERIALS AND METHODS

2.1. Materials

Dynorphin A (1–17) was purchased from Peptide Institute, Inc. (Minoh, Japan). U50488H was a gift from Upjohn Company (Kalamazoo, USA). DAMGO and DPDPE were from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Bremazocine was a gift from Sandoz A.G. (Basel, Switzerland). (–)-[9-³H(N)]bremazocine (30 Ci/mmol) was obtained from DuPont-New England Nuclear.

2.2. Molecular cloning of rat opioid receptors

PCR primers were designed based on the sequence of mouse δ -opioid receptor [2]. The sequences of forward and reverse primers synthesized are 5'-GCGC(ATGC)AAGTACTT(ATGC)ATGGAAAC(ATGC)TGG-3' and 5'-CAGACGATGACGAA(ATGC)ATGTG(ATGC)AT(ATGC)GG-3', respectively. Total RNA from rat dorsal root ganglia was used for RT-PCR as a template. The procedure of RT-PCR was previously described [5]. Briefly, total RNA was reverse-transcribed with MMLV reverse-transcriptase (BRL, Gaithersburg, USA). An aliquot of the reaction mixture was served as a template in 30 cycles of PCR with 0.5 min of denaturation at 95°C, 0.7 min of annealing at 65°C and 1.5 min of extension at 72°C on a Zymoreactor (Atto Corp., Tokyo, Japan). The PCR product was electrophoresed on a 1.5% TAE gel. The DNA fragment was extracted from the gel and subcloned into pCR II (Invitrogen, San Diego, USA) and sequenced. The sequence of the clone (pR11) was 70.8% homologous to the corresponding region of mouse δ -opioid receptor cDNA. Rat thalamus cDNA was prepared by a random-priming method and a cDNA library was constructed using SuperScript choice system (BRL) and λ ZAP II phage vector (Stratagene La Jolla, USA). This library (1 × 10⁶ clones) was screened by hybridization with the insert of pR11. Hybridization was carried out at 65°C in 5 × SSC containing 5 × Denhardt's solution and 0.5% SDS, and then filters were washed at 65°C in 1 × SSC containing 0.1% SDS. Seven positive clones were

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Abbreviations: PCR, polymerase chain reaction; RT, reverse transcription; DPDPE, [D-Pen², D-Pen⁵]enkephalin; DAMGO, [D-Ala, MePhe⁴, Gly(ol)⁵]enkephalin; G protein, GTP binding protein.

isolated and named pMOPR1-5 and pKOPR1,2 based on their sequences and the results of preliminary *in situ* hybridization histochemistry

2.3. cDNA expression in COS-7 cells and binding assay

The *Pst*I–*Spe*I fragment of pKOPR2 was subcloned into the *Eco*RV–*Not*I site of pcDNA1/Amp (Invitrogen), an eukaryotic expression vector, using adequate adaptors. The resultant plasmid DNA (pcDNA1/KOPR) was transfected to COS-7 cells by the DEAE-dextran method [6]. After cultivation for 65 h, the cells were harvested and homogenized in the following buffer: 50 mM Tris (pH 7.4), 10 mM MgCl₂, 1 mM EDTA and 0.25 M sucrose. After centrifugation for 10 min at 500 × *g*, the supernatant was centrifuged for 20 min at 230,000 × *g*. The pellet was resuspended in 50 mM Tris (pH 7.4) containing 10 mM MgCl₂ and 1 mM EDTA. Binding experiments were carried out with 5 nM [³H]bremazocine and various concentrations of unlabeled subtype-specific opioid ligands

2.4. Functional expression in *Xenopus* oocytes and electrophysiological measurements

*Bst*XI-linearized pKOPR2 was transcribed *in vitro* by T7 RNA polymerase in the presence of the cap analogue, 7-methyl GpppG, using an mCAP RNA capping kit (Stratagene). The transcript was microinjected into defolliculated *Xenopus* oocytes. Oocytes were incubated at 22°C for 1–2 days. Transmembrane current was detected by the two-electrode voltage-clamp method [7].

2.5. Northern blot analysis

Total RNA was extracted from rat various brain regions with RNAzol (Biotec Laboratories, Inc., Houston, USA), and poly(A)⁺ RNA was purified using an mRNA purification kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). Poly(A)⁺ RNA (4 µg) from each brain region was fractionated by electrophoresis on a 1% agarose gel containing 6% formaldehyde, transferred to a nylon membrane (Bio-dyne; Pall, Glen Cove, USA) and baked at 80°C for 2 h. The membrane was prehybridized and then hybridized with the ³²P-labeled antisense RNA probe at 65°C. The membrane was washed twice in 2 × SSC/0.1% SDS for 5 min and then twice in 0.1 × SSC/0.1% SDS for 30 min at 65°C and exposed to X-ray film at –80°C using an intensifying screen.

3. RESULTS AND DISCUSSION

Preliminary *in situ* hybridization histochemistry using pR11 as a probe showed that the hybridization signal was very prominent in the thalamus. Screening of the rat thalamus cDNA library with pR11 gave seven positive clones. Sequencing of these clones showed that the nucleotide sequences of five clones (pMOPR1–5) were the same with that of pR11 in the corresponding region, except for one base. This difference was probably due to the misincorporation by Taq DNA polymerase during the PCR procedure. The nucleotide sequences of other two clones (pKOPR1,2) showed 68.5% homologies to those of pR11 and mouse δ -opioid receptor cDNA in the corresponding region. Because only pKOPR2 is thought to contain a full-length coding region, further investigation was carried out for pKOPR1 and 2 in this study.

Fig. 1 shows nucleotide and deduced amino acid sequences of pKOPR1 (nucleotides 131–2371) and pKOPR2 (nucleotides –110–1262). pKOPR2 contained a 1140 bp open reading frame. The nucleotide sequence surrounding the initiation codon agrees with the

–110	TCCAGCCCTGCTGCACAGGCAAGTTTGTCTCTCTGGTCCAAATCTGTC	–61
	CTCTGCTCTCGAGCCCGGCAAGTGCATCGTCTCGGTTTCCAGCTGCAGCACTGACC	–1
	ATGGAGTCCCCATCCAGATTTTCGGGGAGAGCCAGGCCCTACCTGTGCTCCAGTGCT	60
	M E S P I Q I F R G E P G P T C A P S A	20
	TGCCTACTCCCAACAGCAGCTCTTGGTTCGCCAACTGGGCCAATCGGACAGCAATGGC	120
	C L L P N S S S W F P N W G S D S N G	40
	AGTTTGGGCTCCGAGGACAGCAGCTGGAGCCCGGCACATCTCTCCAGCCATCCCTGTT	180
	S L G S E D Q Q L E P A H I S P A I P V	60
	ATCATCCCGCTGTCTACTCTGTGGTGTGGTGGGCTAGTGGGCAATTCCCTGGT	240
	I T A V Y S V V E V V I S I D V Y	80
	ATGTTTGTTCATCATCGATACACAAAGATGAAGACCGCAACACATCTACATATTAAAC	300
	M F V I I R Y T K M K T A T N I Y I P N	100
	CTGGCTTTGGCAGATGCTTTGGTTACTACCACTATGCCCTCCAGAGTGCTGTCTACTTG	360
	L A L A D A L V T T T M P F Q S A V Y L	120
	ATGAATCTTGGCCTTTGGAGATGTTCTGTGCAAGATGTGATTTCCATTGACTACTAC	420
	M N S W P F G D V L C K I V I S I D V Y	140
	AACATGTTTACAGCATATTCACCTTGACCATGATGAGTGTCGACCGCATCTTGGCGTG	480
	N M F T S I E T I T M M S V D R Y I A V	160
	TGCCACCTGTGAAGCTTTGGATTTCGCAACCTTTGAAAGCAAAGATCATCAACATC	540
	C H P V K A L D F R T P L K A K I L N I	180
	TGCATCTGGCTATCGCATCATCTGTTGTATATCAGCATAGTCTCGAGGCAACAA	600
	C I W L L A S S V G I S A I V L G G T K	200
	GTCAGGAAGATGTGGATGTCATTGAATGCTCTTGCAGTTTCTGATGATGAATATCC	660
	V R E D V D V I E C S L Q F P D D E Y S	220
	TGGTGGACCTCTTCATGAAGATCTGTGCTCTCGTCTTTGGCTTTGTTATCCCTGTCTTA	720
	W D F L M K I C V F V F A F V I P V L	240
	ATCATCATCTGTCTGTACACCTGATGATCGTGGCTTGAAGAGTGTCGGGCTCTCTCG	780
	I I I V C Y T L M I L R L K S V R L L S	260
	GGCTCTCGAGAGGACCGAAATCTCCGCGGATCACCAAGCTGGTGTCTGTAGTGTT	840
	G S R E K D R N L R I T K L V L V V V	280
	GCAGTCTTCATCATCTGTGTGACCCCATCCACATCTTTATCTCTGGTGGAGCTCTAGGC	900
	A V F I I C W T P I H I F I L V E A L G	300
	AGCACCTTCACAGCAGCTGTCTCTCTAGCTATTACTCTGCAATGCTTGGGTTAT	960
	S T S H S T A V L S S Y F C I A L G Y	320
	ACCAACAGCAGCTTGAATCCCTGTTCTCTATGCTCTTGTATGAAATCTCAAGCGGTGT	1020
	T N S L N P V L Y A F L D E N F K R C	340
	TTTAGGACTCTGTCTCCCAATTAAGATGCAATGGAGCGGACAGGACAAACAGAGTT	1080
	F R D F C F P I K M R M E R Q S T N R V	360
	AGAAACACAGTTCAGGATCTGCTTCCATGAGGATGTGGTGGGATGAATAAGCCAGTA	1140
	R N T V Q D P A S M R D V G G M N K P V	380
	TGACTAGTCATGAAATGTCTCTCTATTGTTCTCCGGGTAGAGAAAGTCAATGATCTT	1200
	GGTTTAAACAGATTTACCACTGCTGCTGAGGAGAAAGATGAGGTATTAATACTTAG	1260
	CCATGTTATGCAATCTAAAGTTCAGGGACATAGTGATCTAGGCTGAGTAGGGGACG	1320
	AAGTGTGAAGAACAGAGACATGCTCTGGCAACATACACCTCTTCTAGGACAGCAAT	1380
	GAGGCAATCTAACCTCAACCTTCGATAACAGACAGCAGCTCTTCTTGTGGTCCCGTT	1440
	GATTTACTGCACCTCCATCTGCTGGCTTTCTGTGTAACATAGTTCGAAAGCTCTAGAG	1500
	AAGAAATGAAGAAAGTGCATTGATGCAAACTTACTGGGACCCCACTCTGCTGCT	1560
	TAAACAGGCAAAACCACTTCGATACATAGGCGCATCAAGGGTCATATCCAACTC	1620
	ACTTTCTGCTGTGATGATCTTATGTCGATGAGAGTGAACCTTGAAGAACCTGGA	1680
	CTCAGACCTTCAGACTTGGGGAGAGCAGCATATGACATTTGTGAACTATTTAAAGTTC	1740
	TGGGTGTTCTGTGCTCAGAGTGTCTAATGCCCTTGAAGAACTACAGTTGCTCTTAAGTTC	1800
	CTGTGTTTATGATGCTATTCTAGGAAGATAATCTCTGAGAAACATGAACTGATATTA	1860
	AAAGTTTGAAGCTTAATACAGCAAGTGTGTGTAATTTCTCTGTAATAGTGGTCTGT	1920
	ATATAAATAAGGACAGGTTTCTCTGTCAGGCTGATCAATTTCTCAAGGATGCCGTAGAC	1980
	ACACCCCTGGAGGATGAAAGTTTCTGCTGGGATATTTTCTGCTCACTATAAGCTTACTTT	2040
	CTTGATTTGGTCTGTGTGATTTCTACTAGATTACTCAAACTATTTTACTCTAACCT	2100
	GATCAATGCTTGGTGTAAACATTTCCCAAACTTTGAATTCATTTCAAGTGTATGACAT	2160
	GATCAAACTCTCTTTGGTAGCATCTGTTTGAACACACACATATGTCAGATTTCTCT	2220
	ACTCAGGTAGAGAAAGTTGCTTGTATCATGTACACCTTCAAAATGTTATGCTTGGCTTTC	2280
	CACAGAAAGTGAATTTTCAAAATGCATGCTGAAAGAAATAGGATTTAGATGGC	2340
	TTAGCACAAATTCATGTTTATGAGTAAGAG	2371

Fig. 1 Nucleotide and deduced amino acid sequences of rat κ -opioid receptor cDNA. The deduced amino acid sequence is shown beneath the nucleotide sequence using single letter code. Positions of the putative transmembrane segments I–VII are indicated by underlines beneath the amino acid sequence. Stars and closed circles show potential N-glycosylation sites in the extracellular regions and potential phosphorylation sites, respectively.

Kozack nucleotide sequence ACCATGG [8]. The predicted protein sequence consists of 380 amino acid residues with a calculated molecular weight of 42,702. The hydrophobicity profile determined by the Kyte and Doolittle method [9] and the sequence homology analy-

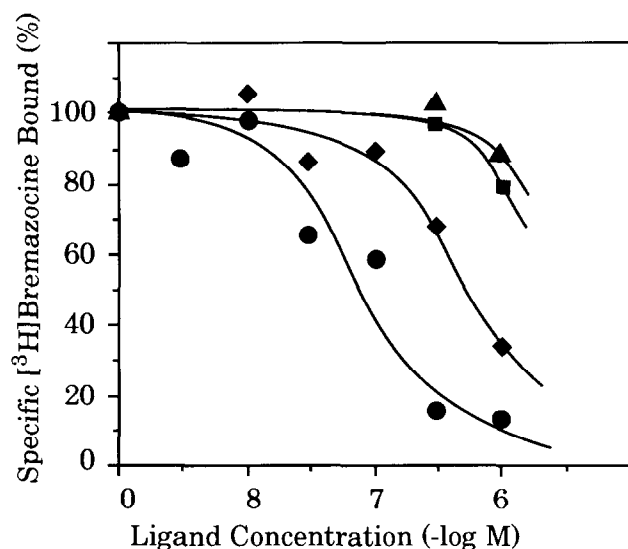


Fig. 2. Displacement of the specific binding of [3 H]bremazocine to the membrane of COS-7 cells transfected with rat κ -opioid receptor cDNA. Unlabeled opioid ligands used are as follows; Dynorphin A (1–17) (●), U50488H (◆), DAMGO (■), DPDPE (▲).

sis indicated that it possesses seven hydrophobic segments and shares a considerable sequence similarity with other G protein-coupled receptors, especially with mouse δ -opioid receptor (58.6%). Like other G protein-coupled receptors, potential *N*-glycosylation sites are seen at the NH_2 -terminal domain. A pair of cysteine residues proposed to form a disulfide bond occur in the first two extracellular loops. Compared to mouse δ -opioid receptor, consensus sequences for potential phosphorylation sites are almost conserved. These characteristics in the primary structure of pKOPR2 suggest that this clone encodes an opioid receptor.

We next examined the binding of [3 H]bremazocine to the membrane of COS-7 cells transfected with pcDNA1/KOPR, which is a eukaryotic expression vector containing the *Pst*I–*Spe*I fragment of pKOPR2 as an insert, and found that the radioactive ligand bound specifically to the membrane with a dissociation constant (K_d) of 0.37 nM. No binding was detected to the membrane of COS-7 cells transfected with pcDNA1/Amp, which is the vector without any insert (data not shown). As shown in Fig. 2, the binding of [3 H]bremazocine was displaced by unlabeled opioid ligands, the displacement efficiency being dynorphin A (1–17) > U50488H >> DAMGO $\approx$ DPDPE. The κ -selective opioid ligands dynorphin A (1–17) and U50488H at 300 nM inhibited the binding by 85 and 33%, respectively. At the same concentration the μ -selective ligand DAMGO and the δ -selective ligand DPDPE did not inhibit the binding at all. These results indicate that pKOPR2 encodes the κ -opioid receptor.

We observed that opioid receptors could link to

phosphatidylinositol turnover and Ca^{2+} mobilization via inositol trisphosphate in *Xenopus* oocytes injected with poly(A) $^+$ RNA derived from rat brain (unpublished data). The rise of intracellular Ca^{2+} can be detected as an inward current induced by opening of Ca^{2+} -activated Cl^- channel [10]. Fig. 3 shows electrophysiological responses of a *Xenopus* oocyte injected with the mRNA transcribed in vitro with pKOPR2 as a template. The oocyte exhibited an inward current in response to 1 μM of U50488H. No response was evoked by DAMGO nor DPDPE at the same concentration. These results demonstrate that pKOPR2 encodes a functional κ -opioid receptor.

Fig. 4 compares the deduced amino acid sequences of rat κ -opioid and mouse δ -opioid receptors. As a whole the two sequences are 58.6% identical with each other. Especially, the putative transmembrane and intracellular regions are 68.2% and 68.3% identical, respectively. Such high homology in the transmembrane and intracellular regions suggests that both receptors link to similar second messenger systems. On the other hand, lower homology (36.4%) is observed in extracellular regions which are thought to be involved in the ligand binding. The second extracellular loop of the κ -opioid receptor is rich in negatively charged amino acid residues, compared to the δ -type. This finding, together with the fact that dynorphin A, an endogenous κ -opioid receptor ligand, has more arginine residues than any other endogenous opioid peptides, suggests that the sec-

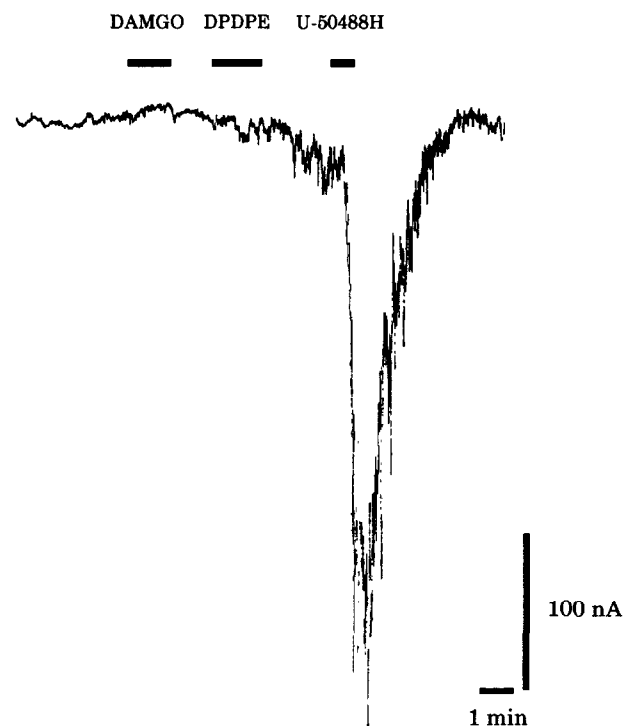


Fig. 3. The current trace from the *Xenopus* oocyte injected in vitro transcribed rat κ -opioid receptor mRNA. A downward deflection indicates an inward current. Bars above the traces show the duration of application of each drug at 1 μM .

		<u>I</u>	
rKOPR	MESPIQIFRGEPTCAPSACLLPNSSSWFPNWAESDSNGSLGSEDQQLEPAHISPAIPV		60
	** * * * *		
mDOPR	ME-----LVPSARAEIQSSPLVNLSDAFPSAFPSAGANASGS---PGARSASSLALAI		50
		<u>II</u>	
rKOPR	IITAVYSVVFVGLVGNLSVMFVIIRYTKMKTATNIYIFNLALADALVTTTTPFQSAVYL		120
	*** ** *		
mDOPR	AITALYSAVCAVGLLGNVLMFGIVRYTKLKTATNIYIFNLALADALATSTLPFQSAKYL		110
		<u>III</u>	
rKOPR	MNSWPFQDVLCKIVISIDYNNMFTSIFTLTMSVDRYIAVCHPVKALDFRTPLKAKIINI	<u>IV</u>	180
	* * * * *		
mDOPR	METWPFGEILLCKAVLSIDYNNMFTSIFTLTMSVDRYIAVCHPVKALDFRTPAKAKLINI		170
		<u>V</u>	
rKOPR	CIWLLASSVGISAIVLGGTKVREDVDVIECSLQFPDDEYSW-WDLFMKICVFVFAFVIPV		239
	*** ** *		
mDOPR	CIWVLASGVGPIMVMAVTQPR-DGAV-VCMLQFP--SPSWYWDTVTKICVFLFAFVVPPI		226
		<u>VI</u>	
rKOPR	LIITVCYTLMLRLKSVRLLSGSREKDRNLRRITKLVLVVVAVFIICWTPIHIFILVEAL		299
	*** ** *		
mDOPR	LIITVCYGLMLRLRSVRLLSGSKEKDRSLRRITRMVLVVVGAFVVCWAPIHIFIVVWTL		286
		<u>VII</u>	
rKOPR	-GSTSHSTAVLSSYYFCIALGYTNSSLPVLYAFLDENFKRCFRDFC-----FPIKMR		351
	* * * * *		
mDOPR	VDINRRDPLVVAALHLCIALGYANSSLPVLYAFLDENFKRCFRQLCRTPCGRQEPGSLR		346
rKOPR	MERQSTNRVRNTVQDPASMRDVGGMNKPV		380
	* * * * *		
mDOPR	RPRQATTRERTACTP-SDGPGGGRAAP-		373

Fig. 4. Comparison of the amino acid sequences of rat κ -opioid receptor (rKOPR) and mouse δ -opioid receptor (mDOPR). The amino acid sequences of rat κ -opioid (upper) and mouse δ -opioid (lower) receptor are aligned to achieve maximal homology. Asterisks indicate exact matches between the two sequences.

ond extracellular loop of this receptor is important to selective recognition of its ligands.

Because pKOPR and pMOPR are highly homologous to each other in the coding regions, 3'-noncoding region of pKOPR1 (nucleotides 1220–2371) was used as a template for synthesis of the 32 P-labeled antisense RNA probe. Upon northern blot analysis, all the regions of rat brain tested gave a single positive band at 5.8 kilobases (Fig. 5). The difference in length between the band and the cloned cDNA is due to that we could not obtain full-length of 3'-noncoding region because of using random-priming method to construct a cDNA library and that poly A tail is usually added to 3'-end of mRNA in a eukaryotic cell. As can be seen in Fig. 5, expression of κ -opioid receptor mRNA was intensive in the striatum and hypothalamus, moderate in the thalamus, midbrain and pons-medulla, and weak or slight in the cerebral cortex, hippocampus and cerebellum. This distribution pattern is consistent with that of κ -opioid binding sites [11]. A study of the detailed distribution of κ -opioid receptor mRNA in rat brain is under way in our laboratory using an in situ hybridization technique.

In this study, we presented for the first time the cloning of the κ -opioid receptor cDNA and its complete

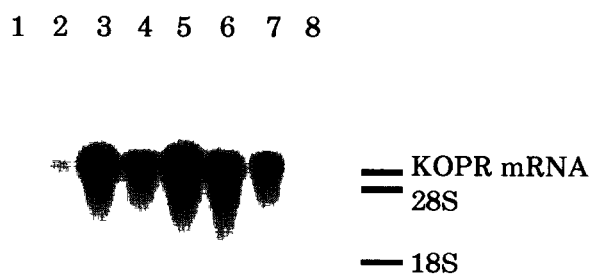


Fig. 5. Northern blot analysis of RNAs isolated from various regions of rat brain. Four μ g of poly(A)⁺ RNA was applied to each lane. Lane 1, cerebral cortex; lane 2, hippocampus; lane 3, striatum; lane 4, thalamus; lane 5, hypothalamus; lane 6, midbrain; lane 7 pons-medulla; lane 8, cerebellum.

amino acid sequence. This work will be useful for molecular studies on the functions of the opioid receptors through which endogenous opioid peptides and opiate drugs manifest their physiological and pharmacological effects. The expression and characterization of pMOPR are now in progress.

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