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cDNA cloning and regional distribution of a novel member of the opioid receptor family

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Abstract

We have cloned a cDNA for a novel member of the opioid receptor family, designated as ROR-C, from the rat cerebrum cDNA library using the probe derived from the δ-opioid receptor subtype cDNA. The deduced amino acid sequence of ROR-C shows high homology with those of ROR-A (rat δ-opioid receptor subtype), ROR-B (rat μ-subtype) and ROR-D (rat κ-subtype). RNA blot hybridization and in situ hybridization analysis revealed that ROR-C mRNA is expressed in discrete regions of the rat central nervous system.

Key words: G-protein-coupled receptor; Opioid receptor; cDNA cloning; RNA blot hybridization analysis; Hybridization analysis (in situ); Rat brain

1. Introduction

G-protein-coupled receptors constitute essential components of the signalling systems in nervous systems [1]. A growing number of G-protein-coupled receptors have been identified by means of recombinant DNA technology [2]. Recently, primary structures of the μ-, δ- and κ-opioid receptor subtypes, that were known to mediate a variety of cellular responses through the action of G-proteins [3], have been elucidated by cloning and sequencing the cDNAs [4–16]. Analysis of the deduced amino acid sequences showed that these opioid receptor subtypes possess characteristic structural features of G-protein-coupled receptors and have ~60% amino acid sequence identity to each other.

This investigation deals with cloning and sequencing a cDNA, designated as ROR-C, from rat cerebrum cDNA library by cross-hybridization using the δ-opioid receptor subtype cDNA as a probe. The results obtained suggest that ROR-C represents a novel member of the opioid receptor family with a high degree of amino acid sequence homology to the so far cloned opioid receptors. However, ligand-binding studies demonstrated no specific binding of opioid ligands or sigma ligands to membranes prepared from COS-7 cells transfected with the ROR-C cDNA. RNA blot hybridization and in situ hybridization analysis using rat brain tissues showed that hypothalamus and brainstem contain ROR-C mRNA in discrete cell groups. These results suggest that ROR-C may be involved in the modulation of neurotransmission in the noradrenergic system and/or in the regulation of the autonomic nervous system and the neuroendocrine system.

2. Materials and methods

2.1. Cloning of cDNA

A rat cerebrum cDNA library was screened with the mouse δ-opioid receptor subtype cDNA probe as in [8,10]. cDNA inserts of three clones including pROR13 and pROR30 share a common restriction endonuclease map (*Sac*I, *Pst*I, *Sma*I and *Apa*I) different from those of ROR-A (δ-subtype), ROR-B (μ-subtype) and ROR-D (κ-subtype), and are indicated to be derived from identical mRNA species (ROR-C). pROR13 and pROR30, carrying the entire protein-coding sequence of ROR-C, were used for sequence analysis [17,18].

2.2. Expression of cDNA and ligand-binding assay

The ~1.7-kilobase pairs (kbp) *Hin*dIII fragment from pROR30, containing the entire protein-coding sequence of ROR-C, was cloned into the *Hin*dIII site of an expression plasmid pKCRH2 [19] in the same orientation with respect to SV40 promoter to yield pRORS30–2. The ~4.2-kbp *Sac*I fragment from pROR13 was blunted with T_4 DNA polymerase and ligated with the synthetic *Hin*dIII linkers to yield pRORS13–1. The ~1.2-kbp *Hin*dIII fragment from pRORS13–1 was cloned into the *Hin*dIII site of pKCRH2 in the same orientation with

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Abbreviations: G-proteins, guanine nucleotide-binding regulatory proteins.

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respect to SV40 promoter to yield pRORS13-3. COS-7 cells were transfected [20] with pRORS30-2 or pRORS13-3 and harvested after 2–3 days. Crude membranes were prepared and assayed for ligand binding as in [8,10].

2.3. RNA blot hybridization analysis

Total RNA and poly(A)$^+$ RNA prepared from tissues of adult Wistar rats were analyzed as in [8,10]. The hybridization probe used was the ~1.6-kbp cDNA insert of pROR30. The probe was labelled with [α-^{32}P]dCTP by the random primer method [21]. Autoradiography was performed at −80°C for 9 days with an intensifying screen. An RNA ladder (Bethesda Research Laboratories) and the *Hin*dIII cleavage products of phage λ DNA were used as size markers.

2.4. In situ hybridization analysis

The ~1.3-kbp *Eco*RI fragment from pROR13 was cloned into pBluescript SK(−) to yield pRORS13-4. Sense or antisense riboprobe RNA was synthesized in vitro in the presence of digoxigenin-11-UTP, using *Bam*HI- or *Eco*RV-cleaved pRORS13-4 as a template, respectively. Under intraperitoneal pentobarbital anesthesia, adult male Sprague–Dawley rats were perfused transcardially with a fixative consisted of 4% paraformaldehyde and 0.12 M sodium phosphate (pH 7.3). The brains were soaked overnight in a solution containing 30% sucrose and 0.12 M sodium phosphate (pH 7.3), and cut into coronal sections of 50-μm thickness on a freezing microtome. Free-floating sections were treated with hydrochloric acid, Triton X-100 and proteinase K, and incubated with the hybridization solution (50% deionized formamide/0.75 M NaCl/25 mM EDTA/25 mM PIPES/0.2% SDS/1 × Denhardt's solution/250 $\mu g \cdot ml^{-1}$ salmon sperm DNA/250 $\mu g \cdot ml^{-1}$ yeast RNA/4% dextran sulfate) for 1 h. Hybridization was carried out overnight at 55°C in the hybridization solution added with 1 ng $\cdot \mu l^{-1}$ digoxigenin-labelled riboprobe. Hybridized sections were then rinsed in 4×, 2×, 1× and 0.5× standard saline citrate (SSC), RNase A was added, and finally washed with 0.1× SSC. The sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:200 dilution; Boehringer-Mannheim) and reacted with chromogenic substrates, 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. They were then placed onto gelatin-coated glass slides, dried and embedded in Crystalmount.

3. Results

cDNA library derived from rat cerebrum poly(A)$^+$ RNA was screened by hybridization with a DNA fragment comprising a part of the mouse δ-opioid receptor subtype cDNA [4,5]. A novel class of cDNA clones (ROR-C) was isolated and nucleotide sequence analysis revealed an open reading frame of 1,101 nucleotides encoding a sequence of 367 amino acids. Fig. 1 shows the deduced amino acid sequence of the ROR-C protein aligned with those of ROR-A (rat δ-opioid receptor subtype), ROR-B (rat μ-subtype) and ROR-D (rat κ-subtype). Amino acid sequence comparison reveals 53%, 52% and 51% identity between ROR-C/ROR-A, ROR-C/ROR-B and ROR-C/ROR-D pairs, respectively. The ROR-C molecule shares characteristic structural features with G-protein-coupled receptors [2]. Seven transmembrane segments are predicted in the ROR-C molecule, and three consensus N-glycosylation sites [22] occur in the amino-terminal region preceding the predicted transmembrane segment, TM-I. Furthermore, the carboxy-terminal region just following TM-VII contains two conserved cysteine residues that may be modified by palmitoylation [2]. To establish evolutionary relation-

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ROR-C   MESLF------PAPYWEVLYGSHFQGNLSLLNETV-------PHHLLLNAS-------------------------HSAFLPLGLKVTIVGLYLAVCIG  61
ROR-A   MEPV-------PSARAELQFSLL--ANVSDTFPSA-------FPSASANASGSPG-------------------ARSA-SSLALAIAITALYSAVCAV  62
ROR-B   MDSS---------------TGP---GNTSDCSDPLAQASCSPAPGSWLNLSHVDGNQSDPCGLNRTGLGGNDSLCPQTGS-PSMVTAITIMALYSIVCVV  81
ROR-D   MESPIQIFRGEPGPTCAPSACLL--PNSSSWFPN--------WAESDSNGSVGSEDQQ-----------------LEPAHISPAIPVIITAVYSVVFVV  72
                                                                                                    TM-I

ROR-C   GLLGNCLVMYVILRHTKMKTATNIYIFNLALADTLVLLTLPFQGTDILLGFWPFGNALCKTVIAIDYYNMFTSTFTLTAMSVDRYVAICHPIRALDVRTS  161
ROR-A   GLLGNVLVMFGIVRYTKLKTATNIYIFNLALADALATSTLPFQSAKYLMETWPFGELLCKAVLSIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTP  162
ROR-B   GLFGNFLVMYVIVRYTKMKTATNIYIFNLALADALATSTLPFQSVNYLMGTWPFGTILCKIVISIDYYNMFTSIFTLCTMSVDRYIAVCHPVKALDFRTP  181
ROR-D   GLVGNSLVMFVIIRYTKMKTATNIYIFNLALADALVTTTMPFQSAVYLMNSWPFGDVLCKIVISIDYYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTP  172
                                    TM-II                                  TM-III

ROR-C   SKAQAVNVAIWALASVVGVPVAIMGSAQVEDEE--IECLVEIPAPQD-YWGPVFAICIFLFSFIIPVLIISVCYSLMIRRLRGVRLLSGSREKDRNLRRI  258
ROR-A   AKAKLINICIWVLASGVGVPIMVMAVTQPRDGA--VVCTLQFPSPSW-YWDTVTKICVFLFAFVVPILIITVCYGLMLLRLRSVRLLSGSKEKDRSLRRI  259
ROR-B   RNAKIVNVCNWILSSAIGLPVMFMATTKYRQGS--IDCTLTFSHPTW-YWENLLKICVFIFAFIMPVLIITVCYGLMILRLKSVRMLSGSKEKDRNLRRI  278
ROR-D   LKAKIINICIWLLASSVGISAIVLGGTKVREDVDVIECSLQFPDDEYSWWDLFMKICVFVFAFVIPVLIIIVCYTLMILRLKSVRLLSGSREKDRNLRRI  272
                    TM-IV                                  TM-V

ROR-C   TRLVLVVVAVFVGCWTPVQVFVLVQGLGVQPGS-ETAVAILRFCTALGYVNSCLNPILYAFLDENFKACFRKFCCAS-------SLHREMQVSDRVRSIA  350
ROR-A   TRMVLVVVGAFVVCWAPIHIFVIVWTLVDINRRDPLVVAALHLCIALGYANSSLNPVLYAFLDENFKRCFRQLCRAPCGGQEPGSLRRPRQATARERVTA  359
ROR-B   TRMVLVVVAVFIVCWTPIHIYVIIKALITIPET-TFQTVSWHFCIALGYTNSCLNPVLYAFLDENFKRCFREFCIPTSSTIEQQNSTRVRQNTREHPSTA  377
ROR-D   TKLVLVVVAVFIICWTPIHIFILVEALGSTSHS-TAVLSSYYFCIALGYTNSSLNPVLYAFLDENFKRCFRDFCFPI-------KMRMERQSTNRVRNTV  364
                  TM-VI                                  TM-VII

ROR-C   KDVGLGCKTSETVPRPA----  367
ROR-A   CTPSDGPGGGAAA--------  372
ROR-B   NTVDRTNHQLENLEAETAPLP  398
ROR-D   QDPASMRDVGGMNKPV-----  380
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Fig. 1. Deduced amino acid sequence of ROR-C (first line) and its alignment with those of ROR-A (rat δ-opioid receptor subtype) (second line), ROR-B (rat μ-subtype) (third line) and ROR-D (rat κ-subtype) (fourth line). Sequence data for ROR-A, ROR-B and ROR-D have been taken from [8,10]. The one-letter amino acid notation is used. Gaps (–) have been inserted to achieve maximum homology. Amino acid residues are numbered from the initiating methionine and numbers of the residues at the right-hand end of the individual lines are given. The predicted transmembrane segments (TM-I-VII) are indicated; the termini of each segment are tentatively assigned. The nucleotide sequence of ROR-C appears in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D16438.

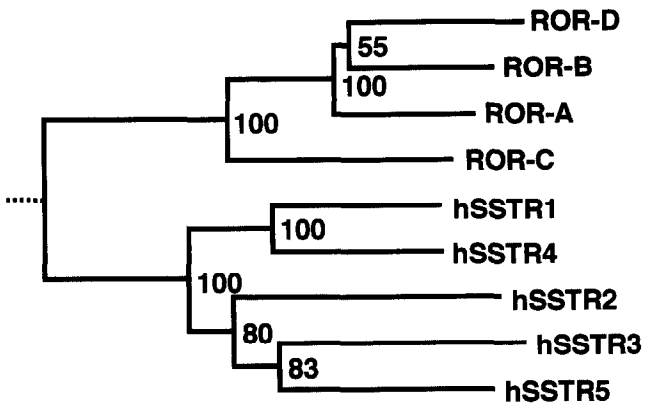

Fig. 2. Phylogenetic tree of the opioid receptors and the somatostatin receptors. On the basis of the distance matrix between aligned amino acid sequences, the tree was inferred by the neighbor-joining method [23]. The deepest root was determined by including distantly related subfamilies in comparison. Branch lengths are proportional to the numbers of amino acid substitutions. The number at each branching point indicates the bootstrap probability that two lineages are joined together to form a single cluster [27]. The members of the somatostatin receptor family included in this analysis are human SSTR1 [28], human SSTR2 [28], human SSTR3 [29], human SSTR4 [30,31] and human SSTR5 [31].

ships among ROR-A-D and the somatostatin receptors, which show the highest degree of homology to the opioid receptors among the G-protein-coupled receptors [5,7,12,15,16], a phylogenetic tree was constructed by the neighbor-joining method [23] (Fig. 2). The phylogenetic tree shows that the opioid receptor family forms a single cluster which is separated from the cluster of the somatostatin receptor family. Judging from the bootstrap probability between lineages leading to ROR-C and three other RORs, it is highly likely that ROR-C is included in the cluster of the opioid receptor family and thus is a novel member of this family.

In order to identify ligands for ROR-C, we performed ligand-binding studies. Membranes prepared from COS-7 cells tranfected with the ROR-C cDNA showed no specific binding activity for opioid ligands [24] including [D-Ala^2, D-Leu^5]enkephalin, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, [D-penicillamine^2, D-penicillamine^5]-enkephalin, ethylketocyclazocine, U69,593, bremazocine, naloxone and naltrindole. We also tested ligands for the sigma receptors including haloperidol, SKF- 10,047 and 1,3-di-*O*-tolylguanidine, because these receptors were originally classified as a subtype of the opioid receptors [25] and could be expected to have high amino acid sequence homology with the opioid receptors. However, no specific binding of sigma ligands could be detected for membranes prepared from COS-7 cells transfected with the ROR-C cDNA.

Poly(A)$^+$ RNA preparations from rat tissues were subjected to blot hybridization analysis (Fig. 3a). Cerebrum and brainstem contain three RNA species of ~15.5 kilobases (kb), ~8.8 kb and ~3.7 kb hybridizable with the ROR-C cDNA probe; the multiple RNA species may arise from polyadenylation at different sites and/or from alternative splicing. A very small amount of ROR-C mRNA is detectable in cerebellum. No signal is observed for kidney. Blot hybridization analysis of total RNA isolated from various regions of rat brain (Fig. 3b) shows that a moderate amount of the ~15.5-kb RNA species hybridizable with the ROR-C cDNA probe is present in hypothalamus and brainstem and small amount in thalamus and cerebral cortex. No signal is detectable in spinal cord, olfactory bulb, caudate-putamen, hippocampus and cerebellum.

Next we analyzed rat brain tissues for ROR-C mRNA by in situ hybridization with riboprobes (Fig. 4). Consistent with the result obtained by RNA blot hybridization analysis (see above), brainstem and forebrain are found to contain specific hybridization signals in discrete cell groups. In brainstem, high expression is localized to nu-

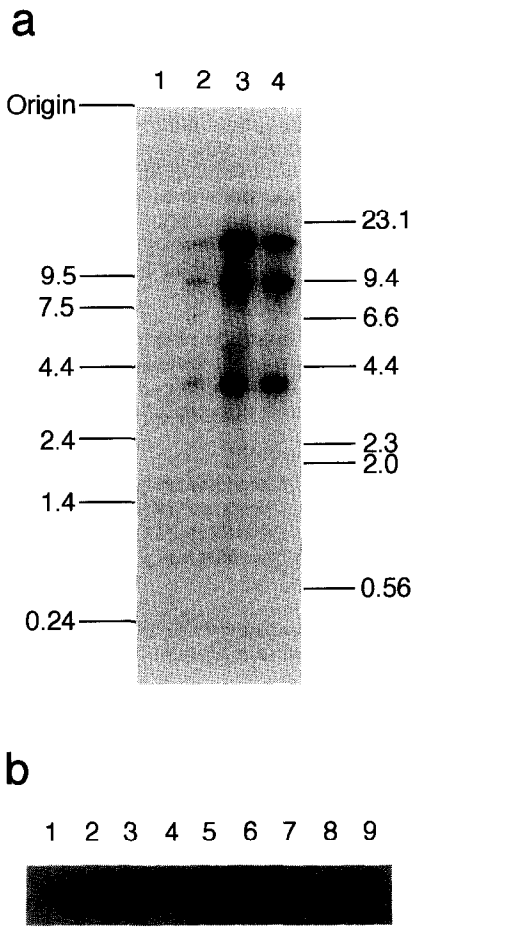

Fig. 3. Autoradiograms of blot hybridization analysis of poly(A)$^+$ RNA (a) and total RNA (b) from rat tissues with the probe derived from the ROR-C cDNA. (a) Each lane contains 30 μg of poly(A)$^+$ RNA; lanes: 1, kidney; 2, cerebellum; 3, brainstem; and 4, cerebrum. The size markers are indicated in kb. (b) Each lane contains 30 μg of total RNA; lanes: 1, spinal cord; 2, olfactory bulb; 3, hypothalamus; 4, thalamus; 5, caudate-putamen; 6, hippocampus; 7, brainstem; 8, cerebellum; and 9, cerebral cortex. The ~15.5-kb RNA species hybridizable with the ROR-C cDNA probe is shown.

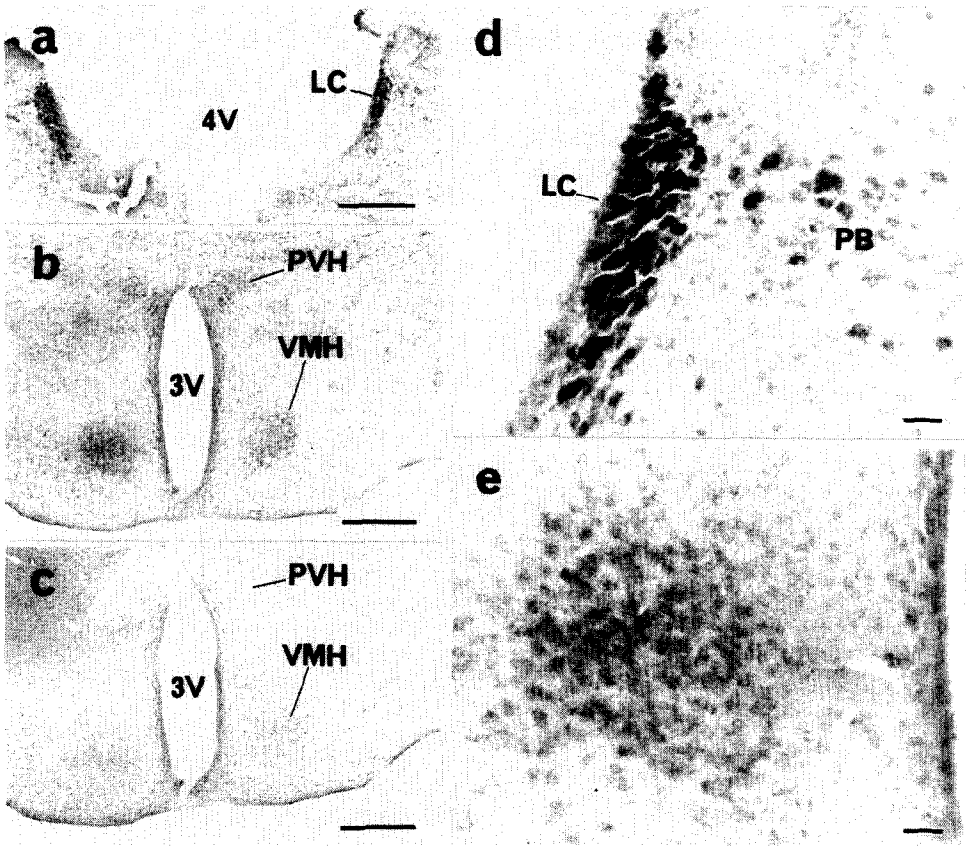

Fig. 4. In situ hybridization analysis of rat brainstem (a and d) and hypothalamus (b, c and e) using the digoxigenin-labelled antisense (a, b, d and e) and sense (c) riboprobes for ROR-C. Bright field photomicrographs show signal-containing cells in the locus coeruleus (LC; a and d), the ventromedial nucleus of hypothalamus (VMH; b and e), and the paraventricular nucleus of hypothalamus (PVH; b). In brainstem (a and d), some hybridization-positive cells are also observed in the parabrachial nucleus (PB; d). Hybridization with the sense riboprobe (c) reveals no appreciable labelling in VMH and PVH. 3V, third ventricle; 4V, fourth ventricle. Scale bars = 500 μm (a–c), 50 μm (d and e).

merous neurons in the locus coeruleus and also to some neurons scattered in the parabrachial nucleus (Fig. 4a and d). In forebrain, high expression is observed in two hypothalamic nuclei, the ventromedial nucleus and the paraventricular nucleus (Fig. 4b and e). The hybridization signal in these hypothalamic nuclei is weaker than that found in the brainstem nuclei. In the paraventricular nucleus, neurons expressing ROR-C mRNA have small cell bodies and are localized to its dorsomedial portion. A sense riboprobe failed to yield specific hybridization signals in any of these brain sites (Fig. 4c).

4. Discussion

The primary structure of a novel member of the opioid receptor family, designated as ROR-C, has been deduced by cloning and sequencing the cDNA. Amino acid sequence comparison reveals 51–53% identities between ROR-C and the members of the opioid receptor family including ROR-A, ROR-B and ROR-D. Although the close homology of the opioid receptor with the somatostatin receptor has been reported [5,7,12,15,16], the phylogenetic tree shows that ROR-C can be classified as a member of the opioid receptor family rather than that of the somatostatin receptor family. From these observations, we assumed that ROR-C might represent a G-protein-coupled receptor for opioid ligands or ligands closely related to opioids. However, our cDNA expression studies could not detect any specific binding of opioid ligands or sigma ligands to ROR-C. Thus, agonists and antagonists for ROR-C remain to be identified.

RNA blot hybridization analysis showed that ROR-C mRNA is expressed in the rat central nervous system, predominantly in brainstem and hypothalamus. Furthermore, by in situ hybridization analysis, ROR-C mRNA was demonstrated to be expressed in neurons in the ventromedial nucleus and the paraventricular nucleus of hypothalamus, and in the locus coeruleus and the parabrachial nucleus in brainstem. Since δ-, μ- and κ-opioid receptor subtype mRNAs have been reported to be expressed in a variety of brain sites including hypothalamus and brainstem [6,11,13,14,26], comparison of our results with previous data may provide information as to the identification of ROR-C as a novel species of

the opioid receptor. The expression of δ-opioid receptor subtype mRNA is widespread in forebrain and the extent of expression in hypothalamus is broader than that shown here for ROR-C [11]. The level of expression of μ-opioid receptor subtype mRNA has been reported to be high in the locus coeruleus and the parabrachial nucleus, while the two hypothalamic nuclei seem to contain a negligible amount of μ-opioid receptor subtype mRNA [13]. The expression of κ-opioid receptor subtype mRNA has more or less been shown in the ventromedial and paraventricular nuclei of hypothalamus and in the locus coeruleus as well as in several other brain sites [6,14,26]. Thus, the pattern of regional expression of the κ-opioid receptor subtype is apparently closest to that of ROR-C mRNA. However, as shown in [6,26], hybridization signals of the κ-opioid receptor subtype mRNA in the paraventricular nucleus of hypothalamus appear to predominate over the more extensive region including the ventrolateral or magnocellular portion, in contrast to the distribution pattern of ROR-C mRNA. Hence, the results obtained by in situ hybridization analysis indicate that ROR-C has least similarities in regional expression of mRNA to any of the so far cloned opioid receptors. The locus coeruleus contains a great number of noradrenergic neurons; and the hypothalamus is a center for regulation of the autonomic nervous system and the neuroendocrine system. Therefore, the tissue distribution of ROR-C mRNA suggests that ROR-C may play a role in the modulation of neurotransmission in the noradrenergic system and/or in the regulation of the autonomic nervous system and the neuroendocrine system.

During preparation of this manuscript, we learned that nucleotide sequences similar to that of ROR-C have appeared in the Nucleotide Sequence Databases under the accession numbers U01913 and U04952.

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