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ORL1, a novel member of the opioid receptor family

Cloning, functional expression and localization

Catherine Mollereau[a], Marc Parmentier[b], Pierre Mailleux[c], Jean-Luc Butour[a], Christiane Moisand[a], Pascale Chalon[d], Daniel Caput[d], Gilbert Vassart[b], Jean-Claude Meunier[a,*]

[a]*Institut de Pharmacologie et de Biologie structurale, Centre National de la Recherche Scientifique UPR 8221, 205 route de Narbonne, 31077 Toulouse Cedex, France*

[b]*IRIBHN and* [c]*Unité de Recherche sur le Cerveau, ULB Campus Erasme, 808 route de Lennik, 1070 Bruxelles, Belgique*

[d]*Sanofi Elf Bio-Recherches, 31676 Labège, France*

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Abstract

Selective PCR amplification of human and mouse genomic DNAs with oligonucleotides encoding highly conserved regions of the δ-opioid and somatostatin receptors generated a human DNA probe (hOP01, 761 bp) and its murine counterpart (mOP86, 447 bp). hOP01 was used to screen a cDNA library from human brainstem. A clone (named hORL1) was isolated, sequenced and found to encode a protein of 370 amino acids whose primary structure displays the seven putative membrane-spanning domains of a G protein-coupled membrane receptor. The hORL1 receptor is most closely related to opioid receptors not only on structural (sequence) but also on functional grounds: hORL1 is 49–50% identical to the murine μ-, δ- and κ-opioid receptors and, in CHO-K1 cells stably transfected with a pRc/CMV:hORL1 construct, ORL1 mediates inhibition of adenylyl cyclase by etorphine, a 'universal' (nonselective) opiate agonist. Yet, hORL1 appears not to be a typical opioid receptor. Neither is it a somatostatin or σ (*N*-allylnormetazocine) receptor. mRNAs hybridizing with synthetic oligonucleotides complementary to mOP86 are present in many regions of the mouse brain and spinal cord, particularly in limbic (amygdala, hippocampus, septum, habenula, ...) and hypothalamic structures. We conclude that the hORL1 receptor is a new member of the opioid receptor family with a potential role in modulating a number of brain functions, including instinctive behaviours and emotions.

Key words: G protein-coupled membrane receptor; Etorphine; Diprenorphine; Cyclic AMP; Hybridization, in situ; Brain; Spinal cord

1. Introduction

Opioid substances act in the central (CNS) and peripheral nervous systems to produce numerous pharmacological effects which are conveniently classified as 'acute' and 'chronic'. The acute effects of opioids (analgesia, respiratory depression, miosis, constipation, ..., sensation of well-being) appear to be of neuromodulatory origin and may reflect in most if not all cases, presynaptic depression of neurotransmitter release as examplified by the one of substance P in relation with spinal analgesia [1]. As for the chronic effects (tolerance and dependence), they may reflect neuronal adaptative changes analogous to those seen in cell learning and memory [2]. In practice, the unwanted effects of opioids delicate their widespread use as therapeutic agents and makes them a major class of abused drugs.

At the molecular level, opioids act primarily via three major types, μ, δ and κ of specific membrane receptors which display clear in vitro binding preference for morphine, enkephalins and benzomorphans, respectively [3]. These receptors couple with pertussis toxin-sensitive, guanine nucleotide-binding regulatory proteins (G proteins) either to inhibit adenylyl cyclase [4] and/or calcium channels [5] or to stimulate potassium channels [6].

The cDNAs of the δ- [7,8], μ- [9–11] and κ- [12–15] opioid receptors have recently been cloned and found to encode monomeric proteins having the structure of G protein-coupled membrane receptors (GPCRs). Since it was initially noticed [8] that the δ-opioid receptor, the first to have been cloned, resembles somatostatin receptors, we applied the PCR amplification strategy of Libert et al. [16] to clone new members of the opioid receptor family. Selective PCR amplification of human genomic DNA with oligonucleotides encoding highly conserved regions of the δ-opioid and somatostatin receptors gen-

*Corresponding author. Fax: (33) 6117-5994.

Abbreviations: CNS, central nervous system; G protein, guanine nucleotide-binding regulatory protein; GPCR, G protein-coupled membrane receptor; ORL1, opioid receptor-like receptor or cDNA; TMS, transmembrane segment.

The sequence presented in this paper has been deposited in the EMBL Nucleotide Sequence Database (accession no. X77130).

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erated a DNA probe of 761 bp which we used to screen a cDNA library from human brainstem. Here, we report the isolation of a cDNA clone encoding hORL1 (human Opioid Receptor-Like 1), a protein of 370 amino acids whose primary structure is also typically that of a GPCR. The hORL1 receptor exhibits substantial sequence identities with opioid receptors and, once stably transfected into CHO-K1 cells, mediates inhibition of adenylyl cyclase by etorphine, a nonselective opiate agonist. ORL1 transcripts are expressed in many regions of the mouse CNS, especially in the limbic areas, hypothalamus, brainstem and spinal cord.

2. Experimental procedures

2.1. DNA probe, cDNA library and library screening

Human, mouse and rat genomic DNAs were subjected to low-stringency PCR amplification using pairs of degenerate, synthetic oligonucleotides corresponding to consensus sequences of the second (forward priming) and sixth or seventh (reverse priming) membrane-spanning domains of the mouse δ-opioid and somatostatin receptors. The amplification products were cloned into phage M13 and sequenced. One human product (hOP01, 761 bp long; see Fig. 1) was found, except for a short medial intron of 118 bp, to encode a sequence of 207 amino acids displaying 57.5% identity with the homologous domain of the δ-opioid receptor. Likewise, one murine product (mOP86, 447 bp long) also contained a very short (77 bp) putative intron and encoded the same amino acid sequence as did hOP01, indicating that the human and murine probes corresponded to species variants of the same gene. hOP01 was ^{32}P-labeled with Boehringer's appropriate kit and used to screen a sized (2–7 kb) cDNA library from human brainstem in Pharmacia's pTZ18R phagemid. Twelve × 30,000 recombinant bacterial colonies were tested, in duplicate, by overnight incubation at 42°C with ^{32}P-labeled hOP01 (500,000 cpm/ml) in hybridization buffer (0.25% nonfat dried milk, Régilait; 0.5% SDS; 5 mM EDTA; 40% formamide and 6 × SSC) followed by two 20-min washes with 2 × SSC/0.1% SDS at 60°C and one 30-min wash with 0.1 × SSC/0.1% SDS at 42°C. Autoradiographic analysis of the filters revealed a single positive clone, hereafter designated hORL1 which, upon a second round of screening as above, was isolated.

2.2. Sequencing

Sequencing on both strands of cDNA insert hORL1 was performed by the dideoxynucleotide chain termination method [17] after partial subcloning in M13mp derivatives, using an automated DNA sequencer (Applied Biosystems 370A).

2.3. Expression in cell lines

A *Hin*dIII-*Pst*I fragment of 1,953 bp containing the entire coding region of hORL1 was subcloned in plasmids pSVL (Pharmacia) and pRc/CMV (Invitrogen). pSVL:hOLR1 was used to transfect COS-7 cells and pRc/CMV:hOLR1 to transform CHO-K1 cells. *COS-7 cells* were grown to ≤ 50% confluency in 60 mm dishes in Dulbecco's modified Eagle medium (Gibco) and transfected with 2–5 μg/dish of pSVL:hOLR1 by the method using DEAE-dextran and chloroquine [18]. 72 h later, the cells were rinsed with phosphate-buffered saline, scraped off their matrix into Tris-HCl buffer (50 mM, pH 7.4) and processed as otherwise described [19] to yield a crude membrane fraction. The COS cells membranes were examined for expression of binding activity with radioactive ligands by the method of rapid filtration on glass fiber disks. The radiolabeled ligands were: [15,16(*n*)-^{3}H]diprenorphine and [15,16-^{3}H]etorphine (Amersham), [^{125}I]β-endorphin (NEN), ^{125}I-Labeled somatostatin-14 and SMS 201-995 (gifts from Dr. Ch. Susini, INSERM, Toulouse), [5-^{3}H]DTG (NEN) and [^{3}H]JO-1784 (Jouveinal Laboratories, Fresnes). *CHO-K1 cells* grown to near confluency in 60 mm dishes in Ham's F12 medium (Gibco) were transfected with 5 μg/dish of pRc/CMV:hOLR1 by a modified calcium phosphate precipitate method [20]. 24 h after transfection, the cells were treated with trypsin and diluted 1:10. The following day, selection of transfectants was initiated by addition of 400 μg/ml G418 (Gibco). Fifteen days later, individual resistant clones were isolated, cultured separately and checked for the presence of hORL1 transcripts by Northern blotting. These cells were used to screen a number of unlabeled ligands for ability to increase basal or decrease forskolin-induced intracellular cAMP. Cyclic AMP levels were assayed for in aliquots of 2×10^5 cells pre-incubated for 1 h at 37°C with 0.6 μCi [^{3}H]adenine (23 Ci/mmol, Amersham) in Ham's F12. Stimulation of cAMP production was for 10 min at 37°C in 0.2 ml HEPES buffered Krebs-Ringer saline with 10 μM forskolin in the presence of 0.1 mM IBMX (Sigma) and 0.1 mM Ro-20, 1724 (Biomol Res.). The reaction was stopped by addition of 20 μl 2.2 N HCl and cAMP was isolated by the single column procedure of Alvarez and Daniels [21].

2.4. In situ hybridization

Coronal sections 15 μm thick from frozen mouse brain and spinal cord were thaw-mounted onto poly-L-lysine coated slides, fixed with paraformaldehyde and examined for in situ hybridization as previously described [22] with the oligonucleotides:

5′-GTGCCCTGGAAGGGCAGTGTCAGCAAGACCAGGGTATC-AGCCAGT-3′

5′-GATAGCAATGACCGTCTTGCACAGTGCATTCCCAAATGG-CCAGAA-3′

complementary to mOP86$_{1-45}$ and mOP86$_{62-106}$, respectively. The two 45-mers were synthesized on an Applied Biosystems 381A DNA synthesizer. They were mixed in a 1:1 ratio and radiolabeled with [α-^{35}S]-dATP to ≥ 1,000 Ci/mmol at their 3′ end with terminal deoxynucleotidyltransferase. Nonspecific hybridization was assessed for with oligonucleotides of same length, G-C content and specific radioactivity but complementary to unrelated mRNAs. Hybridization of the sections with the labeled probes and a 100-fold excess of cold probes or unrelated probes as well as pretreatment of the sections with RNAse (20 μg/ml) were also performed.

3. Results

Fig. 1 shows part of the nucleotide sequence of the hORL1 insert (3.2–3.3 kb) with a long open reading frame of 1,110 bases encoding a protein of 370 amino acids (M_r 40,690). Fig. 1 also shows, for comparison, the partial sequences obtained by PCR from mouse (mOP86) and rat (rOP19) genomic DNA. ORL1 appears to be highly conserved in mammalian species: along the 366 nucleotide overlap between the human and mouse coding sequences, each interrupted by a short intron, the identity amounts to 87%. When comparing the amino acid sequences, the identity increases to 99%, with a single substitution in 123 amino acids. The hORL1 protein contains the seven stretches of predominantly hydrophobic amino acid residues which are thought to represent membrane-spanning domains [23–25]. As opioid receptors and most GPCRs do, hORL1 displays many putative consensus sites for post-translational modifications. These include: (i) three asparagine residues within Asn-X-Ser motifs for N-linked glycosylation near its NH_2-terminus; (ii) a pair of conserved cysteine residues which may connect exofacial loops 1 (C_{123}) and 2 (C_{200}) by a disulfide bridge [26,27]; and (iii) putative palmitoylation sites (C_{329}, C_{334} and C_{335}) downstream of transmembrane segment (TMS) 7 [28,29]. All cytoplasmic domains contain a number of serine and threonine residues representing potential sites of phosphorylation, a process which is known to be involved in homologous and/or

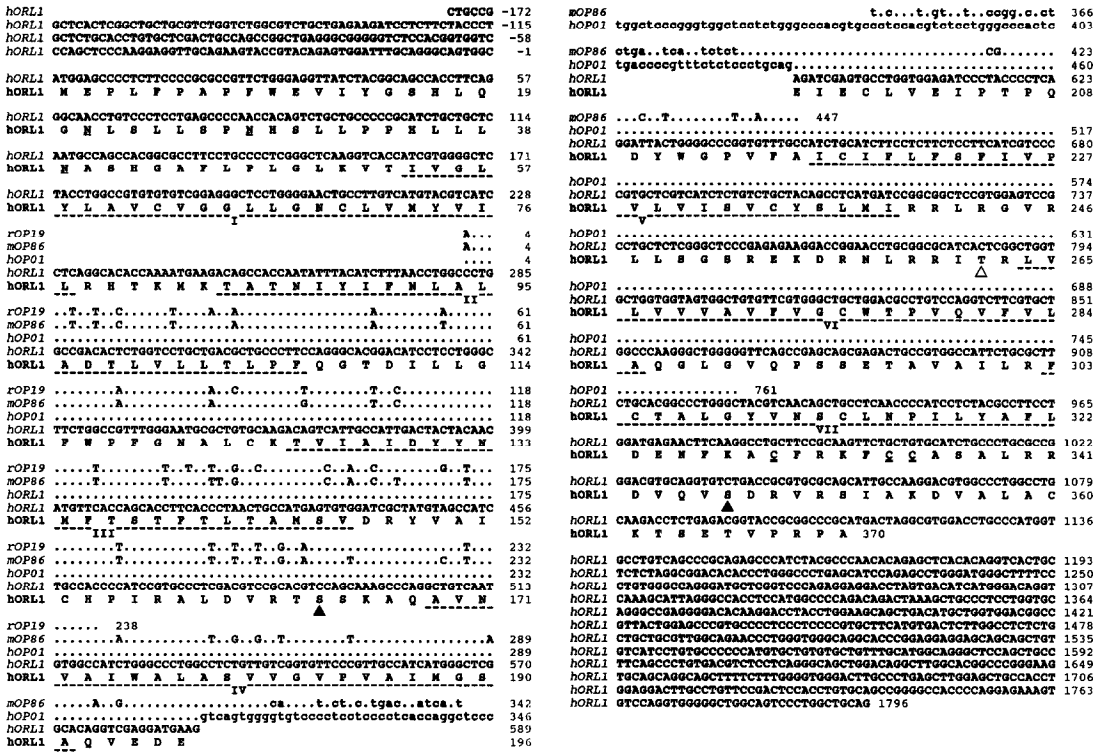

Fig. 1. Nucleotide sequence of ORL1 receptor cDNA. The partial nucleotide sequence of the human cDNA (*hORL1*) encoding the ORL1 receptor and the deduced amino acid sequence (hORL1) are represented. The sequence of the genomic fragment amplified by low stringency PCR and used as probe (*hOP01*) is aligned, showing the location of the short intervening sequence (lowercase characters), as well as PCR-generated genomic sequences from mouse (*mOP86*) and rat (*rOP19*). Identities with the human cDNA (*hORL1*) are represented by dots. The base substitutions in the mouse and rat sequences do not change the amino acid sequence, with the exception of the G_{416} in the mouse sequence that changes a threonine into an alanine. The putative glycosylation (Asn) and palmitoylation (Cys) sites are underlined once and twice, respectively. The potential sites for phosphorylation by proteine kinases A (R/K,R/K,X,S/T) and C (S/T,X,R/K) are indicated by open and closed triangles, respectively. The dashed lines below the hORL1 amino acid sequence situate the membrane-spanning domains, numbered I–VII.

heterologous desensitization of the β_2-adrenergic and other GPCRs [30,31].

Fig. 2 shows that hORL1 is related to somatostatin and, most closely so (see dendrogram), to opioid receptors. Similar amino acid residues in hORL1 and opioid receptors are unevenly distributed along the sequence of amino acids: they are particularly abundant in the putative TMSs (67% identity), especially in the second (82%), third (82%) and seventh (80%), as well as in the cytoplasmic loops and the first dozen of amino acids adjacent to TMS7 in the C-terminal segment (75% identity). In contrast, hORL1 and opioid receptors differ profoundly in their N-terminal, second and third extracellular and C-terminal domains. Moreover, hORL1 contains two aspartate residues: D_{130} in TMS3 and D_{97} in TMS2 which may represent key determinants for agonist binding and allosteric regulation thereof, respectively. Such two residues are highly conserved among GPCRs for catecholamines and other protonated amines (acetylcholine, opiates, ...) and there is evidence that the one n TMS3 is the anionic subsite for the agonist [32] while the other (in TMS2) might support selective sensitivity of agonist binding to Na^+ ions [33,34].

Pharmacological identification of hORL1 was first attempted on membranes from COS-7 cells transfected with pSVL:hORL1 as well as from CHO-K1 cells transfected with pRc/CMV:hORL1, using the following radiolabeled ligands: the two potent, non-selective, 'universal' opiate ligands, [^{3}H]diprenorphine (antagonist) and [^{3}H]etorphine (agonist), [^{125}I]β-endorphin, the putative natural agonist of the ill-defined ε-opioid receptor [35], ^{125}I-labeled SS14 and SMS, somatostatin receptor agonists and σ (non-PCP) receptor ligands: [^{3}H]DTG and [^{3}H]JO-1784. These ligands where chosen on the basis of sequence homology of hORL1, opioid and somatostatin receptors and of pharmacological homology between opioid receptors and the σ receptor whose prototypical ligand is the benzomorphan *N*-allylnormetazocine [36]. Actually, none of these ligands could be shown to specifically bind membranes of COS: and CHO:hORL1 cells at concentrations up to 10 nM. In contrast, when COS cells were transfected with the rat β_2-adrenergic receptor cDNA (control transfection), they expressed, as expected, high levels of high affinity binding for [^{125}I]cyanopindolol (not shown). It was therefore concluded that hORL1 is probably not a typical opioid, somatostatin or σ receptor.

Next, a variety of unlabelled drugs was screened for

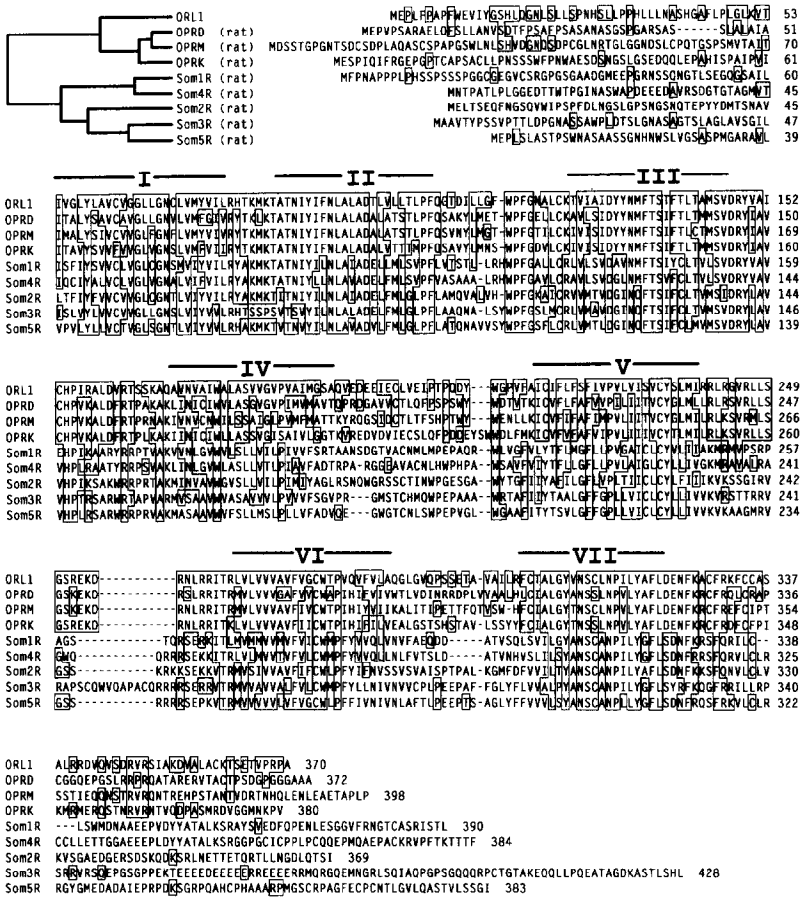

Fig. 2. Alignment of ORL1, opioid and somatostatin receptors. Alignment of the amino acid sequence of ORL1 with those of the three rat opioid receptor types σ, μ and κ (OPRD, OPRM and OPRK, respectively) and of the five rat somatostatin receptors. Similarities with the ORL1 sequence are boxed. The putative membrane-spanning domains are numbered I to VII. The dendrogram represents sequence similarities between the receptors whose sequences are shown. It was constructed by using the Clustal software [44].

their ability to stimulate or inhibit adenylyl cyclase in CHO-K1 cells stably transfected with the pRc/CMV:hORL1 construct. The choice of adenylyl cyclase was dictated by the facts that: (i) ORL1 resembles most opioid receptors in domains that are thought to interact with G proteins; and (ii) opioid receptors have long been known to be (negatively) coupled with adenylyl cyclase [37,38]. The drugs, all tested first at the concentration of 10 μM, included nonselective (etorphine and ethylketocyclazocine), μ-preferring (morphine, fentanyl, [Met^5]enkephalin and [D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin), δ-selective ([D-Pen^2,D-Pen^5]enkephalin) and κ-selective (tifluadom, dynorphin A_{1-8} and [D-Pro^{10}]dynorphin A_{1-11}) opioid agonists as well as the two opiate antagonists naloxone and diprenorphine. Also included in this test were the σ/PCP receptor agonist, phencyclidine, the σ (non-PCP) receptor ligands, pentazocine (also an opioid receptor agonist) and haloperidol, somatostatin-14, ibogaine, an indole alkaloid that is being claimed as a 'quick fix' (Endabuse) for addiction [39] and F8Fa, an amidated octapeptide with anti-opioid activities [40]. If none of these elicited increase of intracellular levels of cyclic AMP, one,

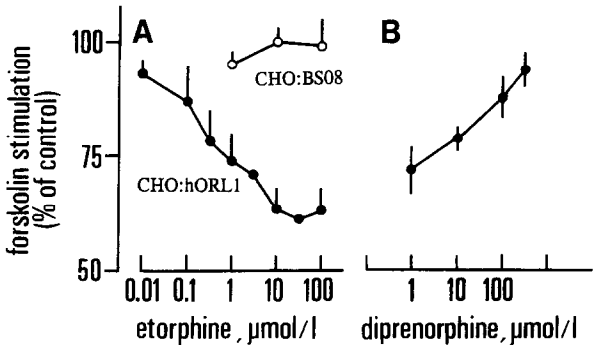

Fig. 3. Forskolin-induced accumulation of cAMP in CHO-K1 cells stably transfected with a pRc/CMV:ORL1 construct. (A) Inhibition by etorphine. Control cells were CHO-K1 cells transfected with the human cannabinoid receptor cDNA (BS08) in the same expression vector. Etorphine produced up to 40% inibition with an IC_{50} of about 0.7 μM in CHO:ORL1 cells and was essentially inactive in control cells at concentrations up to 100 μM. Each value is the mean ± S.D. of the values from 3 or 4 independent experiments. (B) Reversion by diprenorphine of etorphine's inhibitory action. Etorphine was used at the fixed concentration of 3 μM in the presence of increasing doses of diprenorphine. Inhibition in the absence of diprenorphine was 28% relative to control (no etorphine). Each value is the mean ± S.D. of a triplicate assay.

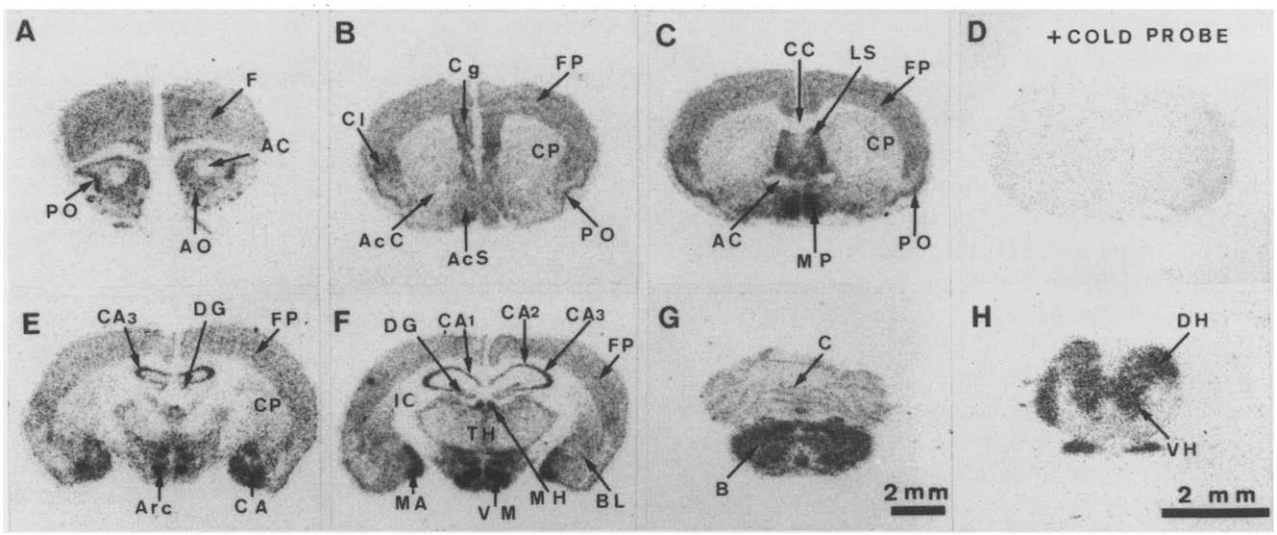

Fig. 4. Hybridocytochemical detection of ORL1-like RNA transcripts in the mouse brain. Autoradiograms were generated by hybridization with ^{35}S-labelled oligonucleotides complementary to mOP86$_{1-45}$ and mOP86$_{62-106}$ in coronal sections of the mouse brain and spinal cord. The distribution is presented in rostral to caudal sections (A–C, E–H). D: hybridization with labeled probes and a 100-fold excess of cold probes. *Abbreviations:* AC, anterior commissure; AcC, core of accumbens; AcS, shell of accumbens; AO, anterior olfactory nucleus; Arc, arcuate nucleus; B, brain stem; BL, basolateral amygdala; C, cerebellum; CA, central amygdala; CA1–3, sectors 1–3 of hippocampal Ammon's horn; CC, corpus calosum; Cg, cingulate cortex; Cl, claustrum; CP, caudate-putamen; DG, dentate gyrus; DH, dorsal horn of spinal cord; F, frontal cortex; FP, fronto-parietal cortex; IC, internal capsule; LS, lateral septum; MA, medial amygdala; MH, medial habenular nucleus; MP, medial preoptic area; PO, primary olfactory cortex; Th, thalamus; VH, ventral horn of spinal cord; VM, ventromedial nucleus.

namely etorphine, was found to inhibit forskolin-stimulated production of cAMP in two independent clones of transfected CHO cells. Fig. 3A shows that etorphine's inhibition of forskolin-elicited increase of cAMP was dose dependent ($ED_{50} \approx 0.7$ μM) and amounted to up to 40% of control (no drug added) values. In control cells, i.e. CHO cells stably transfected either with a human cannabinoid receptor cDNA [41] in pRc/CMV or with pSV2neo, 10 μM etorphine had no effect. Significantly enough, diprenorphine (Fig. 3B) and, however less potently so, naloxone (not shown) were found to antagonize the inhibitory action of etorphine in the CHO:hORL1 cells.

Finally, while attempting to identify the hORL1 receptor, we examined the CNS of the mouse for specific in situ hybridization with the two oligonucleotide 45-mers complementary to distinct regions of mouse genomic PCR product mOP86, the murine counterpart of hOP01. Selected results are shown in Fig. 4. High levels of hybridization were observed in the septum (lateral, medial, septo-hippocampal and septo-hypothalamic nuclei), the horizontal and vertical limbs of the diagonal band of Broca, the hypothalamus (preoptic anterior and medial, arcuate, paraventricular, ventromedial nuclei), the subfornical organ, the hippocampus (Ammon's horn CA3 and other areas), the medial habenular nucleus, the medial amygdala, throughout the brainstem (including the periaqueductal gray matter) and in the dorsal as well as in the ventral horn of the spinal cord. Moderate hybridization signals were seen in the olfactory nuclei, throughout the superficial and deep layers of the cerebral cortex (including the primary olfactory, cingulate, claustrum and fronto-parietal regions), the shell of the nucleus accumbens, the islets of Calleja, the thalamus and the hippocampal dentate gyrus. Low levels of hybridization were visible in the caudate-putamen, the core of the nucleus accumbens and cerebellum. No signal was present in the white matter of the anterior commissure, corpus callosum and internal capsule.

4. Discussion

We report here the cloning of hOLR1, a new G protein-coupled receptor which belongs to the newly characterized opioid receptor subfamily. Indeed, the closest relatives of hOLR1 are the three types of opioid receptor with whom it shares many structural traits, especially in the membrane-spanning and cytoplasmic domains. The high similarities within the latter, in particular the third cytoplasmic loop, suggest that hORL1 is capable of activating the same G proteins as do opioid receptors: G_i to inhibit adenylyl cyclase [4], G_K to stimulate a K^+ channel [6] and G_o to inhibit a Ca^{2+} channel [5]. Accordingly, we have found that, in CHO cells transfected with a pRc/CMV:hORL1 construct, hORL1 mediates inhibition of adenylyl cyclase by the 'universal' opiate agonist, etorphine. Moreover, this effect is abolished by diprenorphine, a 'universal' opiate antagonist of the same chemical family. In these cells, however, etorphine is far less potent ($IC_{50} \approx 0.7$ μmol/l) than in the NG 108-15 hybrid cell line in which, by acting on the σ-opioid receptor, it inhibits the enzyme at concentrations in the range 1–10 nmol/l [42]. The high concentration of these

ligands required here for biological activity, and the lack of activity of other opioid substances suggest that hORL1 is not a classical opioid receptor. The low potency of etorphine and diprenorphine can also be correlated with the absence of detectable binding of up to 10 nmol/l concentrations of the tritiated ligands in membranes from transfected COS cells. For comparison, [^{3}H]diprenorphine and [^{3}H]etorphine bind mammalian and amphibian [43] types of opioid receptors with K_ds in the range 0.1–1 nmol/l. Thus, the detailed pharmacological profile of hORL1 will have to be determined, as well as the nature of its natural ligand which is not expected to be one of the prototypical endogenous opioid peptides (β-endorphin, enkephalin and dynorphin), since these were not active in our bioassay.

We also report here that ORL1 transcripts are discretely yet widely expressed in the CNS of the mouse, suggesting that the ORL1 receptor is potentially endowed with a variety of central functions. Schematically, these may include regulation of neuroendocrine secretion in the hypothalamo-pituitary axis, motricity in the ventral horn of the spinal cord, nociception in the central gray and dorsal horn of the spinal cord, instinctive behaviors, emotions and possibly, learning and memory in the limbic areas. Hence the urgency of discovering the natural agonist(s) of this new receptor which, because it has been cloned out of a cDNA library from human brain stem, is also expressed in the human brain.

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