Cloning, pharmacological characterization and tissue distribution of an ORL1 opioid receptor from an amphibian, the rough-skinned newt *Taricha granulosa*

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Abstract

We have cloned and characterized an opioid receptor-like (ORL1; also referred to as NOP) receptor from a urodele amphibian, the rough-skinned newt *Taricha granulosa*. The cDNA clone encodes a protein of 368 amino acids that contains the seven hydrophobic domains characteristic of G-protein-coupled receptors, and has the highest sequence identity to the frog (*Rana pipiens*) nociceptin-like and human ORL1 opioid receptors (79.6 and 68.4%, respectively). Saturation binding assays on membranes from COS-7 cells transiently expressing the newt ORL1 (nORL) receptor revealed a single, high-affinity (estimated K_d , 0.1974 nM) binding site for the ORL1-specific agonist [3 H]orphanin FQ analog ([3 H]oFQ). In competition binding assays, the [3 H]oFQ-binding site, like the mammalian ORL1 receptor, had no affinity for the non-selective opioid receptor antagonist naloxone, the κ -selective agonists U69593 and U50488, or the μ - and δ -selective opioid receptor agonists DAMGO and DPDPE, respectively. However, the nORL receptor displayed higher affinities for the κ -selective agonists dynorphin A (1–13), dynorphin B, and dynorphin A (1–8) (K_i values, 2.8, 151.8, and 183.0 nM, respectively) than its mammalian homologue. The tissue distribution of the nORL receptor, as determined by reverse transcriptase PCR, was also found to differ from reports on the mammalian ORL1 receptor, with mRNA detected in brain, spinal cord, and lung, but not detected in a number of other peripheral tissues reported to express the receptor in mammals. This is the first report describing the expression and characterization of an amphibian ORL1 receptor, and contributes to our understanding of the evolution of the opioid system.

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Introduction

The opioid receptor-like (ORL1; also called NOP) receptor is a G-protein-coupled receptor (GPCR) that shares high sequence identity with the three classically recognized opioid receptor types, μ , δ , and κ (Mollereau *et al.* 1994). The endogenous peptide for the ORL1 receptor, known as orphanin FQ (oFQ; Reinscheid *et al.* 1995) or nociceptin (Meunier *et al.* 1995), is a heptadecapeptide whose N-terminal tetrapeptide sequence is related to that of the opioid peptides (FGGF or YGGF), and whose basic core is similar to dynorphin (Dyn), the endogenous peptide for the κ receptor. In spite of these similarities, the ORL1 receptor does not recognize the majority of opioid ligands, and oFQ has low affinity for the classic opioid receptors (Reinscheid *et al.* 1995, Meng *et al.* 1996a). The ORL1 receptor couples to the same G_i/G_o protein-mediated, second-messenger systems as the opioid receptors and produces the inhibition of adenylate cyclase, activation of an inwardly rectifying K^+ conductance, and inhibition of voltage-sensitive Ca^{2+} channels (for review, see Meunier 1997). These intracellular effectors generally act to inhibit cellular

excitability, and suggest a role for the oFQ/ORL1 system in the modulation of neuronal activity.

Complete cDNA sequences for the δ -, μ -, κ -, and ORL1 opioid receptor types have been obtained for mammals (held in the GenBank nucleotide sequence database), but sequence information for non-mammalian vertebrates is limited. Full-length opioid receptor cDNA sequences have been reported for two species of fish: in the zebrafish *Danio rerio*, δ (Barallo *et al.* 1998, Rodriguez *et al.* 2000), ORL1 (AY148348), μ (Barallo *et al.* 2000), κ (AF285173); and in the white sucker *Catostomus commersoni*, μ (Darlison *et al.* 1997). Also in the white sucker, partial cDNA sequences for κ -, δ -, and ORL1 receptors have been obtained (Darlison *et al.* 1997). In addition, partial fragments for δ - and μ -like opioid receptors have been amplified from genomic DNA in a more ancient lineage of fish, the pacific hagfish (*Eptatretus stoutii*; Li *et al.* 1996), indicating multiple opioid receptor types were present early in vertebrate evolution. Reports on partial genomic DNA sequences for opioid receptors from representatives of different vertebrate classes also include chicken (*Gallus domesticus*; δ , μ , ORL1), striped bass (*Morone saxatilis*; δ , μ , κ), and

thresher shark (*Alopias vulpinus*; δ , μ , ORL1; Li *et al.* 1996). Amphibian opioid receptor sequence fragments have been reported from bullfrog (*Rana catesbeiana*; δ , μ , κ) genomic DNA (Li *et al.* 1996), and full-length cDNA sequences for μ -, δ -, κ -, and ORL1 opioid receptors were recently submitted to GenBank (accession numbers AF530571, AF530572, AF530573, and AY434690, respectively) for the northern leopard frog (*Rana pipiens*).

The definitive cDNA sequences recently submitted to GenBank for the opioid receptors in *R. pipiens* correlate with previous pharmacological and behavioral studies indicating that multiple opioid receptor types are present in amphibians. Early binding assays in toad and frog brains indicated the presence of μ -, δ -, and κ -like opioid-binding sites, and estimated the predominant form of opioid receptor to be κ -like (60–70%), with considerably fewer μ - and δ -like sites (20–30%; Simon *et al.* 1982, 1984). Since these early studies, numerous reports have characterized multiple ORL receptor types (μ , δ , κ) and subtypes (κ 1, κ 2) in amphibians (Ruegg *et al.* 1980, 1981, Simon *et al.* 1985, 1987, Borsodi *et al.* 1986, Makimura *et al.* 1988, Mollereau *et al.* 1988, Benyhe *et al.* 1990, 1992 1994, Wollemann *et al.* 1994, Newman *et al.* 2002).

In addition to the pharmacological evidence, behavioral studies support the presence of multiple opioid receptor types in amphibians. In amphibians, receptor-type-selective opioids have been implicated in the control of nociceptive responses, locomotor activity, and sex behaviors. The opiate morphine sulfate (a μ -receptor agonist) is an analgesic when injected into the spinal cord of *R. pipiens*, and the analgesia is attenuated by naloxone (Pezalla 1983, Stevens & Pezalla 1983). Also in frogs, spinal administration of Dyn, β -endorphin, or Met-enkephalin produces a potent, dose-dependent increase in the nociceptive threshold (Stevens *et al.* 1987). In the rough-skinned newt, *Taricha granulosa*, the κ -agonist bremazocine reduces spontaneous locomotor activity, but has no effect on corticotropin-releasing factor-induced locomotion; whereas, morphine does not affect spontaneous locomotion, but does reduce corticotropin-releasing factor-induced locomotion (Deviche *et al.* 1989, Lowry *et al.* 1990). Morphine also influences locomotion in *R. pipiens*, where it induces explosive motor behavior when administered systemically (Pezalla 1983). Both bremazocine and ethylketocyclazocine (κ agonists) reduce the incidence of sexual behaviors in *T. granulosa*, and the effects of both drugs are reversed by naloxone (Deviche & Moore 1987). These behavioral observations, which are based on the effects of receptor-type-selective opioid agonists, support the presence of multiple opioid receptor types in amphibians.

In the present study we report the cloning and characterization of an amphibian ORL1 opioid receptor. The pharmacology of the newt ORL1 (nORL)

receptor was determined using saturation and competition binding assays on membranes from COS-7 cells transiently expressing the receptor, and was found to differ from the mammalian ORL1 receptor in its affinity for Dyn-derived peptides. The nORL receptor also has a unique distribution as compared with mammals, with expression in brain, spinal cord, and lung. To the best of our knowledge, this is the first characterization of this type of opioid receptor in an amphibian, and adds to the current body of knowledge on the evolution of the opioid system.

Materials and methods

PCR with degenerate primers

Degenerate primers were designed based on highly conserved regions in transmembrane domains (TMs) I and III from multiple opioid receptor types identified in diverse vertebrate phyla (Li *et al.* 1996). The sequences of the sense and the antisense primers were 5'-ACCAAY ATYTACATHHTTYAA-3' and 5'-GGTRAACATRTT RTAGTARTC-3', respectively (Gibco BRL Custom Primers, Carlsbad, CA, USA). Template cDNA was obtained by reverse transcriptase (RT)-PCR (MBI Fermentas, Hanover, MD, USA) on newt brain total RNA. The degenerate primers (0.5 μ M) and newt brain cDNA template were used in PCR reactions with the following cycling conditions: 2 min at 94 °C, 35 cycles of 94 °C for 30s, 50 °C for 30s, and 72 °C for 1 min, followed by a 7 min extension at 72 °C. Appropriately sized products (approximately 152 bp) were ligated into the pGEM T-Easy vector and transformed into JM109 *Escherichia coli* competent cells (Promega, Madison, WI, USA) for amplification. Plasmids were purified by alkaline lysis (Qiagen, Valencia, CA, USA) and sequenced by the Central Services Laboratory at Oregon State University. Sequences were analyzed with a BLAST-N 2.0 (Basic Local Alignment Search Tool) search provided by National Center for Biotechnology Information.

3' and 5' rapid amplification of cDNA ends (RACE)

The partial sequence (152 bp) of the nORL1 receptor obtained by PCR with degenerate primers (above) was used to design a specific sense primer (5'-CTGGCT GATGCGCTGGTGCTCGT-3') for 3' RACE. 3' RACE-ready cDNA, synthesized from newt brain RNA according to the SMART RACE cDNA amplification kit protocol (BD Biosciences Clontech, Palo Alto, CA, USA), was used in conjunction with the nORL gene-specific sense primer (One Trick Pony; Ransom Hill Bioscience, Ramona, CA, USA) and the provided Clontech antisense primer in a 3' RACE PCR reaction according to the kit protocol and recommended cycling

conditions. 5' RACE-ready cDNA was synthesized from newt brain RNA according to the Ambion RACE RLM kit protocol (Ambion, Austin, TX, USA). 5' RACE PCR reactions were carried out using the kit reagents and protocol, the provided Outer RACE sense primer, and a 5' RACE gene-specific antisense primer (5'-TTGGCTT TGTGTGGTGTACGGATGT-3'), with the following cycling conditions: 94 °C for 3 min, four cycles of 94 °C for 45 s, 62 °C for 45 s, and 72 °C for 1 min, 34 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 6 min. The RACE PCR products were subcloned and sequenced as described above. Overlapping the 5' and 3' RACE product sequences produced a contig that contained the entire nORL1 receptor sequence. The receptor was subsequently PCR-amplified for sequence confirmation three separate times with primers designed at the ends of the coding sequence and a proof-reading polymerase (Pfx; Invitrogen, Carlsbad, CA, USA).

Tissue distribution of the nORL1 receptor by RT-PCR

Six rough-skinned newts were cryoanaesthetized and rapidly decapitated. The newts were then dissected, and the following organs were collected and frozen immediately in liquid nitrogen: brain, spinal cord, spleen, small intestine, heart, lung, liver, sperm duct, bladder, and kidney. The tissues were stored at -80 °C. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and had been approved by the Oregon State University Institutional Animal Care and Use Committee. The reagents and protocol provided with RNA STAT-60 (Tel-Test, Friendswood, TX, USA) were used to collect total RNA from the tissues. The purified RNA was reverse-transcribed into cDNA using an oligo-dT primer and the Gibco Superscript System for RT-PCR First-Strand Synthesis kit and protocol (Invitrogen). Up to 5 µg total RNA from each tissue was used per reverse-transcription reaction. The resultant cDNA was stored at -20 °C.

Gene-specific primers were designed for PCR based on the nORL receptor sequence. The sense primer (5'-GTGATGGGGTCTGCTGAAAT-3') corresponds to amino acid residues 184–190 at the start of the putative second extracellular loop of the nORL receptor, and the antisense primer (5'-CACTGCACCAGCAC AAAGAT-3') corresponds to amino acid residues 278–284 at the end of putative TMVI. PCR reactions (20 µl) were set up with the specific primers (0.5 µM) and the cDNA (10–150 ng) from the collected tissues. The quality and quantity of the cDNA was controlled for in PCR reactions conducted simultaneously using β-actin primers. The following cycling conditions were used: 94 °C for 2 min, 35 cycles of 94 °C for 10 s, 60 °C for

30 s, and 72 °C for 1 min, followed by 72 °C for 5 min. The PCR products were examined by 1.5% agarose gel electrophoresis and ethidium bromide staining for the nORL-specific amplicon (303 bp) or the actin-specific amplicon (150 bp).

Transfection

The full-length nORL receptor insert was subcloned into the mammalian expression vector pcDNA3.1/V5-His-TOPO (Invitrogen); individual clones were analyzed for correct orientation with respect to the cytomegalovirus promoter. COS-7 cells (ATCC, Manassas, VA, USA) were cultured in 10 cm tissue-culture plates (Falcon catalog no. 353003) at 37 °C under 5% CO₂ in Dulbecco's modified Eagle's medium (Mediatech Cellgro; Fisher, Pittsburgh, PA, USA) supplemented with 2 mM L-glutamine (Invitrogen) and 10% fetal bovine serum (Hyclone, Logan, UT, USA). Transient expression was achieved by transfecting plates (90–95% confluency) using Lipofectamine 2000 (Invitrogen) diluted with Opti-MEM medium (Invitrogen). 6 h after transfection, the lipofection mixture was aspirated and replaced with fresh growth medium.

Cell harvesting and membrane preparation

At 48 h after transfection, cells were washed twice with room temperature PBS and harvested under homogenization buffer (2 ml/plate) using a cell scraper. Suspended cells were homogenized in chilled buffer (25 mM Hepes, 100 µM PMSF, 100 µg/ml trypsin inhibitor, 0.7 µg/ml leupeptin, and 100 µg/ml bacitracin) using a chilled Dounce homogenizer. Homogenates were centrifuged for 10 min at 45 000 *g* (4 °C). Pellets were resuspended in homogenization buffer (1 ml/plate) and centrifuged a second time. Cell membrane pellets were resuspended in binding buffer (250 µl/plate) containing MgCl₂ (see below), snap-frozen in liquid N₂, and stored at -80 °C until use. In the course of this study, multiple transfections were performed; however, tissues from different transfections were not pooled, and in a given assay all tissues used were obtained from a single transfection. The concentration of total protein in membrane preparations was determined using a colorimetric protein assay (Pierce, Rockford, IL, USA).

Binding assays

Saturation and competition binding assays were performed in binding buffer consisting of 25 mM Hepes (pH 7.45) containing 10 mM MgCl₂, 100 µM PMSF, trypsin inhibitor (100 µg/ml), leupeptin (0.7 µg/ml), and bacitracin (100 µg/ml). Frozen membrane pellets were thawed on ice and diluted with binding buffer to achieve a final protein concentration of 50 µg per binding

reaction. For saturation experiments, variable concentrations of [^3H]oFQ analog (102 Ci/mmol; Multiple Peptide Systems, San Diego, CA, USA) were used in the presence of 10 μM nor-BNI (Tocris, Ballwin, MO, USA). Competition binding experiments were performed using 1 nM [^3H]oFQ analog and various doses (10^{-12} – 10^{-5} M) of non-radioactive competitors. Porcine Dyn A (1–8), porcine Dyn A (1–13) amide, and porcine Dyn B were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA); U50488, U69593, naloxone and [D-Ala 2 , NMe-Phe 4 , Gly-ol 5]-enkephalin DAGO (DAMGO) were purchased from Tocris; [D-Pen 2 , D-Pen 5]-enkephalin (DPDPE) is a product of Multiple Peptide Systems. Non-specific binding was defined as radioactivity remaining bound in the presence of 10 μM non-radioactive nociceptin (TOCRIS). Triplicate samples were run at each dose for both total and non-specific binding. Assays were conducted at 4 °C for 4 h with gentle mixing (80 r.p.m. on an orbital shaker). At the end of the incubation period, bound and free ligands were separated by rapid filtration over GF/C filters (pre-soaked with 0.25% polyethyleneimine for 30 min) under vacuum using a Brandel cell harvester (Brandel, Gaithersburg, MA, USA). Filters were washed twice with 4 ml of chilled (4 °C) Hepes/MgCl $_2$ (pH 7.45) buffer. Radioactivity in the filters was determined by liquid-scintillation counting on a Beckman LS 6500 scintillation counter (GMI, Albertville, MN, USA). Binding data were analyzed using GraphPad Prism software (version 3.0; GraphPad, San Diego, CA, USA).

Results

Sequence analysis

Figure 1 shows the nucleotide coding region and its deduced amino acid sequence for the clone isolated by RT-PCR and 5' and 3' RACE from newt brain (GenBank accession no. AY728087). The cDNA encodes a protein that is 368 amino acids long and contains seven hydrophobic regions that share identity with the TMs of GPCRs. The deduced amino acid sequence also contains a number of putative consensus sites for post-translational modifications common to characterized GPCRs, including three asparagine residues within the N-terminal domain (glycosylation sites), two cysteine residues in the intracellular C-terminal domain (palmitoylation sites), and a cysteine residue in extracellular loops 1 and 2 (disulfide bridge).

Of the characterized GPCRs, the newt clone shares the highest homology with members of the opioid receptor family, and shares the greatest amino acid sequence identity with the ORL1 type of opioid receptor (64.2–79.6%), compared with κ (54.3–56%), δ (51.7–53.6%), and μ (51.2–53.6%). When compared with the human opioid receptor types (ORL1, κ , δ , μ), there is

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      *
      M D P H F S I P S F E A S Y L S K L L N
1  atggaccacatttctctattccgtcttttggagcatcctacttgagcaagcttctgaac 60
      *
      D S S V M N N L S T L P S G W Y F N S T S
61 gactcttcagttatgaacctgagcaccctgcgcagcgctgtgtaactcaactctactccc 120
      TMI
      D T F L P L G I K L T I V I V Y L I V C
121 gacacctctctgccccttgccatcaagctcaacctgctgattgtctactcattgtgtgt 180
      IL1
      V L G L V G N C A V M F V I V R Y T K M
181 gtgctgggactggtggaaactgcgctgtcatgtttgtcattgtcagatataccaagatg 240
      TMI
      K T A T N I Y I F N L A L A D A L V L V
241 aaaaacagccacaacatttatcatctttaacctggcctggctggtcctggtgctcgct 300
      EL1
      T L P F Q G T D T F L G F W P F G N V L
301 acgtgcccctccagggaaacagataaccttcttgggcttttggcccttcgggaatgctcgt 360
      TMI1
      C K I A I A I D Y Y N M F T S T F T L T
361 tgcacaaatcgcaatcgccatcgactactacaacatgttcaccagtaaccttcaactgtgacc 420
      IL2
      M M S V D R Y I A I C H P V K A L D I R
421 atgtgagcgtggtgctgctacatcgccatcgccacccctgcaagcgctggacgcgcgt 480
      TMI2
      T P H K A K I I N V C I W A L A S V I G
481 acacacacaaagcaagatcatcaacgtctgcatctggcgctggcgctcgtcgtcgcc 540
      EL2
      V P V M V M G S A E M E N N E V E C L V
541 gtcccgctcatggtgatggggctgctgaaatggaaacaatgaggtggagtcgctaagt 600
      TMI3
      Q I P V P E D Y W D P V F G I C V F V F
601 cagatccacgtacctgaagattactgggaccagctcttggaaatctgctgcttctgtctc 660
      IL3
      S F V I P V F I I T I C Y S L M I R R L
661 tctctgctcatcccgctcttcttcatcaccatctgctacagctgatgacccgctgacta 720
      K N V R V L L G S K E K D R N L R G I T
721 aagaacgtgcgcttctcttaggtccaaggagaaagaccgttaactgcggggcatcaact 780
      TMI4
      R L V L V V V A V F V I C W T P I Q I F
781 cgctggtgctggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggt 840
      EL3
      V L V Q C L G A K P D S E A K V V I L H
841 gtgctggtgcagtgctggtggcgccagccgacagcgaggccaaagtggtgatcctgcac 900
      F C I A L G Y V N S S L N P I L Y A F L
901 tctctgcatgcccctaggctatgtcaacagcagcctcaacctattctcaagccttctgt 960
      D E N F K A C F R K F C F P A A F R S E
961 gagcagaacttcaaggctgtcttcgcgaagtctgtctcccgctgcttcaaggtctgag 1020
      L Q M S N R M C S I A K D V A Y A C K N
1021 ctacagatgtccaacccgcatgtgcagcatagccaaggtatgtggcctatgcatgcaagaac 1080
      S D G T N N P A *
1081 tccgatgggactaacaaccccgcatga 1107

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Figure 1 The cDNA nucleotide sequence (GenBank accession no. AY728087) for the coding region of the nORL1 receptor is shown aligned with its deduced amino acid sequence. Underlined regions, putative transmembrane domains (TMs); IL, intracellular cytoplasmic loop; EL, extracellular loop; *, potential glycosylation sites; cysteine residues with light-gray shading, disulfide-bridge formation; cysteine residues with black shading, potential palmitoylation sites.

high sequence identity in the TM regions (66–77%) and the intracellular loops (70–81%). However, the identity between the newt clone and the human ORL1 receptor in the extracellular loops (60%) is definitively higher than its identity to μ , δ , and κ in these regions (28–37%). Furthermore, the second extracellular loop (EL2) of the newt receptor contains a high number of acidic residues, a characteristic shared only by the ORL1 and κ -receptor types.

The deduced amino acid sequence for the nORL1 receptor clone matches a number of residues in the mammalian ORL1 receptor that have been implicated in ligand binding and receptor function by previous studies (Fig. 2). Among these are Gln 286 at the C-terminus of TMVI, Asp 130 and Tyr 131 in TMIII,

nORL	-----MDPHFSIPSFEASYLSKLLNDSSVMN--LSTLP SGWYFNST-----D	41
hORL1	-----E.L.PA.FW.VI.G.H.QGNL.LLSPNH.L..PHLLL.ASH-----G	43
rORL1	-----ESL.PA.YW.VL.G.HFQGNL.LL---E.V.HHLLL.ASH-----S	40
rKOR	MES-----PIQIFRGE.GPTCAPSAC..PN..SWFPNWAESD.NGSVG.EDQQ---LEP	51
rDOR	-----MEPV..AR.ELQFS..ANV.DTFPSAFPSA.ANASG.PG-----AR	41
rMOR	MDSSTGPGNTS.CSDPLAQASC.PAPGSWNLN.L.HVDGNQ.DPCGLNRTGLGGNDSLCPQT	60
	TMII IL1 TMII	
nORL	TFLPLGIKLTIVIVYLIVCVLGLVGNCAVMFVIVRYTKMKTATNLYIFNLALADALVLVT	101
hORL1	A....L.V...GL..A..G..L...L..Y..L.H.....T...L.	103
rORL1	A....L.V...GL..A..IG..L...L..Y..L.H.....T...L.	100
rKOR	AHISPA.PVI.TA..SV.F.V....SL...I.....TT.	111
rDOR	SASS.ALALIA.TAL.SA..AV..L.VL...G.....L.....ATS.	101
rMOR	GSPSMVTAL..MAL.S...V..F..FL..Y.....ATS.	120
	EL1 TMIII IL2	
nORL	LPFQGTDTFLGFWPFGNVLCIAIAIDYNNMFTSTFTLTMMSVDRYIAICHVPKALDIRT	161
hORL1	IL.....A...TV.....A.....V.....IR..V..	163
rORL1	IL.....A...TV.....A.....V.....IR..V..	160
rKOR	M...SAVYLMNS...D...V.S.....I.....V.....F..	171
rDOR	...SAKYLME...EL...AVLS.....I.....V.....F..	161
rMOR	...SVNYLM.T...TI...V.S.....I...CT.....V.....F..	180
	TMIV EL2 TMV	
nORL	PHKAKIINVCIWALASVIGVPVMVMSAEMENNE--VECLVQIPVPED-YWDPVFCICVF	218
hORL1	SS...QAV..A.....V...AI...QV.DE.--I...E..T.Q...G...A..I.	220
rORL1	SS...QAV..A.....V...AI...QV.DE.--I...E..A.Q...G...A..I.	217
rKOR	.L.....I...L..SV.ISAI.L.GTKVREDVDVI..SL.F.DD.YSW..LFMK...	231
rDOR	.A...L..I...V...GV...I..AVTQPRDGA--V.TL.F.S.SW--T.T.K...	218
rMOR	.RN...V...N.I.S.A..L...F.ATTKYRQGS--ID.TLTFSH.TW--ENLLK...	237
	IL3 TMVI	
nORL	VFSEVIVPVFIITICYSLMIRRLKNVRVLGSGKEKDRNLRRITRLVLVVAVFVICWTFIC	278
hORL1	L...IV..LV.SV.....RG..L...R.....G...V.	280
rORL1	L...I...L..SV.....RG..L...R.....G...V.	277
rKOR	..A.....L..IV..T...L...S..L...R.....K.....I...H	291
rDOR	L.A..V..IL...V..G..LL..RS..L.....S.....M.....GA..V..A..H	278
rMOR	I.A.IM..L...V..G..L...S..M.....M.....IV...H	297
	EL3 TMVII	
nORL	IFVLVQCLGAKP-DSEAKVVIILHFCIALGYVNSSLNPILYAFLDENFKACFRKFCFPAAF	337
hORL1	V...A.G..VQ.-S..TA.A..R..T.....C.....CAS.L	339
rORL1	V...G..VQ.-G..TA.A..R..T.....C.....CASSL	336
rKOR	..I...EA..STS-H.T.VLSSYY.....T.....V.....R...D...IKM	350
rDOR	...I.WT.VDINRRDPLV.AA..L...A.....V.....R...QLRAPCG	338
rMOR	..Y.IIKA..ITI.-ETTFQT.SW...T...C...V.....R...E..I.TSS	356
nORL	RSELQMSNRMC-SIAKDVAYACKNS-----DGTNNPA-	368 (100%)
hORL1	.RDV.V.D.VR-.....L...T-----ETVPR.-	370 (68.4%)
rORL1	HR.M.V.D.VR-.....GLG..T-----ETVPR.-	367 (67.9%)
rKOR	.M.R.ST..VR-NTVQ.P.SMRDVG-----M.K.V-	380 (56.4%)
rDOR	GQ.PGSLR.PRQAT.RERVT..TP-----DGPG.GAAA--	372 (53.8%)
rMOR	TI.Q.N.T.VRQNTREHPST.NTVDRTNHQLENLEAETA.LP	398 (49.5%)

Figure 2 The nORL receptor amino acid sequence is shown aligned with the human ORL1 (hORL1), rat ORL1 (rORL1), rat kappa (rKOR), rat delta (rDOR), and rat mu (rMOR) receptor sequences. Predicted transmembrane domains are boxed and labeled TMI–TMVII. Residues known to be important for ligand affinity or signal transduction from mutagenesis studies on the hORL1 and conserved in the nORL are highlighted in gray (Mouledous *et al.* 2000). Mutating four residues of the rORL1 to the conserved classical opioid receptor counterparts (Val-Gln-Val^{276–278} to Ile-His-Ile in TMVI and Thr³⁰² to Ile in TMVII) enables the receptor to bind prodynorphin peptides with subnanomolar affinity (Meng *et al.* 1996a); the nORL matches the classical opioid receptors at three of the four residues (black highlights). The addition of a fifth mutation to the rORL1 (Ala to Lys in TMV) increases its affinity towards selective opioid antagonists by two or three orders of magnitude (Meng *et al.* 1998); the nORL has a unique residue at this position (Gly²¹⁴; black highlight). Percentage amino acid identities to the nORL are listed at the end of the alignment. EL, extracellular loop; IL, intracellular loop. The included mammalian receptor sequences were obtained from GenBank: ORL1 *Homo sapiens*, accession no. NP_872588; ORL1 *Rattus norvegicus*, NP_113757; KOR *R. norvegicus*, NP_058863; MOR *R. norvegicus*, NP_037203; DOR *R. norvegicus*, NP_036749.

Figure 3 Distribution of the nORL receptor as determined by RT-PCR. The nORL receptor-specific amplicon (303 bp) was detected in the brain, spinal cord, and lung. +, Positive control; -, no-template control.

Phe²²⁰ and Phe²²⁴ in TMV, and Trp²⁷⁶ in TMVI (human ORL1 numbering; Mouldous *et al.* 2000). Also, the newt receptor shares identity with the classic opioid receptors at three residues (Ile²⁷⁷, Ile²⁷⁹, and Ile³⁰³; newt numbering) shown to be important in the recognition of κ ligands that are not conserved in the mammalian ORL1 receptor (Meng *et al.* 1996a, 1998).

nORL receptor distribution

The expression of the nORL receptor in various tissues was determined by RT-PCR on newt brain, spinal cord, spleen, small intestine, heart, lung, liver, sperm duct, bladder, and kidney. The cDNA generated from the tissues was tested in PCR reactions using nORL receptor-specific primers. Appropriately sized amplicons (303 bp) were produced in reactions containing cDNA from brain, spinal cord, and lung (Fig. 3). Negative controls were reactions in which water was substituted for template cDNA. The cDNA from each tissue was also tested in PCR reactions with β -actin primers to ensure that the template was of sufficient quality and quantity to amplify. The β -actin primers successfully amplified the cDNA from each tissue (results not shown).

Characterization of the cloned receptor transiently expressed in COS-7 cells

Saturation binding of [³H]oFQ analog, an ORL1-specific agonist, to membranes of transfected COS-7 cells revealed the presence of a single, high-affinity binding site with an estimated K_d value of 0.1974 nM (Fig. 4). At a dose equivalent to the estimated K_d for [³H]oFQ, specific binding was 94.5% of total binding. No specific binding was detected in membranes prepared from untransfected (control) cells. In competition experiments designed to assess the ability of various opioid ligands to displace [³H]oFQ, the κ opioid receptor-selective agonist Dyn A (1–13) had high affinity

Figure 4 Saturation analysis of [³H]oFQ analog binding to membranes isolated from COS-7 cells transiently expressing nORL receptor. Non-specific binding for each ligand dose was determined in the presence of 10 μ M nor-BNI. At a dose equivalent to the estimated K_d for oFQ analog, specific binding was 94.5% of total binding. Each data point represents the mean $\pm$ S.E.M. from triplicate measurements. The inset shows Scatchard analysis.

(K_i , 2.76 nM), and the κ agonists Dyn B and Dyn A (1–8) displayed sub-micromolar affinities (K_i , 151.81 and 183.03 nM, respectively). In contrast, the κ -selective agonists U50488 and U69593, the non-selective opioid receptor antagonist naloxone, the μ -selective agonist DAMGO, and the δ -selective agonist DPDPE, did not compete for the [³H]oFQ-binding site (Fig. 5 and Table 1).

Discussion

The present study reports the molecular cloning and pharmacological characterization of an amphibian GPCR. The results indicate that the GPCR cloned from newt brain is the homologue of the mammalian ORL1 opioid receptor. This conclusion was reached based on sequence analysis and binding studies with an ORL1-selective agonist, [³H]oFQ. To the best of our knowledge, this is the first reported characterization of an ORL1 opioid receptor from an amphibian.

Initial BLAST searches on the nucleotide and deduced amino acid sequence of the newt clone indicated it belonged to the GPCR superfamily. The following characteristics of the deduced amino acid sequence supported the GPCR classification: the presence of seven hydrophobic regions that share

Figure 5 Inhibition of specific [^3H]oFQ analog binding to membranes from COS-7 cells transiently expressing nORL1 receptor. 1 nM [^3H]oFQ analog was used in all experiments; non-specific binding was defined as radioactivity remaining bound in the presence of 10 μM non-radioactive nociceptin. Each data point represents the mean $\pm$ S.E.M. for triplicate measurements.

sequence identity with the membrane-spanning domains of GPCRs (Minami & Satoh 1995, Law *et al.* 1999); asparagine residues within the N-terminal domain that function as potential sites for N-linked glycosylation (Minami & Satoh 1995); cysteine residues in the intracellular C-terminal domain that are potential palmitoylation sites (Ovchinnikov *et al.* 1988, O'Dowd *et al.* 1989); and two cysteine residues, located in extracellular loops 1 and 2, that may connect the loops by forming a disulfide bridge (Dixon *et al.* 1987, Karnik *et al.* 1988). In addition, there are a number of serine and threonine residues in the putative cytoplasmic regions, presenting potential sites for phosphorylation by protein kinases. The phosphorylation of homologous sites in GPCRs is a process thought to affect receptor regulation, namely desensitization (Lefkowitz *et al.* 1990, Law *et al.* 2000).

Overall, the newt receptor shares >50% amino acid sequence identity with the four main opioid receptor types (μ , δ , κ , ORL1) cloned from other species, and has the highest identity to the ORL1 receptors (>64.2%).

Table 1 Summary of inhibition binding data obtained in experiments designed to assess the ability of various opioid ligands to displace [^3H]oFQ analog from membranes of COS-7 cells transiently expressing nORL1 receptor

Ligand	IC ₅₀ (nM)	K _i (nM)
ORL-selective		
Nociceptin	2.8	0.5
κ -Selective		
Dynorphin A (1–13)	13.3	2.8
Dynorphin B	728.8	151.8
Dynorphin A (1–8)	916.3	183.0
U50488	> 10 000	–
U69593	> 10 000	–
μ -Selective		
DAMGO	> 10 000	–
δ -Selective		
DPDPE	> 10 000	–
Non-selective		
Naloxone	> 10 000	–

Of the cloned full-length ORL1 receptors, the newt receptor has the highest amino acid identity to frog (79.6%) and human (68.4%), and the lowest to zebrafish (64.2%). These sequence identities led us to conclude that the newt cDNA codes for an ORL1 receptor. This conclusion was further supported by the high sequence identity (60%) between the human and nORL1 receptors in the putative extracellular loops, in contrast to substantially lower sequence identity in these regions when compared with the other opioid receptor types (28–37%). The extracellular loops may be responsible for the receptor's ability to recognize and discriminate between similar ligands (Wang *et al.* 1994, 1995, Meng *et al.* 1996b, Mollereau *et al.* 1994).

Based on sequence analysis, the nORL1 receptor shares with its mammalian counterpart a number of key residues involved in ligand affinity and receptor activation. The transmembrane domains of opioid receptors form an opioid-binding pocket, a region where specific ligand–receptor interactions cause receptor activation (for review see Waldhoer *et al.* 2004). The conservation of identity in these regions (66–77%) suggests the nORL1 receptor possesses a transmembrane binding pocket similar to the alkaloid binding pocket of the opioid receptors. In particular, five residues within the opioid binding pocket of the human ORL1 receptor are known to greatly influence ligand affinity: Asp¹³⁰ and Tyr¹³¹ in TMIII, Phe²²⁰ and Phe²²⁴ in TMV, and Trp²⁷⁶ in TMVI (Mouldous *et al.* 2000). Mutation to alanine of each of the aforementioned residues has an adverse effect on receptor affinity and reactivity towards oFQ, the endogenous ligand of the ORL1 receptor (Mouldous *et al.* 2000). The nORL1 receptor has the mammalian ORL1 receptor residues at these five sites,

indicating their functional importance may be highly conserved. In addition, a residue located at the end of TMVI (Gln²⁸⁶), which plays a pivotal role in the transduction of signal in the human ORL1 (Mouldous *et al.* 2000), is also conserved in the nORL receptor.

Although the nORL receptor has the highest overall sequence identity to the ORL1 receptors, it also shares a number of residues with the classical opioid receptors that are implicated in κ ligand recognition. The rat ORL1 receptor was rendered capable of binding Dyn-derived peptides with high affinity after mutating as few as four residues to the conserved classical opioid receptor counterparts (Val-Gln-Val^{276–278} to Ile-His-Ile in TMVI, and Thr³⁰² to Ile in TMVII; Meng *et al.* 1996a). These amino acid substitutions created a mutant receptor capable of binding both oFQ and prodynorphin products with sub-nanomolar affinity. The addition of a fifth mutation to the rat ORL1 receptor construct (Ala²¹³-Lys in TMV) caused its affinity towards selective opioid antagonists to increase two or three orders of magnitude (Meng *et al.* 1998). Interestingly, the nORL receptor matches the classical opioid receptors at three of the aforementioned residues targeted for mutation (Ile³⁰³ in TMVII, Ile²⁷⁷ and Ile²⁷⁹ in TMVI; nORL amino acid numbering). Therefore, we predicted that the nORL receptor pharmacology may differ from its mammalian counterpart in that it may be capable of recognizing both oFQ and Dyn peptides with high affinity.

The results from saturation and competition binding assays performed on membranes from COS-7 cells expressing the nORL receptor supported our prediction that this receptor would have a unique pharmacology. In saturation binding assays, the ORL1-selective agonist [³H]oFQ displayed specific, high-affinity (K_d , 0.1974 nM) binding to the transfected membranes; oFQ does not bind to the classical opioid receptors, so this result further confirms the identity of the expressed newt clone as an ORL1 receptor. Untransfected cells displayed no specific binding. Results from competition experiments proved interesting. The non-selective antagonist naloxone did not compete with [³H]oFQ for the binding site, which is consistent with previous reports on mammalian ORL1 receptors. However, the κ opioid receptor-selective agonist, Dyn A (1–13), competed with nanomolar affinity (K_i , 2.76 nM) for the [³H]oFQ-binding site. This is in contrast to previous reports demonstrating that Dyn A can bind to the mammalian ORL1 receptor with only moderate affinity (Butour *et al.* 1997). The high-affinity binding of Dyn A (1–13) to the nORL1 receptor is similar to that seen in the rat ORL1 mutation study (Meng *et al.* 1996a) described above, and indicates that the same amino acids in the newt receptor may be responsible for ligand specificity. Another Dyn A analog (Dyn A 1–8) displayed moderately high affinity for the binding site (K_i , 183.03 nM), as did the κ -selective

agonist Dyn B (K_i , 151.81 nM). However, two additional κ -selective agonists, U50488 and U69593, did not compete for the binding site. Therefore, the pharmacological profile of the nORL1 receptor is unique among the mammalian profiles thus far reported.

It has been hypothesized that the four opioid precursor genes (proenkephalin, proopioidmelanocortin, prodynorphin, and proorphanin) arose by sequential duplication of a common ancestral gene over the past 500 million years, and that the emergence of the cognate receptor proteins (δ , μ , κ , ORL1) paralleled the divergence of the peptide genes (Douglass *et al.* 1984, Does *et al.* 1993, Danielson & Does 1999). Recently, the first complete non-mammalian vertebrate proorphanin cDNA gene sequence was obtained from the sturgeon, *Acipenser transmontanus* (Danielson *et al.* 2001). The sturgeon oFQ peptide retains the classical opioid peptide core sequence (YGGF), whereas all mammalian oFQ peptides have a modified opioid-like core (FGGF). The sturgeon oFQ, with the N-terminal tyrosine, shows affinity for the mammalian ORL1 as well as the μ , δ , and κ opioid receptors. The authors suggest that the sturgeon oFQ represents an intermediate stage in the evolution of the proorphanin gene, and a “transitional state in the eventual functional isolation of the orphanin heptadecapeptide from the classical opioids” (Danielson *et al.* 2001). If this is true, then receptor coevolution may have produced cognate receptor intermediates in some species capable of recognizing oFQ and the classical opioid peptides. Based on the pharmacological profile for the nORL receptor reported here, we could speculate that it is potentially an evolutionary intermediate between the κ - and ORL1 receptor types.

The results of the RT-PCR distribution study indicate that nORL receptor mRNA is expressed in brain, spinal cord, and lung. The detection of nORL receptor mRNA in brain and spinal cord was expected, since previous studies have documented high ORL1 receptor densities in the central nervous system (see Mollereau & Mouldous 2000). However, the peripheral distribution of the nORL receptor is unique when compared with the distribution of the mammalian opioid receptor types. nORL1 receptor mRNA expression was not detected in a number of peripheral tissues shown to express the ORL1 receptor in mammals, including intestine, liver, vas deferens (sperm ducts), and spleen in rat (Wang *et al.* 1994), and kidney in pig (Osinski *et al.* 1999). High-affinity [³H]nociceptin-binding sites on rat heart (Dumont & Lemaire 1998), and the ability of oFQ to inhibit contractions of rat bladder (Giuliani *et al.* 1998), also suggest ORL1 receptor expression in these mammalian organs, although none was detected by RT-PCR in the newt. In the present study, the lung was the one peripheral tissue found to contain nORL receptor mRNA. The ORL1 receptor may be expressed in the lungs of mammals as well, since the oFQ peptide

can inhibit contractions of guinea-pig bronchus when applied to the isolated organ (Rizzi *et al.* 1999). Based on the limited peripheral distribution of the nORL receptor, it appears its primary role in this species is in central nervous system processes. Future studies on the specific distribution of the nORL1 receptor in brain and spinal cord will provide additional useful information on interspecific differences.

The results of the present study indicate that an opioid-like receptor cDNA with high sequence identity to the mammalian ORL1 receptor is present in an amphibian. The expression of the nORL receptor demonstrated that it is capable of binding both oFQ and prodynorphin peptides with high affinity; a unique pharmacology compared with previous reports on the mammalian ORL1 receptors. In addition to being expressed in brain and spinal cord, nORL receptor mRNA is also localized in the lung; a peripheral distribution that is novel as compared with mammals. The results of the binding assays, sequence information, and distribution studies indicate that the nORL1 receptor may represent an intermediate stage in the evolution of the opioid receptors, and contributes to the current body of knowledge regarding this important neuropeptide system.

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