Characterization of a new duplicate δ -opioid receptor from zebrafish

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Abstract

A new full-length cDNA (ZFOR4) that encodes an opioid receptor has been isolated from the teleost zebrafish. The encoded polypeptide is 375 amino acids long and shows high sequence similarity to other δ -opioid receptors, including ZFOR1, the other δ -opioid receptor from zebrafish previously characterized by us. *In situ* hybridization studies have revealed that ZFOR4 mRNA is highly expressed in particular brain areas that coincide with the expression of the δ -opioid receptor in other species. Pharmacological analysis of ZFOR4 shows specific and saturable binding with [³H] diprenorphine, displaying one binding site with $K_D = 3.42 \pm 0.38$ nM and a receptor density of 6231 ± 335 fmol/mg protein. Competition-binding experiments were performed using [³H]diprenorphine and several unlabelled ligands (peptidic and non-peptidic). The order of affinity obtained is Met-enkephalin > Naloxone > Leu-enkephalin > Dynorphin A >> BW373U86 > Morphine >>> [D-Pen², D-Pen⁵]-Enkephalin, U69,593. [³⁵S]GTP γ S stimulation studies show that the endogenous ligands Met- and Leu-enkephalin and the non-peptidic δ agonist BW373U86 were able to fully activate ZFOR4. Our results prove the existence of two functional duplicate genes of the δ -opioid receptor in the teleost zebrafish.

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Introduction

At present, morphine, the major active alkaloid of opium, remains one of the most important compounds used in medicine for the treatment of chronic pain. Nevertheless, opioid administration produces, besides its positive effects, severe side effects like hypotension, constipation and hypothermia (Pasternak 1988, Craft *et al.* 2000). In addition to this, the illegal abuse of opioids, heroin in particular, represents one of the devastating social problems of our time. Hence, the elucidation of the mechanisms involved in opioid activity should be considered a priority in this field of research.

Although the main body of research on opioid receptors has been performed on mammalian models, the presence of opioids and their receptors in other phyla was suggested shortly after their discovery (Pert *et al.* 1974, Simantov *et al.* 1976). Pharmacological and immunohistochemical data point towards the existence of an opioid system in birds, teleosts (Bird *et al.* 1988, Does *et al.* 2002) and even in invertebrates (Harrison *et al.* 1994), but the role that opioid receptors play in the biology of such organisms remains obscure. It has been proposed that the opioid system arose as an

immunomodulatory system in invertebrates and that the analgesic properties exhibited by these molecules were developed later in evolution, when pain was recognized as an alerting process (Stefano *et al.* 1998).

An approach to the problem of understanding why opioid drugs can cause tolerance and dependence is to develop and use new animal models, which would provide findings that could be extrapolated to higher vertebrates and ultimately to humans. The zebrafish has been widely and successfully used in molecular and developmental biology (Ingham 1997, Fishman 2001, Gollig *et al.* 2002, Pichler *et al.* 2003) and has also been proved as a valid model to study the effects of some drugs of abuse, such as cocaine (Darland & Dowling 2001) and ethanol (Dlugos & Rabin 2003). Our group has studied the zebrafish opioid system and we have characterized several receptors (Barrallo *et al.* 2000, Rodriguez *et al.* 2000) and five opioid propeptide genes (Gonzalez-Núñez *et al.* 2003a,b,c) so far. Our previous results indicate that the zebrafish receptors as well as the endogenous peptides are similar to their mammalian homologues, thus indicating that the opioid system has been well conserved throughout the evolution of vertebrates. Hence, we propose the zebrafish as an

organism, where the study of the opioid system and its interactions with different drugs can be easily evaluated in basic research and that the results can be applied to the mammalian opioid system.

Opioid receptors are classified according to their pharmacological profile into three different types, namely μ , δ and κ . Several investigators have shown that the analgesic effect of morphine mainly depends on its action on the μ -opioid receptor, although morphine also seems to bind to the δ - and κ -opioid receptors (for review, see [Waldhoer et al. 2004](#)).

In an attempt to shed light on the biological role of the δ -opioid receptor and to investigate its phylogeny, we have isolated a full-length cDNA from the zebrafish *Danio rerio*, which corresponds, from molecular comparison, to the δ -type of opioid receptor. We present here the sequence, genomic structure, expression, pharmacology and G-protein activation of this new duplicate of the δ -opioid receptor that has been conserved during the course of vertebrate evolution and that may represent a new model for studying opioid mechanisms that control pain and drug-induced behaviour.

Materials and methods

Animals

Adult zebrafish *D. rerio* were obtained from a local pet supplier, maintained at 25–28 °C and fed once a day. In all experiments fish from both sexes were used. Animals were handled according to the guidelines of the European Communities Council directive of 24 November 1986 (86/609/EEC) and in all cases, were treated in accordance with the declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and the Current Spanish Legislation (BOE 67/8509-12, 1998).

Cloning techniques

To clone the ZFOR4 receptor, we have followed the methodology prescribed by [Barrallo et al. \(2000\)](#). Briefly, a κ -opioid receptor fragment was cloned by reverse transcriptase (RT)-PCR using total RNA from mouse brain as template and labelled with [³²P]dCTP by the random primer method (Redi prime II Ready to Go Labelling kit; Amersham Pharmacia Biotech). A ZIPLOX cDNA library from adult zebrafish brain (GIBCO-BRL) was screened using the above-described fragment as a probe. Approximately 5×10^5 pfu were plated and transferred on duplicate nylon membranes (Hybond-N; Amersham). After a 2 h prehybridization step at 65 °C, hybridization was performed for 16 h at 65 °C. Filters were washed twice for 30 min at

39 °C in 1 × SSC, SDS 0.1% and exposed for 24 h at –80 °C to an autoradiography film (Kodak-X-Omat-AR) in the presence of an intensifying screen. Positive clones were purified and DNA isolated following standard procedures ([Sambrook & Russell 2001](#)). A λ -DASH II genomic library from adult zebrafish (courtesy of B Jones and M Petkovich, Queen's University, Canada) was screened using the cDNA previously obtained as a probe. Labelling and hybridization was performed as described previously.

The DNA obtained was digested with the restriction endonucleases EcoRI, BamHI, HindIII and SacI (Promega) and analysed by electrophoresis in agarose gels. Fragments of interest were purified from agarose and ligated in Bluescript plasmid (Stratagene, Madrid, Spain). Inserts were sequenced by dideoxynucleotide chain terminators method (T7 Sequencing kit; Pharmacia).

NCBI PubMed and EMBL websites were visited to obtain information about homologous sequences to the opioid receptors, as well as to search the zebrafish genome. DNA sequences were analysed with Edit View (ABI Automated DNA Sequence Viewer 1.0, Perkin-Elmer, Madrid, Spain) and DNA Strider 1.1 (Institut de Recherche Fondamentale) software. Oligonucleotides were designed with Oligo 4.05 Primer Analysis Software (National Biosciences, Inc., Plymouth, MN, USA). Homology studies were performed with FASTA or BLAST programs from EMBL website and ClustalW program was used to perform the alignments with other homologous genes.

Expression studies

To determine the expression pattern of ZFOR4 in the central nervous system (CNS) of zebrafish, we used the *in situ* hybridization techniques (ISH), using the same methodology that we have previously used to study the expression of ZFOR1, a putative duplicate of ZFOR4 ([Porteros et al. 1999](#)). Briefly, adult zebrafish (*D. rerio*) of both sexes obtained from a local supplier were deeply anaesthetized with tricaine methanesulphonate (MS-222; Sigma) and fixed overnight by immersion in freshly prepared 4% paraformaldehyde, soaked in 30% sucrose, serially sectioned on a cryostat (Leica, Microsystems, Barcelona, Spain) in 20 μ m thickness and finally thaw-mounted onto gelatine-coated microscope slides. Coronal sections were fixed in 4% paraformaldehyde, followed by one wash in 1 MPBS (pH 7.4), and dehydration in graded ethanol series (60, 80, 90 and 100%).

Sense (5'-CAC TTA ATT AGA AGG CGT TCG ACA TAT AGG GGA-3') and antisense oligonucleotides (5'-TCC CCT ATA TGT CGA ACG CCT TCT AAT TAA GTG-3') were designed from the cDNA sequence of ZFOR4 (Genbank database Accession number AY262256) at the 5' region ([Fig. 1](#)), the most specific zone for ZFOR4 in comparison with the rest of opioid

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-93                                     atgatcttacaccttttcattttaaa
caaacactttcaacattttacagtttcacttaattagaaggcggttcgacatataggggagcgagcata

1  ATGGAGCCTCCAACAGTACTGTCAGCGATTTTCCGAGCGTTATCCTTTATTCTGCAC
1  M E P P T V T V S D F S E R Y P L F L H
61  AACTCGAGCTTTCTGGAGGAGCCTGCGGGACTTCTGTCCAACTGGAGCGGAGGAAGCAGT
21  N S S F L E E P A G L L S N W S G G S S
121 GAGCTGAAGGCGGTGAGAGGCAGCAGTGCGGTCGCTATCGCTGTCTCCATCACTGCGCTA
41  E L K A V R G S S A V A I A V S I T A L
TM I
181 TACTCGGTTCATCTGCGTTGTGGGACTCGTTGGAAATGTGCTGGTTATGTACGGAGTGGTC
61  Y S V I C V V G L V G N V L V M Y G V V
241 AGGTACACGAAGATGAAAACGGCAACCAACATATACATCTTCAACCTGGCATTAGCAGAT
81  R Y T K M K T A T N I Y I F N L A L A D
TM II
301 GCGTTGGCCACCACTACATTGCCCTTCCAGAGCGCCAAATACCTCATGGGCACCTGGCCG
101  A L A T S T L P F Q S A K Y L M G T W P
TM III
361 TTCGCGAGCTCTTGTGTAAAGTGGTCATTGCTATTGACTATTACAACATGTTTACCAGT
121  F G E L L C K V V I A I D Y Y N M F T S
421 ATTTTCACGCTGACGATGATGAGTGTGGACCGCTATATTGCCGTGTGTATCCGGTGAGG
141  I F T L T M M S V D R Y I A V C H P V R
481 GCCCTGGACTTCCGCACGCGCTGTCAAAGCCAAGATCATCAACATCTGCGTCTGGATTCTG
161  A L D F R T P V K A K I I N I C V W I L
TM IV
541 TCCTCCGCTGTGCGCTTCCCGTCATGGTGTATGGCTGTTACTAAAGAATTAGACTCAGGC
181  S S A V G F P V M V M A V T K E L D S G
#
601 AAAACAATCTGCATGTGAAATTCCTCCGACCCAGAATGGTACTGGGATACAGTGACCAAA
201  K T I C M L K F P D P E W Y W D T V T K
TM V
661 ATCTCGGTTTTTATTTTGCCTTTGTGTTTCCCGTCCTGGTGATCACCGTCTGCTACGGC
221  I C V F I F A F V F P V L V I T V C Y G
721 CTGATGATCCTGCGGCTGAAGAGCGTGCGCCTGCTCTCCGGCTCCAAAGAGAAGGACAGG
241  L M I L R L K S V R L L S G S K E K D R
TM VI
781 AACCTCCGCGCATCACCCGCATGGTTTTAGTGGTGGTGGCGGCATTCATCTGCTGG
261  N L R R I T R M V L V V V A A F I I C W
841 ACCCCATCCACATCTTCATCATCGTCAAGACAGTGGTAGAGATCGACCAGAAGAACCTG
281  T P I H I F I I V K T V V E I D Q K N L
901 CTGGTGGTGGCCTGTGCGCATCTGTGCATCGCTCTGGGTACATGAACAGCAGCCCTCAAC
301  L V V A C W H L C I A L G Y M N S S L N
*
961 CCGGTCTCTACGCTTTTCTAGATGAGAACTTCAAGAGGTGCTTCAGAGAGTTCTGCTTG
321  P V L Y A F L D E N F K R C F R E F C L
Y
1021 CCTTTTCGCATCGCATAGAGCAGAACAGCTTTTCCAAAGCCCGCAGCGTCATCAGAGAG
341  P F R T R I E Q N S F S K A R S V I R E
Y
1081 CCCATCTCTGTCTGCGCCAAATCAGAATCAATCAAGCAGCCACA TGA
361  P I S V C A K S E S I K Q P T STOP

ctagacctggacgggatgactcctagtacacagaaagaccgcagtcogaggttaaccagatctctac
aactgagttttaagcttatctggccataaaactgctggcagaatgtcatgttggggcattatttatat
tttattctcatcgttcttctgactgaaaagatggatggccctgaaatgatctcgagatattttcctaa
gtgaggaatgagatgatctggaccatcaagatggtaatcatgcattggaaaagacatcaaaagacctg
accaacaggaattttacattcattagcggatatagagccaatacacgagattccctaagagatTTTT
tttttatattgcaagttaatgcatttccatgggaattgtgggaattgtgggatttatgatatactga
ataatcaattctgcttttgaacaacaaaagtgaagctgaagatttttctacaagagcttgttacaca
gaaaaatgaaatgactcttttgaagctcctgtttagacaataaaaagttttttttcttaaaaac
caagacatatgggagaaacattggaatttagaattggacattcccttgtttttcatggatgcaagcat
gttcttcttttgaacaataaactctgagcattatcatttccatgtgtttataaagttcaattgtg
aaatttggctactagtggaaggtgttttaaaacctttagctatgaagtataaaagcttttttagctctt
tagtatttagtggacgaaaactctgcatttcttggcattggcgagaaatggctgttttatgacctt
cttcaaatcttaagtgtaagtgaacatttaacgaatcgtctcatgatgttagtgttgatttaaaatg
tcagcttactgttaaaggagatggttttagtttcttgagctgtaattaggaaactttttccagccaagt
gtgtttatgtatttagtttcttctgtattctgcttcaactgatctgttctcagcaatctattttta
tggacgatggaaattagacatcacaaactatctgatctgccagattttgtgtgaaagttcagaaaa
aataattaaaatcattctgtagattaaaaaaaaaaaaaacccgccggcgagatctcct 2370

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Figure 1 Nucleotide and deduced amino acid sequences for the ZFOR4 cDNA, a δ -opioid receptor from zebrafish. The predicted transmembrane domains are underlined and written in bold, the two cysteines that are forming a disulphide bond are marked with #, the Asn residues that can be glycosylated with ●, the potential Ser/Thr phosphorylation sites with ¥ and the putative palmitoylation site with *. The oligonucleotide sequence used as a probe in the expression studies has been italicized and underlined.

receptor genes in zebrafish. Also, in order to compare the expression of both δ -opioid receptor-like duplicates, ZFOR1 and ZFOR4, the same oligonucleotides (5'-ACC AGT GCG ATG CAA GTG CCA GCT A-3' and 5'-GCA GAC TGT TGT ATT CTG ATT TGT CAC TCT AGT GA-3') used by our group to describe the expression of ZFOR1 (available in EMBL DataBase under Accession number AJ001596) in the CNS of adult zebrafish were used (Porteros *et al.* 1999). All these oligonucleotides were labelled at 5' end with digoxigenin and used at a final concentration of 10 ng/ μ l.

Tissue was prehybridized in hybridization buffer (Omnibuff, Wak Chemie, Medical GMBH) for 30 min at room temperature and hybridized overnight at 37 °C with the oligonucleotide probes. Sections were then washed in PBS for 10 min, once at 37 °C and twice at room temperature. Mouse antidigoxigenin antibody diluted in TBS (Tris 50 mM, NaCl 800 mM (pH 7.4)) was used at 4 °C overnight for immunological amplification. After washing for 5 min at room temperature in TBS, sections were incubated in a humid chamber with biotinylated goat antimouse antiserum in TBS for 40 min and then washed in TBS. Streptavidin-peroxidase conjugate and 3-3'-diaminobenzidine were used for immunological detection. Finally, after dehydration, the slides were mounted with Entellan (Merck). To ensure the specificity of the method, adjacent sections were used as controls. The following approaches were performed: pretreatment with RNase, incubation with sense probe, incubation without probe and incubation prior to hybridization with an excess of non-labelled oligodeoxynucleotide and then hybridization with a labelled one. Absence of signal in adjacent sections hybridized as negative controls confirmed the specificity of the hybridization.

Cell culture and transfection

The complete cDNA sequence of ZFOR4 was ligated in the mammalian expression vector pcDNA3 and HEK 293 cells were transfected using lipofectamine (Invitrogen) and selected with geneticin 0.5 μ g/ μ l (GibCo BRL). After 2 months of selection period, RT-PCR was used to confirm that the positive clones were expressing the ZFOR4 receptor. Stably transfected HEK 293 cells expressing the ZFOR4-opioid receptor were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, glutamine 2 mM, penicillin 100 U/ml, streptomycin 0.1 mg/ml and geneticin 0.25 μ g/ μ l at 37 °C under 5% CO₂ atmosphere (Biotech Galaxy, RS Biotech., Irvine, Scotland, UK).

Pharmacological studies

Cells were grown to 80% confluence, harvested in PBS (pH 7.4) containing EDTA 2 mM and collected by

centrifugation at 500 g. The cell pellets were resuspended in Tris-HCl buffer 50 mM (pH 7.4; assay buffer) with protease inhibitors bacitracin 0.1 mg/ml, captopril 3.3 μ M and thiorphan 0.33 μ M (Sigma). The cell suspensions were homogenized with a Kinematika polytron in assay buffer, the homogenates were centrifuged at 6000 g for 15 min at 4 °C and the pellets were washed once in assay buffer, homogenized and centrifuged again. Membranes were resuspended in ice-cold assay buffer with protease inhibitors and protein concentration was determined by Lowry method (Onishi and Barr Modification).

To perform the pharmacological studies, the radioligands [³H]diprenorphine (50 Ci/mmol), [³H]DPDPE (50 Ci/mmol) and [³⁵S]GTP γ S were purchased from Perkin-Elmer, Dupont and Perkin-Elmer respectively. Morphine was obtained from the Spanish Ministry of Health, naloxone, BW373U86, Met-enkephalin; Leu-enkephalin, DPDPE, dynorphin A and U69,593 were purchased from Sigma Aldrich and D-Ala-D-Leu-enkephalin from Tocris. For saturation-binding assays, 25–50 μ g protein were incubated with different concentrations of the radioligands [³H]diprenorphine and [³H]DPDPE for 3 h 45 min at 25 °C in a final volume of 250 μ l. Ten micromolars of naloxone were used to determine non-specific binding. After incubation, the reaction was stopped by adding 4 ml ice-cold assay buffer, the mixture was rapidly filtrated using a Brandel Cell Harvester and washed twice onto GF/B glass-fibre filters that were presoaked with 0.2% polyethylenimine (Sigma) for at least 1 h. The filters were placed in scintillation vials and incubated overnight at room temperature in EcoScint A scintillation liquid. Radioactivity was counted using a Beckman Coulter scintillation counter. All experiments were performed in duplicate and repeated twice.

In competition-binding assays, [³H]diprenorphine was displaced by several unlabelled compounds at a concentration range from 0.3 nM to 10 μ M, using 10 μ M naloxone to determine non-specific binding. All experiments were performed in duplicate and done thrice.

Agonist stimulation of [³⁵S]GTP γ S binding was performed as described by Traynor & Nahorski (1995). Twenty micrograms of protein were incubated in a buffer with Tris-HCl 50 mM (pH 7.4), NaCl 100 mM, MgCl₂ 5 mM, EDTA 1 mM, DTT 1 mM, BSA 0.1%, GDP 10 μ M and [³⁵S]GTP γ S 0.1 nM in the presence of varying concentrations of opioids ranging from 0.1 nM to 10 μ M in a final volume of 200 μ l for 1 h at 30 °C. Basal [³⁵S]GTP γ S binding was defined in the absence of agonist, and non-specific [³⁵S]GTP γ S binding was defined as binding in the presence of 10 μ M unlabelled GTP γ S. Bound and free [³⁵S]GTP γ S were separated by vacuum filtration through GF/B glass-fibre filters and quantified by liquid scintillation counting. All experiments were performed in triplicate and done thrice.

Specific binding was defined as the difference between total binding and non-specific binding (measured in the presence of 10 μ M naloxone for saturation and competition-binding assays and in the presence of 10 μ M unlabelled GTP γ S for [35 S]GTP γ S stimulation assays). Data were analysed using the Graph Pad Prism software (Graph Pad, San Diego, CA, USA) and affinity constant (K_D), receptor density ($B_{\max}$), inhibition constant (K_i) and mean effective dose (EC_{50}) values were obtained for each ligand. In saturation-binding assays, data were fit to the non-linear function and to the linear transformation (Scatchard-plot: Bound/Free versus Bound). K_i values were calculated using the correction of Cheng and Prusoff, which corrects for the concentration of radioligand used in each experiment as well as the affinity of the radioligand for its binding site (K_D).

Results

Cloning of a δ -opioid-like receptor from zebrafish

A DNA sequence that codes for the third exon of the mouse κ -opioid receptor was cloned by RT-PCR using total RNA from mouse brain as template. This fragment, which codes from the first to the fourth transmembrane domains (from nucleotides 448 to 781 of the sequence published by Yasuda *et al.* 1993), was used as a probe to hybridize a ZIPLOX cDNA library from adult zebrafish brain and a full-length cDNA of 2370 bp was obtained (Fig. 1). The corresponding nucleotide sequence has been deposited under Accession number AY262256. Sequence analysis of this cDNA presents an open reading frame (ORF) of 375 amino acids, with an approximate molecular weight of 42.18 kDa. The encoded protein shows a high sequence similarity to mammalian δ -opioid receptor (64% identity to human (Knapp *et al.* 1994), 63% to mouse (Evans *et al.* 1992, Kieffer *et al.* 1992) and 62% to rat δ -opioid receptor (Wang *et al.* 1993)), amphibian homologues (69% identity to *Taricha granulosa* (Bradford *et al.* 2006) and 68% to *Rana pipiens* δ -opioid receptor (Stevens 2004)) and to the other δ -opioid receptor from zebrafish ZFOR1 (71% identity (Barrallo *et al.* 1998)). On the other hand, the degree of homology with the mammalian μ - and κ -opioid receptors only reaches 52% identity. Hydrophobic analysis reveals that this receptor presents seven potential transmembrane domains, thus confirming that ZFOR4 can be classified as a member of the G-protein-coupled receptor (GPCR) superfamily. Besides, this receptor has two consensus sites for N-glycosylation on the N-terminal extracellular domain (Asn²¹ and Asn³⁴), two conserved cysteine residues that can form a disulphide bridge between the first and second extracellular loops (Cys¹²⁶ and Cys²⁰⁴), eleven consensus sites for phosphorylation by protein kinase A or C (Ser²⁴⁸, Ser²⁵³, Ser²⁵⁵ and Thr²⁶⁶

in the third intracellular loop, and Thr³⁴⁴, Ser³⁵⁰, Ser³⁵², Ser³⁵⁶, Ser³⁶³, Ser³⁶⁸ and Ser³⁷⁰ in the carboxyl-terminal domain) and a putative palmitoylation site in the C-terminal domain that renders a fourth intracellular loop (Cys³³⁹).

To determine the genomic structure of ZFOR4, we have used its complete cDNA sequence as a probe to hybridize a zebrafish genomic library (courtesy of B Jones and M Petkovich, Queen's University, Canada). Restriction and sequence analysis showed that ZFOR4 is formed by at least three exons: the first exon comprises the 5'UTR region and the first 243 bp of the ORF (Met¹–Arg⁸¹), the second exon contains 357 bp of the coding region (Tyr⁸²–Gly²⁰⁰) and the third exon includes the last 528 bp of the ORF (Lys²⁰¹–Thr³⁷⁵) and the 3'UTR region. The genomic organization of the ZFOR4 gene is highly conserved and similar to the one displayed by the mammalian δ -opioid receptors (Augustin *et al.* 1995).

Expression studies

The neuroanatomical atlas from Wullmann *et al.* (1996) has been used for the description of the results. We detect the expression of ZFOR4 specifically localized in the CNS of the zebrafish. Expression is found in all main subdivisions of the brain (telencephalon, diencephalon, mesencephalon and rhombencephalon) and in the spinal cord (Table 1). Specific labelling was observed from rostral to caudal levels, with higher intensity in the hypothalamus (Fig. 2J), periventricular layer of the optic tectum (Fig. 2D and F), granular layer of the cerebellum (Fig. 2G), medium intensity in the dorsal telencephalic areas (Fig. 2A), torus semicircularis (Fig. 2D), reticular formation (Fig. 2I) and facial lobe (Fig. 2H) and with lower intensity in other regions such as the olfactory bulb, thalamus (Fig. 2C) and spinal cord (Table 1). Also, we confirmed the results obtained by our group for the expression of ZFOR1 (Porteros *et al.* 1999; Fig. 2K).

Pharmacological studies

To establish the pharmacological profile of ZFOR4, the complete ORF that codes for this receptor was cloned in the mammalian expression vector pcDNA3 and the construct was used to transfect the HEK293 cell line. After obtaining clones that stably express this receptor, membranes were extracted from these cells and radioligand-binding assays were performed. The non-specific antagonist [3 H]diprenorphine was used for saturation-binding assays (Fig. 3), displaying one binding site with an affinity constant of $K_D = 3.42 \pm 0.38$ nM and a receptor density of $B_{\max} = 6231 \pm 335$ fmol/mg protein. The specific binding was displaced by naloxone, thus confirming the opioid nature of these sites.

Table 1 Relative density of ZFOR4 mRNA-labelled cells in the main subdivisions of the zebrafish CNS. Density has arbitrarily been assigned to values as follow: +, low abundance; ++, moderate abundance; +++, high abundance; + + + +, very high abundance

	ZFOR4
CNS region	
I Telencephalon	
Olfactory bulb	++
Dorsal telencephalic area	++
Ventral telencephalic area	++
II Diencephalon	
Preoptic area	+++
Epithalamus	++++
Dorsal thalamus	+++
Ventral thalamus	+++
Posterior tubercle	++
Hypothalamus	++++
Synencephalon (NMLF)	+
Pretectum	++
III Mesencephalon	
Optic tectum	++++
Torus semicircularis	+++
Tegmentum	++
IV Metencephalon	
Valvula cerebelli	++++
Corpus cerebelli	++++
Lobus vestibulolateralis	++++
V Myelencephalon	
Octavolateralis area	++
Reticular formation	+++
Raphe nuclei	++
Vagal lobe	++
Glossopharyngeal lobe	++
Facial lobe	+++
VI Spinal cord	
Ventral horn	+
Dorsal horn	+

The δ -selective ligand [^3H]DPDPE (δ) has also been used to perform saturation-binding assays on ZFOR4 homogenates, but it did not display any saturable binding (data not shown), hence indicating that this highly selective ligand for mammalian δ receptors has no affinity for ZFOR4.

To analyse the affinity of different ligands for the ZFOR4 receptor, competition-binding experiments were performed using [^3H]diprenorphine as radioligand and several unlabelled compounds were used at a concentration range from 0.3 nM to 10 μM . The results are shown in Fig. 4 and summarized in Table 2. In all cases, the experimental data fitted better to the one-site competition model, as determined by an *F*-test. The ligands tested here can be classified into three categories: (a) the peptidic ligands Met-enkephalin, Leu-enkephalin and dynorphin A and the non-specific antagonist naloxone, which are able to displace almost all the [^3H]diprenorphine-specific binding with equilibrium K_{ds} (K_{i} values) in the nanomolar range. (b) The synthetic

δ agonist BW373U86 and morphine, which cannot totally displace all the specifically bound [^3H]diprenorphine and whose K_{i} values are in the micromolar range. (c) The highly selective ligands for mammalian opioid receptors DPDPE (δ) and U69,593 (κ), which are unable to show an effective displacement.

To confirm if the ZFOR4 receptor is functional, then the agonist activity of several opioid ligands was evaluated using the [^{35}S]GTP γ S stimulation assay. The maximal [^{35}S]GTP γ S binding obtained for each ligand when used at a concentration of 10 μM is represented in Fig. 5. Our results indicate that the endogenous ligands Met- and Leu-enkephalin and the non-peptidic δ agonist BW373U86 are able to fully activate this receptor (more than 100% of the maximal [^{35}S]GTP γ S stimulation), while D-Ala-D-Leu-enkephalin, DSLET and morphine only produce a lower response (around 80% [^{35}S]GTP γ S stimulation).

Discussion

Molecular characterization of ZFOR4

ZFOR4 is a new opioid receptor from the teleost zebrafish, which can be molecularly classified as a δ -opioid receptor, since it presents a higher degree of homology with the δ receptors (around 65% identity) than with the μ and κ receptors (52% identity). This new receptor is a duplicate of another δ receptor of zebrafish, ZFOR1, which we have previously cloned (Barrallo *et al.* 1998), as shown in the phylogram presented in Fig. 6. Both duplicate receptors maintain their opioid activity as demonstrated in our pharmacological characterization. The degree of homology is not uniform along the sequence, as it is higher at the transmembrane domains and at the intracellular and the extracellular loops and amino- and carboxyl-terminus are more divergent (Fig. 7). The first extracellular loop of ZFOR4 is homologous to its mammalian counterparts, since it only has a substitution of the conserved residue Glu¹¹² by a Gly¹¹⁷. Remarkably, this glycine is also present in the same position in the μ and ORL receptors (Gly¹³³ and Gly¹¹³ in the human δ and ORL receptors respectively). Nevertheless, it seems that the change of Glu¹¹²Gly in the δ -opioid receptors does not affect the ability of different ligands to bind to these receptors (Fukuda *et al.* 1995).

The second and third extracellular loops are involved in the selective binding of different ligands to the opioid receptors (Metzger & Ferguson 1995). The second extracellular loop of ZFOR4 possesses more positive and negative charges than its counterparts in the mammalian and amphibian δ receptors, which can explain why the highly selective δ ligands show lower or no affinity for ZFOR4. The third extracellular loop is the least homologous, exhibiting significant changes in charge and amino acid size or hydrophobicity.

Figure 2 Localization of ZFOR4 mRNA expression by *in situ* hybridization in the adult zebrafish brain (coronal sections) (A–I). (A) Telencephalon (D, dorsal telencephalic area; V, ventral telencephalic area), (B) anterior diencephalon (PPa, anterior part of the parvocellular preoptic nucleus), (C) diencephalon (Nomarski micrograph; CP, central posterior thalamic nucleus; Cpost, commissura posterior; PPv, ventral part of the periventricular pretectal nucleus; TPp, periventricular nucleus of posterior tuberculum), (D) mesencephalon (DTN, dorsal tegmental nucleus; TeO, optic tectum; TSc, central nucleus of the torus semicircularis; Va, valvula cerebelli), (E) a coronal section at the same level, close to D, processed for negative control, does not show any labelling, (F) optic tectum (Nomarski micrograph). High density of cells was observed in the periventricular layer (open arrow) and scarce cells with different morphologies were observed in other strata (filled arrows), (G) metencephalon. Abundant cells were labelled in the eminentia granularis (EG) and corpus cerebelli (CCe) and scarce cells (arrows) were also observed in the caudal lobe of the cerebellum (LCa), (H) myelencephalon (MLF, medial longitudinal fascicle; LVII, facial lobe; LX, vagal lobe; RV, rhombencephalic ventricle), (I) high magnification of labelled cells in the inferior reticular formation (IRF), showing the variety of sizes and shapes of positive cells. (Nomarski micrograph). (J–L) Coronal sections of adult zebrafish brain showing the comparative *in situ* hybridization signal expression for both δ -opioid-like receptors from zebrafish, (J) ZFOR4 and (K) ZFOR1 in the ventral mesencephalon. (L) Absence of signal is shown at the same level, close to A and B, processed for negative control. It can be observed that the expression for ZFOR4 is more widespread and the number of positive cells is greater than that observed for ZFOR1 (Porteros *et al.* 1999). Abbreviations: CM, corpus mamilare; DIL, diffuse nucleus of the hypothalamic inferior lobe; Hc, caudal zone of the hypothalamic periventricular nucleus; Hd, dorsal zone of the hypothalamic periventricular nucleus; TeO, optic tectum; TLa, torus lateralis. Scale bar: 50 μ m in I; 100 μ m for F, G and H; 200 μ m for A–E and J–L.

Figure 3 Saturation-binding assays of [3 H]diprenorphine in membrane homogenates from stably transfected HEK293 cells expressing ZFOR4. Inset: Scatchard-plot. Results are from a representative experiment performed in duplicate and repeated thrice.

Although the Val³⁰² and Val³⁰³ are conserved in the same positions as in the mammalian receptors (Val²⁹⁶ and Val²⁹⁷), the Trp²⁸⁴ is replaced by Lys²⁹⁰, which plays a crucial role for the binding of DPDPE and deltorphin (Valiquette *et al.* 1996). Also, instead of the Arg²⁹¹ and Arg²⁹² of the mammalian δ receptors, ZFOR4 presents a Gln²⁹⁷ and Lys²⁹⁸, which together with the existence of a Trp³⁰⁶, can explain the loss of affinity for the highly selective ligands for the mammalian δ receptors (Wang *et al.* 1995, Pepin *et al.* 1997).

Although the two δ -opioid receptors from zebrafish are homologues with high sequence similarity, they present some divergences in their amino acid sequences that can explain the pharmacological differences. The second extracellular loop of ZFOR4 has more charged residues than ZFOR1, which means that this loop from ZFOR resembles more than the ones from the mammalian δ receptors. Some exceptions are the conserved Asp¹⁹⁸ in ZFOR4 (equivalent to Asp¹⁹³ in

mammalian receptors), which is substituted by Asn¹⁹⁶ in ZFOR1, and the Gly²⁰⁰ in ZFOR4 (equal to Gly¹⁹⁴ in mammals) that is replaced by Asn¹⁹⁸, a bulky residue, in ZFOR1. The third extracellular loops of ZFOR1 and ZFOR4 are quite similar among themselves, but as we have described previously, they differ significantly from the ones of the mammalian δ receptors. However, there are some substitutions in one of these two duplicate receptors: the conserved Pro²⁹⁴ in the mammalian receptors and in ZFOR1 (Pro²⁹⁸) is replaced by Leu³⁰⁰ in ZFOR4, and conversely, the conserved Leu²⁹⁵ in the mammalian receptors and in ZFOR4 (Leu³⁰¹) is exchanged by Phe²⁹⁹ in ZFOR1.

Expression studies

We have analysed the distribution of ZFOR4 in the zebrafish CNS by means of non-radioactive *in situ* hybridization using specific oligonucleotides labelled with digoxigenin at its 5' end. We have detected specific signal in the same regions, where ZFOR1 (Porteros *et al.* 1999) was found to be expressed in the CNS of the zebrafish (Table 1), but the signal is less intense in this case although most of the structures that show expression, such as dorsal telencephalic areas (D; Fig. 2A), hypothalamus (Fig. 2J and K), granular layer of the cerebellum (Fig. 2G), reticular formation (Fig. 2I) and facial lobe (Fig. 2H) present a greater number of positive cells.

The distribution of δ -opioid receptors in fish has been described from binding data (Bird *et al.* 1988) and by *in situ* hybridization in the case of ZFOR1, the duplicate gene previously described by us (Barrallo *et al.* 1998). In mammals, the distribution of δ -opioid receptor mRNA (Mansour *et al.* 1994) shows this receptor being abundant in olfactory bulb, neocortex, striatum, hippocampus, amygdala, pontine nuclei and dorsal horn of the spinal cord, what has been related to analgesia, gastrointestinal motility and hypothalamic

Figure 4 Competition-binding assays of [3 H]diprenorphine and several unlabelled ligands using membrane homogenates from stably transfected HEK293 cells expressing ZFOR4. (A) Unlabelled ligands which are δ -selective. (B) Unlabelled ligands that are not δ -selective. Results represent the mean $\pm$ S.E.M. of three independent experiments performed in duplicate. Non-specific binding was determined using 10 μ M naloxone. Legend: ■, BW373U86; ▲, Met-enkephalin; ●, Leu-enkephalin; ▼, DPDPE; □, dynorphin A; △, U69,593; ▽, naloxone; ○, morphine.

Table 2 K_i values of several ligands in ZFOR4 obtained from competition-binding assays using [3 H]diprenorphine. Values represent mean $\pm$ S.E.M. from three experiments performed in duplicate

Ligand	K_i (nM)	% Displacement ^a	K_i (nM) for mammalian DOR	K_i (nM) for ZFOR1 (Rodríguez <i>et al.</i> 2000)	K_i (nM) for amphibian DOR (Bradford <i>et al.</i> 2006)
BW373U86	1020.53 $\pm$ 377.74	83.58 $\pm$ 4.17	0.16 $\pm$ 0.02 (Meng <i>et al.</i> 1996) 1.63 (Befort <i>et al.</i> 1996)		
Met-enkephalin	45.77 $\pm$ 9.62	93.49 $\pm$ 2.06	3.0 $\pm$ 1.8 (Meng <i>et al.</i> 1996) 5.01 (Befort <i>et al.</i> 1996)		24.9
Leu-enkephalin	175.20 $\pm$ 69.45	90.08 $\pm$ 2.49	1.80 $\pm$ 0.5 (Mansour <i>et al.</i> 1995) 4.2 $\pm$ 2.4 (Meng <i>et al.</i> 1996) 6.52 (Befort <i>et al.</i> 1996)		198
DPDPE	> 10 000	18.67 $\pm$ 10.97	6.19 $\pm$ 1.00 (Mansour <i>et al.</i> 1995) 21 $\pm$ 4 (Meng <i>et al.</i> 1996)	953	499
Dynorphin A	343.47 $\pm$ 114.12	89.45 $\pm$ 3.98	31.7 (Befort <i>et al.</i> 1996) 21.6 (Befort <i>et al.</i> 1996) 1.25 $\pm$ 0.12 (Zhang <i>et al.</i> 1998) 2.64 $\pm$ 0.35 (Mansour <i>et al.</i> 1995)		
U69,593	> 10 000	8.94 $\pm$ 3.42		> 10 000	> 10 000
Naloxone	88.29 $\pm$ 13.33	99.22 $\pm$ 0.24	30.6 (Befort <i>et al.</i> 1996) 44.8 $\pm$ 5.8 (Chaturvedi <i>et al.</i> 2000) 0.92 $\pm$ 0.21 (Mansour <i>et al.</i> 1995)	53.8	3.32
Morphine	1427.67 $\pm$ 302.44	75.40 $\pm$ 3.60	249.7 $\pm$ 41.4 (Chaturvedi <i>et al.</i> 2000) 7.48 $\pm$ 0.59 (Mansour <i>et al.</i> 1995)	223	

^aPercentage of displacement of [3 H]diprenorphine shown at a concentration of 10 μ M of the unlabelled ligand.

regulation. In our case, the abundant ZFOR4 mRNA immunostained cells observed through the inferior hypothalamic lobes and in the periventricular grey of zebrafish lateral recesses (Fig. 2J) could be related with an opioid modulation of visuomotor activities related to prey catching and feeding responses in zebrafish brain. Indeed, electrical stimulation of these hypothalamic areas has been demonstrated in fish (Demski 1973) to elicit feeding, picking up of gravel and aggressive behaviours. On the other hand, although there is evidence that morphine has a modulatory effect in the corticotrophin-releasing activity in fish (Bird *et al.* 1987, Mukherjee *et al.* 1987) and that the opioid peptides interact with the neuroendocrine system of mammals (Grossman & Rees 1983), the involvement of δ -opioid receptor in the modulation of hormone release from the hypothalamic-hypophysary axis seems to be of minor importance in mammals (Mansour *et al.* 1993), although it has also been demonstrated that endogenous opioids, such as β -endorphin, exert a tonic restraint on gonadotrophin-releasing hormone (GnRH) secretion (Pu *et al.* 1997). In teleosts, regional distribution and *in vitro* secretion of GnRH from the brain and pituitary of goldfish (*Carassius auratus*) has also been studied (Rosenblum *et al.* 1994). Indeed, the expression of these δ -opioid receptors from zebrafish, found in neuroendocrine regions, including preoptic area (Fig. 2A) and the hypothalamus (Fig. 2J and K) is likely involved in control of GnRH and luteinizing hormone (LH) release. In this sense, the presence and distribution of a δ -opioid receptor population on a subset of

hypothalamic GnRH neurons in the mouse has recently been demonstrated (Pimpinelli *et al.* 2006).

In the reticular formation of mammals, δ -opioid receptors are involved in analgesic mechanisms (Mansour *et al.* 1993), as might be in teleosts, due to the presence of ZFOR4 and ZFOR1 mRNAs in the whole rostrocaudal extension of the reticular formation (Fig. 1I). ZFOR4 mRNA expression has been detected

Figure 5 Stimulation of [35 S]GTP γ S binding to ZFOR4 membrane homogenates by various ligands at a concentration of 10 μ M. Non-specific binding was determined with 10 μ M GTP γ S. Values represent the mean $\pm$ S.E.M. of three independent experiments performed in triplicate.

Figure 6 Phylogram generated by neighbour joining method, from the alignment of the deduced amino acid sequences of the opioid receptors from zebrafish and its homologues identified in mammals and in amphibians. This analysis was conducted using MEGA version 2.1 (Kumar *et al.* 2001). Whole numbers (bold) indicate bootstrap values and branch distances are given in decimal numbers. Sequences were obtained from GenBank. Accession numbers are: hDOR (*Homo sapiens*), U10504; mDOR (*Mus musculus*), L06322; rDOR (*Rattus norvegicus*), NM_012617; ZFOR1 (*Danio rerio*), NM_131258; ZFOR4, AY262256; rpDOR (*Rana pipiens*), AF530572; nDOR (*Taricha granulosa*), AY751785; hMOR, NM_000914; mMOR, U10558.1; rMOR, NM_013071.1; ZFOR2, AF132813; rpMOR, AY244471; nMOR, AY75178; hKOR, NM_000912.3; mKOR, NM_011011.1; rKOR, NM_017167.1; ZFOR3, AF285173; nKOR, AF285173.

in dorsal and ventral horns of the spinal cord of zebrafish, although to a lesser extent that was reported for ZFOR1 (Porteros *et al.* 1999) and similar to that previously reported in mammals, where δ -opioid receptor binding and mRNA expression have been observed in scattered cells on several laminae of ventral and dorsal horns (Mansour *et al.* 1993).

The occurrence of moderate expression of ZFOR4 mRNA in areas, such as the pretectum and posterior tuberculum, which are sensory-motor integrative centres very well developed in teleosts (Meek & Nieuwenhuys 1998), suggests that the δ -opioid system might be influenced, at least in lower vertebrates, by adaptive mechanisms also related to behavioural responses basic for survival. Also, even when there are evolutive differences between the CNS of mammals and fish (Butler & Hodos 1996), some homologies have been proposed, such as the dorsal telencephalic areas in fish with the cerebral cortex and basal ganglia and the cerebellum, which suggests that if there is correspondence in the distribution then it could also exist as a comparative evolution in the function.

Pharmacological profile of the ZFOR4 receptor

The binding studies allowed us to determine the pharmacological profile of ZFOR4 receptor and to compare it with those established for the δ receptors in other species as well as with its duplicate in zebrafish ZFOR1. The non-specific antagonist [3 H]diprenorphine binds to ZFOR4 with high affinity, and this binding can be displaced by naloxone, hence confirming the opioid nature of this receptor. The affinity constant ($K_D = 3.42 \pm 0.38$ nM) is in the same range to that previously observed for ZFOR1 ($K_D = 3.4 \pm 0.6$ nM) by us (Rodriguez *et al.* 2000), and slightly higher, although in the same order, than those reported for the human δ receptor ($K_D = 1.48 \pm 0.8$ nM (Varga *et al.* 1996), $K_D = 1.8 \pm 0.4$ nM (Zhang *et al.* 1998) and $K_D = 0.75 \pm 0.07$ nM (Cavalli *et al.* 1999)). On the other hand, the δ -selective radioligand [3 H]DPDPE for the mammalian δ receptors ($K_D = 6$ nM (Mansour *et al.* 1995) and $K_D = 3.1 \pm 0.3$ nM (Pepin *et al.* 1997)) does not bind to ZFOR4, and with very low affinity to ZFOR1 ($K_i = 953$ nM; Rodriguez *et al.* 2000). The fact that the δ -opioid receptor of the newt presents a $K_i = 499$ nM for DPDPE (Bradford *et al.* 2006), which is an intermediate value between those established for the mammalian and zebrafish δ receptors, indicates that the DPDPE selectivity is gradually lost when moving down in the evolutionary scale and suggests that this ligand may not be suitable for determining the δ -binding profile in such organisms.

To determine the affinity of different compounds for ZFOR4 receptor, we have performed competition-binding assays using [3 H]diprenorphine as the radioligand. The specific binding was displaced by several unlabelled ligands, some of them are δ selective (DPDPE, BW373U86), κ selective (U69,593), non-selective (naloxone), endogenous peptides (Met-enkephalin, Leu-enkephalin, Dynorphin A) and non-endogenous compounds (morphine). Met-enkephalin, naloxone and Leu-enkephalin are good displacers, since their K_i values are in the nanomolar range and able to displace almost all the specific binding, while dynorphin A, BW373U86 and morphine present lower affinities (K_i values near or in the micromolar range) and only displace about 80–90%, and lastly DPDPE and U69,593 are not able to displace more than 20% of the specifically bound [3 H]diprenorphine. These values obtained for ZFOR4 are higher than the ones reported for mammalian δ receptors (see Table 2), with the less significant differences found for the endogenous peptides and naloxone. Nevertheless, it is important to consider that some of the K_i values reported for the mammalian δ receptors have been obtained using other radioligands different from [3 H]diprenorphine, such as [3 H]ethylketocyclozoline (Meng *et al.* 1996), bremazocine (Meng *et al.* 1996, Chaturvedi *et al.* 2000) and [3 H]DPDPE (Mansour *et al.* 1995), that may recognize different

N-Terminus

[illegible]

Figure 7 Alignment produced with ClustalW program of the deduced amino acid sequences for the δ -opioid receptors from human (Accession number U10504), mouse (L06322), rat (NM_012617), the amphibians newt (AY751785) and frog (AF530572) and the δ -opioid receptors from zebrafish ZFOR1 (NM_131258) and ZFOR4 (AY262256). The transmembrane regions are shaded. Legends: *, identical residues in all of the sequences; :, conserved residues;., similar residues

moieties in the receptor-binding pocket and thus displaying different displacement curves for the unlabelled compounds. When the displacement results for ZFOR4 are compared with those seen for the amphibian δ receptor (Bradford *et al.* 2006), the K_i values are comparable, except for naloxone, which is also the radioligand used by these authors. The study of the competition-binding results of ZFOR1 and ZFOR4 indicates that the K_i values are similar when the same unlabelled ligand is used, with the exception of morphine, which is a better displacer in ZFOR1 than in ZFOR4.

To determine if ZFOR4 is functional, we have used the [35 S]GTP γ S-binding assay to evaluate the effect of different agonists to activate the G-proteins through this receptor. All the ligands used in this test were able to activate ZFOR4 and transduce the signal to the G-proteins, although with different maximal effect. The non-peptidic δ ligand BW373U86 and the endogenous Met- and Leu-enkephalins showed more than 100% stimulation of [35 S]GTP γ S binding to membranes from ZFOR4 expressing HEK293 cells, while in experiments using [D-Ser 2 ,Leu 5]enkephalin-Thr 6 (DSLET), D-Ala-D-Leu-enkephalin and morphine [35 S]GTP γ S stimulation only reached 80%, thus behaving as partial agonists. Interestingly, similar results have been found with its duplicate ZFOR1, where BW373U86 fully activates the receptor and morphine is also behaving as a partial agonist on the [35 S]GTP γ S-binding assay (Rodríguez *et al.* 2000).

Taking into consideration the results obtained from the pharmacological studies of ZFOR4, we can conclude that, as seen in ZFOR1, the non-selective ligands as well as the endogenous peptides can bind to the receptor-binding pocket with high affinity, while the highly selective ligands designed for the mammalian opioid receptors are not recognized by the determinants of selectivity in ZFOR4 receptor. Our findings agree with the hypothesis that the opioid receptor-binding pocket that contains the essential moieties for ligand binding is well conserved through vertebrate evolution, but the structural features implicated in ligand selectivity, mainly located in the second and third extracellular loops (Metzger & Ferguson 1995, Meng *et al.* 1996, Varga *et al.* 1996), have gradually evolved. As a result, this process gave rise to the different opioid receptor types with differential-binding profile between types and among one type (namely μ , δ or κ) in the distinct vertebrate species. The presence of two genes that code for δ -opioid receptors (ZFOR1 and ZFOR4) in the zebrafish could be the outcome of an extra duplication that took place in teleosts.

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