Pharmacological characterization of a nociceptin receptor from zebrafish (*Danio rerio*)

Ángel A Rivas-Boyer^{1*}, M Javier Herrero-Turrión^{2*}, Veronica Gonzalez-Nunez^{1,2*}, Fátima Macho Sánchez-Simón^{1,2}, Katherine Barreto-Valer^{1,2} and Raquel E Rodríguez^{1,2}

¹Department of Biochemistry and Molecular Biology and ²Institute of Neurosciences of Castilla y León (INCYL), University of Salamanca, 37007 Salamanca, Spain

(Correspondence should be addressed to R E Rodríguez; Email: requelmi@usal.es)

* (Á A Rivas-Boyer, M J Herrero-Turrión and V Gonzalez-Nunez contributed equally to this work)

Abstract

The nociceptin receptor (NOP) and its endogenous ligand, nociceptin/orphanin FQ (OFQ), are involved in a wide range of biological functions, such as pain, anxiety, learning, and memory. The zebrafish has been proposed as a candidate to study the *in vivo* effects of several drugs of abuse and to discover new pharmacological targets. We report the cloning, expression, and pharmacological characterization of a NOP receptor from zebrafish (drNOP). The full-length cDNA codes a protein of 363 residues, which shows high sequence similarity to other NOPs. Phylogenetic analysis indicates that NOPs are broadly conserved during vertebrate evolution, and that they stand for the most divergent clade of the opioid/OFQ receptor family. Expression studies have revealed that *drNOP* mRNA is highly expressed in the central nervous system, and low expression levels are also found in peripheral tissues such as gills, muscle, and liver. Pharmacological analysis indicates that drNOP displays specific and saturable binding for [*Leu*yl-3,4,5-³H]nociceptin, with a $K_d = 0.20 \pm 0.02$ nM and a $B_{max} = 1703 \pm 81$ fmol/mg protein. [³H]Nociceptin binding is displaced by several opioid ligands such as dynorphin A (DYN A), naloxone, bremazocine, or the κ -selective antagonist nor-binaltorphimine. [³⁵S] GTP γ S stimulation studies showed that drNOP receptor is functional, as nociceptin is able to fully activate the receptor and DYN A behaves as a partial agonist (50% stimulation). Our results indicate that drNOP receptor displays mixed characteristics of both NOP and κ opioid receptors. Hence, drNOP, which has retained more of the likely ancestral features, bridges the gap between nociceptin and opiate pharmacology.

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Introduction

The opioid receptor-like 1 (ORL1) or nociceptin receptor (NOP) is a G-protein-coupled receptor (GPCR) that exhibits a significant degree of sequence identity with the three opioid receptors: μ (MOR), δ (DOR), and κ (KOR; Waldhoer *et al.* 2004). Despite this sequence similarity, mammalian NOPs display different pharmacological characteristics, since they do not recognize opioid ligands, such as morphine or naloxone (Meunier *et al.* 1995, Reinscheid *et al.* 1995). The endogenous NOP ligand, nociceptin/orphanin FQ (N/OFQ), is a peptide of 17 residues (Meunier *et al.* 1995) which displays high sequence similarity to dynorphin A (DYN A), the prototypical κ peptide. NOP activation has been associated with numerous biological processes, including spinal analgesia, supraspinal hyperalgesia, inhibition of locomotor activity, anxiolytic-like effects, stress, stimulation of feeding, diuresis, antagonism of opioid-induced effects, and depression of the cardiovascular system (Mogil & Pasternak 2001, Waldhoer *et al.* 2004, Chiou *et al.* 2007). Besides, it has been suggested that NOP plays an

important role in many complex processes, such as in learning and memory, attention and emotions, movement and motor processes, homeostasis and neuroendocrine secretion (Meunier 1997). Also, expression analysis has revealed that this receptor shows a widespread distribution in both the central (CNS) and the peripheral nervous system (Mollereau & Mouledous 2000).

To date, several *NOP* genes have been cloned and/or identified from different mammalian species (*Homo sapiens*, human (Mollereau *et al.* 1994); *Rattus norvegicus*, rat (Nishi *et al.* 1993, Fukuda *et al.* 1994, Lachowicz *et al.* 1995); *Mus musculus*, mouse (Pan *et al.* 1996); *Mesocricetus auratus*, golden hamster; *Cavia porcellus*, guinea pig; *Sus scrofa*, pig (Osinski *et al.* 1999); *Canis familiaris*, dog; *Equus caballus*, horse; *Monodelphis domestica*, opossum; and *Ornithorhynchus anatinus*, platypus), chicken (*Gallus gallus*), from three amphibian species (*Taricha granulosa*, rough-skinned newt (Walthers *et al.* 2005); *Rana pipiens*, northern leopard frog (Stevens *et al.* 2007); and *Xenopus laevis*, African clawed frog), and chondrosteian *Acipenser transmontanus*, white sturgeon (McClendon *et al.* 2010).

However, the existence of a functional NOP in teleosts was still unknown.

Our research group has cloned and characterized the opioid receptors from zebrafish (dr: *Danio rerio*): drDOR1, drDOR2, drMOR, and drKOR. Besides, we have also characterized the opioid precursors, namely two proenkephalins, two proopiomelanocortins, a prodynorphin (PDYN), and a pronociceptin (PNOC; for a complete review of the zebrafish opioid system, please refer to [Gonzalez-Nunez & Rodriguez \(2009\)](#)). Interestingly, drPNOC codes for two different nociceptin peptides: drNOC (YGGFIGIRKSARKWNNQ) and drNOC-like (FGGFMKGRHGLRKLVSGRPLQ; [Gonzalez-Nunez et al. 2003](#)). The fact that drNOC contains the classical 'opioid message' -Try-Gly-Gly-Phe- instead of the 'NOP message' -Phe-Gly-Gly-Phe-, and that the synthetic ligand Tyr-nociceptin binds to NOP as well as to κ and μ opioid receptors ([Reinscheid et al. 1996](#), [Lapalu et al. 1997](#)), led us to think that the zebrafish NOP might show high affinity not only for N/OFQ, but also for opiate ligands, especially for the κ agents. This hypothesis was previously postulated by [Danielson et al. \(2001\)](#) in their work with the sturgeon orphanin and by [Walthers et al. \(2005\)](#) in the characterization of the nociceptin receptor from the rough-skinned newt.

Additionally, the teleost zebrafish is a suitable animal model to study development and to gain insight into the molecular mechanisms of some human diseases ([Lieschke & Currie 2007](#)). Besides, it is possible to conduct chemical screenings to establish the *in vivo* effect of novel chemical agents and to perform toxicological investigations ([Zon & Peterson 2005](#)). In addition to this, recent studies have revealed that teleosts are capable of experiencing pain, and a whole issue of the *ILAR Journal* was dedicated to this topic ('Pain and Distress in Fish', vol. 50, 2009). On the other hand, the ethical considerations that arise when using the zebrafish as an alternative model in pain research will stand for a clear advantage as compared with mammalian models.

In the present study, we report the cloning, gene expression, and pharmacological characterization of a NOP from zebrafish (drNOP). Our results indicate that NOP genes have been broadly conserved during the course of vertebrate evolution, although significant changes in their functionality might have occurred.

Materials and methods

Drugs and radioligands

[Leucyl^{3,4,5-3}H]Nociceptin (82.3 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA, USA). Bremazocine (Bre), naloxone (Nx), nor-binaltorphimine (nor-BNI), and porcine DYN A (1–17) (mammalian,

mdYN A) were purchased from Sigma–Aldrich, and nociceptin was purchased from Bachem GmbH (Weil am Rhein, Germany). Zebrafish DYN A (drDYN A) was synthesized as trifluoroacetic derivative by G Arsequell and G Valencia at the Consejo Superior de Investigaciones Científicas (Barcelona, Spain). All other reagents used were of analytical grade.

Animals

Adult zebrafish were obtained from commercial suppliers and kept in aquaria at 25–28 °C with 12 h light:12 h darkness periods of light cycle and fed once a day. Fish were anesthetized with 150 mg/l tricaine methanesulfonate (MS-222, Sigma–Aldrich) in tank water and killed by rapid cervical transection. In all experiments, adequate measures were taken to minimize pain or discomfort, and animals were handled according to the guidelines of the European Communities Council directive of 24 November 1986 (86/609/EEC) and to the current Spanish legislation for the use and care of animals RD 1201/2005 (BOE 252/34367-91, 2005).

Cloning, sequence, and phylogenetic analysis

To clone the drNOP gene, we have followed the standard methodology previously described ([Barrallo et al. 1998](#), [Pinal-Seoane et al. 2006](#)). Oligonucleotides were designed using the Oligo 4.05 Primer Analysis Software (National Biosciences, Inc., Plymouth, MN, USA), DNA sequences were analyzed with Chromas 2.3 (School of Health Science, Griffith University, Australia) software and compared with other nucleotide and/or protein sequence databases using the FASTA or BLAST programs from EMBL and from the NCBI websites (<http://www.ebi.ac.uk/embl/> and <http://www.ncbi.nlm.nih.gov/> respectively). Protein analysis and structure prediction were performed with the SMART server (<http://smart.embl-heidelberg.de>).

The retrieved DNA and protein sequences were aligned with the ClustalW program, setting all the parameters as default ([Thompson et al. 1994](#)). The obtained multiple sequence alignments were then used to construct a neighbor-joining (NJ) tree with Molecular Evolutionary Genetics Analysis (MEGA4.0.2) software ([Tamura et al. 2007](#)) to analyze *P*-distance (calculating the proportion of amino acid differences) with the following parameters: complete deletion and considering a bootstrap value of 1000 replicates. The other settings were given as default by the program. The NJ method has a high degree of accuracy, and it has been previously used to perform the phylogenetic analysis of MOR genes ([Herrero-Turrión & Rodriguez 2008](#)).

Quantitative reverse transcription real-time PCR

Total RNA from whole embryos was extracted using Trizol Reagent (Invitrogen Corporation) following the protocol recommended by the manufacturers. cDNA synthesis was carried out by reverse transcription (RT) of total RNA to cDNA using the Promega Corporation RT kit following the manufacturer's protocol. The cDNA samples were then treated with RNase A (50 ng/μl) during 10 min at 65 °C to avoid RNA contamination of the sample. As cDNA is a DNA–RNA double-stranded hybrid, the RNase A does not act on these molecules. The RNase A was then precipitated using 8 μl of 7.5 M ammonium acetate (30 min incubation at 4 °C and spin at 17 400 *g* for 30 min at 4 °C). In order to eliminate the impurities of the sample as a consequence of these treatments, cDNA was precipitated with 0.5 ml cold absolute ethanol (30 min at –20 °C). The ethanol was discarded, and the cDNA pellet was eluted in diethyl pyrocarbonate-treated water.

The quantification of the PCR products was performed using SYBR-Green Power Master Mix (Applied Biosystems Hispania, Alcobendas, Madrid, Spain) as previously described (Sanchez-Simon & Rodriguez 2008). The amplification of β-actin on 25 ng of the cDNA used in all the experiments was established as the control of cDNA quality. No significant differences were found in the expression of β-actin on the different cDNAs from tissues used. The oligonucleotides used to amplify the *NOP* and β-actin were: *drNOP_F* 5' ccgtctgtcaccggtgaa 3'; *drNOP_R* 5' aagatgcactcgatgct 3'; β-actin_F 5' acgaccagacatcagggag 3'; β-actin_R 5' cctcttgccttgagcctca 3'. The ABI Prism 7300 detection system (Applied Biosystems) was used to amplify *drNOP* and β-actin, with the following conditions: 10 min at 95 °C followed by 36 cycles of 15 s at 95 °C and 1 min at 55 °C. Three PCR reactions were performed for each sample per plate, and each experiment was repeated three times.

The absolute quantitation was achieved through a standard curve. A basic PCR was carried out to amplify the fragment of the transcript of interest (either *drNOP* or β-actin) on zebrafish cDNA with the same pair of primers that were going to be used in the real-time PCR. The fragment was cut from the agarose gel and purified. Serial 1:10 dilutions were made from the purified PCR product, ranging from 10² to 10^{–5} ng/μl. Four out of these eight points were chosen according to their *C_t* values, considering that the perfect amplification is obtained when the difference in the *C_t* values from one dilution to the next is 3.3, which gives a four-point straight line that has a slope of –3.3. The dilutions used in the standard curve of both genes, *drNOP* and β-actin, were 10^{–1}, 10^{–2}, 10^{–3}, and 10^{–4} ng/μl. The number of copies was calculated as previously described (Sanchez-Simon & Rodriguez 2008).

The RT-qPCR results are expressed as mean ± S.E.M. In the analysis of gene expression changes, the mean of transcripts for each tissue was compared with the mean of transcripts of the control tissue (brain) using unpaired Student's *t*-test with Welch correction; *P* ≤ 0.05 was considered statistically significant.

Cell culture and transfection

A 2.6 kb long EcoRI–NotI fragment comprising the complete open-reading frame (ORF) of *drNOP* cDNA was excised from the pZiploc plasmid and ligated into the mammalian expression vector pcDNA3 (Invitrogen). Human embryonic kidney cells (HEK293), obtained from American Type Culture Collection (ATCC CRL-1573, Manassas, VA, USA), were maintained in DMEM supplemented with 10% (v/v) FCS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Gibco-BRL Life Technology, Inc.), at 37 °C in humidified atmosphere containing 5% (v/v) CO₂ in a Forma incubator. The cell line was transfected with the pcDNA3-*drNOP* plasmid using Transfectam reagent (Promega Corp.) according to the manufacturer's instructions. Geneticin (G418, Gibco-BRL Life Technology) was added to a final concentration of 500 μg/ml to obtain cell lines that stably express the *drNOP* transcript. Positive colonies were isolated and tested for *drNOP* mRNA expression by RT-PCR. These colonies were expanded and grown during 3 months before being used in binding studies.

Membrane preparation

Stably transfected HEK293 cells expressing *drNOP* were grown to 80% confluence, harvested in PBS pH 7.4 containing 2 mM EDTA, and collected by centrifugation at 500 *g*. The cell pellets were frozen at –80 °C for at least 1 h and resuspended in 50 mM Tris–HCl buffer pH 7.4 (assay buffer) with protease inhibitors (0.1 mg/ml bacitracin, 3.3 μM captopril, and protease inhibitor cocktail, from Sigma–Aldrich). The cell suspensions were homogenized with a Potter–Elvehjem tissue grinder with teflon pestle in assay buffer, and the homogenates were centrifuged at 2000 *g* for 10 min at 4 °C. The nuclear pellet was homogenized again, centrifuged, and discarded. The two supernatants were combined, homogenized again with the tissue grinder, and the membrane pellet was collected upon centrifugation at 18 000 *g* for 30 min at 4 °C. The crude membrane fraction was resuspended in ice-cold assay buffer with protease inhibitors, and protein concentration was determined by Bradford (Bio-Rad Laboratories).

Saturation-binding assays

A total of 20–25 µg protein was incubated with different concentrations of the radioligand [*Leucyl*-3,4,5-³H] nociceptin for 1 h at 25 °C in a final volume of 250 µl of assay buffer with (0.1 mg/ml) proteinase-free BSA to avoid the adsorption of the radioligand to the walls of the tubes. A total of 2 µM N/OFQ was used to determine nonspecific binding. After incubation, the reaction was stopped by adding 4 ml of ice-cold 50 mM Tris–HCl buffer pH 7.4; the mixture was rapidly filtrated using a Brandel Cell Harvester and washed two times with GF/B glass fiber filters that were presoaked with 0.2% (v/v) polyethylenimine for at least 1 h. The filters were placed in scintillation vials and incubated overnight at room temperature in EcoScint A scintillation liquid (London, UK). Radioactivity was counted using a Beckman Coulter 6500 scintillation counter (Pasadena, CA, USA). All experiments were performed in triplicate and repeated five times.

Competition-binding assays

The following unlabeled ligands were used: N/OFQ (FGGFTGARKSARKLANQ), mDYN A (1–17) (YGGFLRRIRPKLKWQ), drDYN A (1–17) (YGGFMRRIRPKLRWDNQ), Nx, Bre, and nor-BNI. A total of 20–25 µg protein was incubated with different concentrations of unlabeled ligand ranging from 0.3 nM to 10 µM, and using [*Leucyl*-3,4,5-³H]nociceptin as a radioligand (the working concentration was similar to the affinity constant, K_d). Reactions were incubated for 1 h at 25 °C in a final volume of 250 µl assay buffer with 0.1 mg/ml proteinase-free BSA. A total of 10 µM N/OFQ was used to determine nonspecific binding. Experiments were performed as described in ‘Saturation-binding assays’. All experiments were performed in triplicate and repeated three times.

[³⁵S]GTPγS stimulation assays

[³⁵S]GTPγS (guanosine-5'-[γ-thio]triphosphate)-binding assays were conducted as previously described (Befort *et al.* 1996). Briefly, 20 µg protein was incubated in a 50 mM Tris–HCl pH 7.4 buffer with 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% (v/v) BSA, 10 µM GDP, and 0.1 nM [³⁵S]GTPγS in the presence of varying concentrations of activating ligands, ranging from 0.1 nM to 10 µM. The following ligands were used: N/OFQ, mDYN A, and Bre. Reactions were performed in a final volume of 200 µl for 1 h at 30 °C. Nonspecific binding was determined with 10 µM unlabeled GTPγS. Bound and free [³⁵S]GTPγS were separated by vacuum filtration through GF/B glass fiber filters with a Brandel Cell Harvester as described before. Radioactivity was

quantified by liquid scintillation counting. All experiments were performed in triplicate and repeated at least three times.

Data analysis

Specific binding was defined as the difference between total binding and nonspecific binding (measured in the presence of 2 µM for saturation and 10 µM N/OFQ for competition-binding assays, and in the presence of 10 µM unlabeled GTPγS for [³⁵S]GTPγS stimulation assays). Radioligand-binding data were analyzed by computer-assisted nonlinear regression analysis using GraphPad Prism software (San Diego, CA, USA), and K_d , receptor density (B_{max}), inhibition constant (K_i), and mean effective dose (EC_{50}) were obtained for each ligand. In saturation-binding assays, data were fit either to nonlinear function or to the linear transformation (Scatchard plot: bound/free versus bound). The K_i values were calculated using Cheng and Prusoff's equation, which corrects for the concentration of radioligand used in each experiment as well as for the affinity of the radioligand for its binding site (K_d ; Cheng & Prusoff 1973). In all cases, data were fit to the one-site or two-site binding model, and compared by using the nonlinear least-squares curve fitting, which is based upon a statistical *F*-test. [³⁵S]GTPγS stimulation results were fitted to a sigmoidal dose–response curve using the Prism program.

Results

Molecular characterization of drNOP

A full-length cDNA of 2569 bp, named *drNOP* (GenBank accession no. AY148348), was cloned (Fig. 1). Sequence analysis of this cDNA shows an ORF of 363 residues (predicted molecular weight of 40.87 kDa), which is similar to the length of other NOPs (361–370 amino acids). Protein analysis reveals that drNOP shares the common features with other members of the GPCR superfamily (Fig. 1): seven putative transmembrane domains (TM), four consensus sequences for *N*-glycosylation sites (Asn⁵, Asn²⁰, Asn²⁷, and Asn³²) in the N-terminal extracellular domain, two conserved Cys residues (Cys¹¹⁵ and Cys¹⁹³) that can form a disulfide bond between the first and second extracellular loops (ELs), six putative phosphorylation sites on Ser or Thr residues (Ser²³⁷, Ser²⁴², Ser²⁴⁴, and Thr²⁵⁵ in the third intracellular loop (IL3) and Ser³³⁹ and Thr³⁴⁶ in the C-terminal domain), and two potential palmitoylation sites on Cys residues (Cys³²² and Cys³²⁷) in the C-terminal domain.

Using the BLASTn program in the ENSEMBL web server (<http://www.ensembl.org/>), the chromosomal

Figure 1 Nucleotide and predicted amino acid sequences of drNOP. Nucleotides are numbered in the 5'–3' direction, and the residues are shown in one-letter code below the nucleotide sequence. The seven transmembrane domains (TM) are single underlined, the exon–intron boundaries are shaded in gray, and the oligonucleotide sequences used in RT-qPCR experiments are double underlined. (*) Ser and (¶) Thr phosphorylation sites (4/2); (4/2) N-glycosylation sites (4) at the N-terminus; (#) putative palmitoylation sites in Cys at the C-terminus; and (>) disulfide bond between Cys located at the extracellular loops (EL1 and EL2) respectively.

location of *drNOP* gene was assessed in the Zv9 zebrafish genomic assembly. The *drNOP* gene was mapped to 89 039 bp fragment (6024852–6113891) in chromosome 23, which comprises the NW_001878401 genomic clone. As also shown in Fig. 1, the *drNOP* gene is formed in four exons: the first exon is noncoding (227 bp), the second one contains 8 bp of the 5'-untranslated region (UTR) and 209 bp of the coding sequence (Met¹-Arg⁷⁰), the third exon is 358 bp long (Arg⁷⁰-Ser¹⁹⁰), and the fourth exon contains the last 526 bp of the ORF (Ser¹⁹⁰-STOP) and the 1245 bp long 3'-UTR.

Phylogenetic analysis

drNOP protein sequence was aligned to the annotated NOP -orthologs- and opioid (μ , δ , and κ) -paralogs- protein sequences that were retrieved from public databases (Fig. 2). Then, using the NJ method, a phylogenetic tree was created from the multiple sequence alignment (Fig. 3). Four major clusters are evident for NOPs, KORs, DORs, and MORs, which are supported by bootstrap values of 100, 100, 99, and 100% respectively. In addition to this, no significant

differences were found when the trees were constructed with the nucleotide instead of the amino acid sequences (data not shown). Our findings clearly show that drNOP is indeed a NOP ortholog.

Expression analysis of *drNOP* mRNA

The *drNOP* gene expression in different tissues from adult zebrafish was analyzed by direct quantification of the number of *drNOP* transcripts using RT-qPCR techniques (Fig. 4). *drNOP* is expressed at high levels in brain, pituitary gland, and intestine (more than $\sim 3.5 \times 10^5$ copies per 25 ng cDNA), as compared with gills, muscle, and liver (less than $\sim 4 \times 10^4$ copies). Furthermore, low gene expression of *drNOP* was observed in the heart (~ 1300 copies).

Saturation-binding assays of [*Leucyl*-3,4,5- ^3H] nociceptin

To determine whether drNOP binds the N/OFQ peptide, the crude membrane fraction of HEK293 cells stably expressing drNOP was used to perform saturation-binding assays with [*Leucyl*-3,4,5- ^3H]nociceptin. Nonspecific binding was determined in the presence of 2 μM unlabeled N/OFQ. As it can be observed in Fig. 5, radiolabeled N/OFQ displays one single binding site in drNOP with a K_d of 0.20 ± 0.02 nM and a B_{max} of 1703 ± 81 fmol/mg protein. The Scatchard transformation also confirms the presence of one single binding site for N/OFQ on this receptor (Fig. 5, inset). As a negative control, the crude membrane fraction of untransfected HEK293 cells was tested in saturation-binding experiments with [*Leucyl*-3,4,5- ^3H]nociceptin; as expected, no specific binding was observed.

Competition-binding assays using opioid ligands on drNOP

Competition-binding experiments with [*Leucyl*-3,4,5- ^3H]nociceptin were carried out to establish whether opioid ligands were able to bind to drNOP

and thus to displace the bound N/OFQ. Nonspecific binding was determined in the presence of 10 μM unlabeled N/OFQ. Besides, a homologous displacement with N/OFQ was performed in parallel as a positive control. All tested ligands displaced [*Leucyl*-3,4,5- ^3H]nociceptin binding, yet with different affinities (Fig. 6), and in all cases, the experimental data fitted better to the one-site displacement model. Interestingly, drDYN A showed higher affinity for drNOP than N/OFQ itself, whereas the mDYN A peptide, the κ -selective antagonist nor-BNI, and the non-selective ligand Bre competed efficiently, albeit with reduced affinity when compared with N/OFQ. The affinity of Nx was two rank orders lower than the K_i value obtained for N/OFQ. Besides, Nx leaves some residual [*Leucyl*-3,4,5- ^3H]nociceptin binding that could not be effectively displaced even at the highest concentration (maximal displacement, as determined in the presence of 10 μM Nx of $79.15 \pm 2.75\%$).

GTP γ S-binding assay

To determine whether drNOP is functionally coupled to the heterotrimeric G proteins, [^{35}S]GTP γ S stimulation analysis was performed using N/OFQ as well as using opioid ligands. As shown in the Fig. 7, N/OFQ itself was able to stimulate [^{35}S]GTP γ S binding with a EC_{50} of 87.84 ± 18.61 nM and a percentage of maximal stimulation of $85.69 \pm 7.21\%$. The opioid ligands mDYN A and Bre show EC_{50} values in the nanomolar range, but they could only activate G-protein coupling up to 55%, thus behaving as partial agonists.

Discussion

We have cloned and characterized a NOP from zebrafish (drNOP), which displays a high degree of sequence identity to other vertebrate NOPs cloned so far: 58–59% sequence identity to mammalian NOPs (Nishi *et al.* 1993, Fukuda *et al.* 1994, Mollereau *et al.* 1994, Lachowicz *et al.* 1995, Pan *et al.* 1996, Osinski *et al.* 1999) and 64% sequence identity to amphibian NOPs

Figure 2 ClustalW Multiple Sequence Alignment of NOP and opioid receptor (KOR, DOR, and MOR) sequences. The amino acid sequences from the following species were used: (I) Teleosts: zebrafish (drKOR, NM_182886.1; drDOR1, NM_131258.3; drDOR2, NM_212755.1; and drMOR, NM_131707) and *Catostomus commersoni*, white sucker (ccMOR, Y10904). (II) Amphibians: *Taricha granulosa*, rough-skinned newt (tgNOP, AY728087; tgKOR, AY725197.1; tgDOR, AY751785.1; and tgMOR, AY751784), *Xenopus laevis*, African clawed frog (xlNOP, AY7244474), and *Rana pipiens*, northern leopard frog (rpNOP, AY434690; rpKOR, AF530573.2; rpDOR, AF530572.1; and rpMOR, AF530571). (III) Birds: *Gallus gallus*, chicken (ggNOP, XM_417424.2; ggKOR, XM_426087.2; ggDOR, XM_427506.2; and ggMOR (Herrero-Turrión & Rodríguez 2008). (IV) Mammals: *Rattus norvegicus*, rat (rnNOP, NM_031569.2; rnKOR, NM_017167.2; rnDOR, NM_012617.1; and rnMOR, NM_013071), *Mus musculus*, mouse (mmNOP, AK079529; mmKOR, NM_011011.1; mmDOR, NM_013622.3; and mmMOR, NM_011013), and *Homo sapiens*, human (hsNOP, NM_000913.3; hsKOR, NM_000912.3; hsDOR, NM_000911.3; and hsMOR, NM_000914.2). Transmembrane domains are boxed, and intracellular/extracellular loops and N-/C-terminal domains are also indicated. Filled triangles, highly conserved residues in GPCR superfamily; Φ , NOP residues known to influence ligand-binding affinity; Φ , amino acids which are involved in κ -ligand recognition; dots, identical residues to drNOP; dashes, sequence gaps. For further details about the importance of those amino acid residues which are shaded (from #1 up to #6), please refer to Table 1. TM, transmembrane domain; IL, intracellular loop; EL, extracellular loop.

Figure 3 Phylogenetic analysis generated by the NJ method using MEGA4 (Tamura *et al.* 2007) from the multiple sequence alignment shown in Fig. 2. Whole numbers indicate bootstrap values of 1000 replicates, and branch distances are given in decimal numbers when $> 50\%$. Two zebrafish somatostatin receptors (drSSTR1a and drSSTR1b, XM_691574 and XM_680740) were used as an outgroup.

(Walthers *et al.* 2005, Stevens *et al.* 2007). On the other hand, the degree of homology to opioid receptors, either from zebrafish (drMOR, drDOR, and drKOR) or from other vertebrates, only reaches 50–55%. In general, the highest level of sequence identity between the different NOP and opioid receptors is found at the TMs and ILs, whereas the N-terminus, EL2–3, and the C-terminus are the most divergent (Waldhoer *et al.* 2004).

The number of exons that contain the entire coding region of *drNOP* (3) and their lengths are relatively similar to those described in other vertebrates (Nishi *et al.* 1993, Bunzow *et al.* 1994, Chen *et al.* 1994, Fukuda *et al.* 1994, Mollereau *et al.* 1994, Wang *et al.* 1994, Wick *et al.* 1994, Lachowicz *et al.* 1995, Pan *et al.* 1996, Osinski *et al.* 1999, Walthers *et al.* 2005, Stevens *et al.* 2007), whereas the introns of *drNOP*, which have variable lengths, are longer than introns of other mammalian NOPs (Pan *et al.* 1996).

Using the NJ method, a phylogenetic tree was constructed with the aligned amino acid sequences from NOPs and opioid receptors (MORs, DORs, and KORs) from different vertebrates (Fig. 3). Somatostatin receptors of zebrafish (drSSTR1a and drSSTR1b), which are paralogs of the opioid receptors, were used

as the outgroup. drNOP is aligned in the same clade as the rest of vertebrate NOPs; hence, this new cDNA/protein corresponds to a NOP ortholog in zebrafish. In particular, NOP sequences are positioned basal to the rest of the clades of opioid receptors, and NOP is the most divergent clade of the opioid/orphanin receptor family.

RT-qPCR gene expression analysis indicates that *drNOP* mRNA is highly expressed in the CNS (brain and pituitary gland). Previous studies have reported the expression of *PNOC* gene in zebrafish brain (Gonzalez-Nunez *et al.* 2003), and high NOP densities in the rat CNS (Mollereau & Mouldous 2000) and in the amphibian (rough-skinned newt) brain (Walthers *et al.* 2005). We also detect high gene expression of *drNOP* in the intestine, which is similar to expression of *NOP* in the rat small intestine (Wang *et al.* 1994). In relation to this, similar results were also reported by Gonzalez-Nunez *et al.* (2003) for *drPNOC*. On the other hand, lower gene expression levels of *drNOP* mRNAs were detected in other peripheral tissues. The presence of drNOP in gills might depict *NOP* expression in the lungs of the tetrapodian rough-skinned newt (Walthers *et al.* 2005) and mammals (Rizzi *et al.* 1999). The fact that low expression levels of *drNOP* mRNA were found

Figure 4 Absolute quantification of *drNOP* expression by RT-qPCR in adult tissues. Bars (mean ± S.E.M.) represent the number of mRNA copies of *drNOP* mRNA in each adult tissue. Zebrafish β -actin was used as a housekeeping gene. For each tissue, the number of experiments represented in this graph was between four and six; ** $P \leq 0.005$; *** $P \leq 0.001$ (unpaired Student's *t*-test with Welch correction).

in the heart is in accordance with the existence of high-affinity [^3H]N/OFQ-binding sites on rat heart (Dumont & Lemaire 1998). Furthermore, it is the first time that NOP expression has been found in muscle and liver cells, although its role in these organs has not been determined yet.

Binding studies allowed us to determine the pharmacological profile of drNOP. Saturation-binding analyses have revealed that [*Leu*yl-3,4,5- ^3H]nociceptin binds to drNOP with relatively high affinity. The affinity constant ($K_d = 0.20 \pm 0.02$ nM) of this single binding site is in the same range as those reported for mammalian NOPs ($K_d = 0.10$ – 0.20 nM; Meng *et al.* 1996, Mollereau *et al.* 1996, Butour *et al.* 1997, Hashiba *et al.* 2002) and for the rough-skinned newt NOP ($K_d = 0.20$ nM; Walthers *et al.* 2005). These results indicate that drNOP affinity for N/OFQ is conserved throughout the evolutionary scale.

Competition-binding experiments indicated that [*Leu*yl-3,4,5- ^3H]nociceptin binding is displaced by N/OFQ as well as by opioid ligands. Remarkably, the K_i value for drDYN A is lower than the one found for N/OFQ in homologous competition-binding assays, thus implying that drDYN A binds to drNOP with higher affinity than N/OFQ itself. mDYN A, Bre, and nor-BNI competed efficiently (K_i values on the nanomolar range), whereas Nx showed reduced affinity for drNOP receptor. A prior study has reported that PDYN-derived peptides displayed relatively high affinity for the rough-skinned newt NOP (Walthers *et al.* 2005). In contrast, mDYN A (1–17) showed moderate ability to compete in equilibrium binding of [^3H]nociceptin in a crude membrane fraction from Chinese hamster

ovary cells expressing the mammalian NOP (Butour *et al.* 1997). However, the combination of four-point mutations in the rat NOP yielded a chimeric receptor that binds both N/OFQ and mDYN A (1–17) with subnanomolar affinity (Meng *et al.* 1996).

To determine whether drNOP was functional, [^{35}S]GTP γ S stimulation assay was used to evaluate the effect of different agonists to promote G-protein coupling. All ligands tested were able to activate drNOP, yet with different potencies and maximal effects. N/OFQ behaved as a full agonist, displaying nanomolar EC_{50} values, while opioid ligands could partially activate drNOP, thus acting as partial agonists. In contrast, mDYN A had no effect in inhibiting adenylate cyclase via mammalian NOP (Butour *et al.* 1997), and to date, no functional results have been reported for amphibian NOPs.

The fact that different NOPs share a common binding profile indicates that the binding pocket is highly conserved among the evolutionary scale. Particularly, five residues within the binding pocket are known to greatly influence ligand affinity: Asp 130 and Tyr 131 in TM3, Phe 220 and Phe 224 in TM5, and Trp 276 in TM6 (human hsNOP amino acid numbering; New & Wong 2002); these residues are well conserved in drNOP. Furthermore, Asn 133 in TM3 and Gln 286 in TM6, which play a pivotal role in signal transduction (Mouldous *et al.* 2000, Kam *et al.* 2002), are also conserved in drNOP (Fig. 2). Furthermore, the hydrophobic cleft between Gly 182 –Gly 189 in TM4 and Gly 212 –Ile 219 (EL2 and TM5), which is located within the transmembrane helix bundle and that interacts with Gly 6 from N/OFQ (hsNOP amino acid numbering; New & Wong 2002), is partially conserved in drNOP. However, Gly 212 , which is present in all mammalian NOPs, is replaced by Asp in non-mammalian NOPs (e.g. drNOP, Asp 205), KORs, DORs,

Figure 5 Saturation-binding analysis of [*Leu*yl-3,4,5- ^3H]nociceptin in membrane homogenates of HEK293 cells stably expressing drNOP. Inset: Scatchard transformation. Data are from a representative experiment that was repeated five times in triplicate.

Figure 6 Competition-binding experiments using [Leucyl-3,4,5-³H]nociceptin on drNOP membrane homogenates. Relative abilities of N/OFQ and opioid ligands to compete with equilibrium binding of radiolabeled N/OFQ. Data were fit to the one-site competition model, and each point represents the mean ± s.e.m. (capped bars) of three independent experiments performed in triplicate. The table summarizes the *K_i* values (mean ± s.e.m., nM) for several ligands obtained from this competition-binding assay.

Ligand	N / OFQ	mDYN A	drDYN A	Nx	Bre	Nor-BNI
<i>K_i</i> value (nM)	7.99 ± 1.02	32.04 ± 9.78	1.62 ± 0.47	1086 ± 177	94.55 ± 9.78	15.88 ± 2.25

and amphibian MORs, and by Glu in the northern leopard frog NOP (rpNOP) and teleost and mammalian MORs. These amino acid substitutions might explain why mammalian NOPs display reduced affinity for DYN A, whereas non-mammalian NOPs are able to bind PDYN-derived peptides with (sub)nanomolar affinities.

The different pharmacological profiles observed for zebrafish and mammalian NOPs could be explained by the existence of amino acid changes in the protein sequences, most of them located in the ELs. Besides, both NOP and KOR discriminate between their corresponding ligands through a general pattern of attractions and repulsions, which is based on the secondary structure of the ELs (Meng *et al.* 1996). The degree of sequence identity at the EL1 between drNOP and mammalian NOP is 87% and between drNOP and mammalian KOR is 46%. Although it seems that EL1 is not directly involved in ligand recognition and/or specificity, EL2 is thought to play a critical role in N/OFQ binding and receptor activation (Mollereau *et al.* 1999, Vincent *et al.* 2008). As shown in Fig. 2, EL2 of drNOP is quite unique, as compared with mammalian NOP and KOR (degree of sequence identity of 36 and 30% respectively). It has been shown that the negative charges in EL2 are essential for ligand–receptor interaction (Reinscheid *et al.* 1996, Dooley & Houghten 2000). In this line, although the sequence Glu¹⁹⁴-Asp-Glu-Glu¹⁹⁷ does not have a direct role in agonist binding *per se*, it maintains the mammalian NOP in its active conformation after agonist binding

(Mouldous *et al.* 2000). In the case of drNOP, although the degree of sequence identity in EL2 is relatively low, the number of acidic residues is conserved, yet these amino acids are more spread across the EL2 domain. Finally, the degree of sequence identity of EL3 between drNOP and mammalian NOP and KOR is fairly low (35 and 21% respectively); however, some residues known to play a critical role in receptor activation are conserved among NOPs, as it is the case of Gln²⁸⁶ in mammalian NOP (Gln²⁷⁹ in drNOP) (Fig. 2; Mouldous *et al.* 2000).

Site-directed mutagenesis studies have yielded receptors with both NOP and KOR characteristics (Meng *et al.* 1996, Mollereau *et al.* 1996, Meng *et al.* 1998). Interestingly, in drNOP and other non-mammalian NOPs, some of the amino acids, which are conserved in other NOPs, are replaced by prototypical κ residues which are involved in κ-ligand recognition (Table 1). For example, the mutation Thr to Ile in the rat NOP increases the affinity for mDYN A in 20-fold (Meng *et al.* 1996). In this case, non-mammalian NOP contains an Ile in the homologous position (#5 in Table 1 and Fig. 2). Additionally, the four-point mutations Val-Gln-Val to Ile-His-Ile (in TM6-EL3) and Thr to Ile (in EL3-TM7) in the rat NOP yielded a dual receptor, as it binds N/OFQ with the same affinity as the wild-type receptor, but it also recognizes opioid ligands (Meng *et al.* 1996). In fact, this mutant receptor displays

Figure 7 [³⁵S]GTPγS stimulation assays on drNOP. Relative abilities of nociceptin and opioid ligands to promote G-protein coupling and [³⁵S]GTPγS binding in crude membrane fraction of HEK293 (drNOP). Data points represent the mean ± s.e.m. (capped bars) of three different experiments performed in duplicate. The table summarizes the EC₅₀ values obtained for several ligands obtained from this [³⁵S]GTPγS stimulation assays and the percentage of maximal [³⁵S]GTPγS stimulation shown at a concentration of 10 μM of the activating ligand.

Ligand	N/OFQ	mDYN A	Bre
EC ₅₀ (nM)	87.84 ± 18.61	47.47 ± 30.76	283.20 ± 119.70
% Max. Stim.*	85.69 ± 7.21 %	55.63 ± 2.49 %	54.17 ± 3.21 %

* Percentage of maximal [³⁵S]GTPγS stimulation shown at a concentration of 10 μM of the activating ligand.

Table 1 Amino acid motifs of non-mammalian NOPs, which are not conserved in mammalian NOPs and which are replaced by prototypical κ residues (*); interestingly, these residues are involved in κ -ligand recognition. For a better understanding of this table, consideration of Fig. 2 is suggested. Moreover, in the analysis of this table the amino acid sequence of the white sturgeon NOP (GenBank accession number GU228526; McClendon *et al.* 2010) has also been included

	Domain	Non-mammalian NOPs	Mammalian NOPs	KORs
#1	EL2	Val/Gln*/Lys	Glu	Gln*
#2	EL2	Asp*/Glu	Gly	Asp*
#3	EL2-TM5	Val*	Ile	Val*
#4	TM6-EL3	Ile/Val-Gln-Ile*	Val-Gln-Val	Val-His-Ile*
#5	EL3-TM7	Ile*	Thr	Val/Ile*
#6	C-terminal	His/Tyr/Phe*	Cys	Cys/Phe*

TM, transmembrane domain; EL, extracellular loop.

increased affinity for PDYN κ -selective peptides, and the affinity shown for mDYN A (1–17) is in the subnanomolar range (Meng *et al.* 1996). As shown in Table 1 (#6 in Table 1 and Fig. 2), non-mammalian NOPs also contain Ile-Gln-Ile (in TM6-EL3) and Ile (in EL3-TM7), as expected in an opioid receptor.

The message–address theory states that opioid peptides can be divided into two clearly distinct domains. The ‘message’ would be the common structural moiety, which is located at the N-terminus, namely ‘Try-Gly-Gly-Phe’; the rest of the peptide would form the ‘address’ (Chaykin & Goldstein 1981). The ‘message’ is where the biological activity of the peptide resides, and hence it binds to the receptor-binding pocket. The ‘address’ would interact with different domains of the ELs, thus determining the affinity and selectivity of the ligand for a given receptor (Metzger & Ferguson 1995). Also, it has been suggested that KOR and NOP derived from the same ancestral gene through a series of duplication and divergence events that run in parallel with the evolution of PDYN and PNOC genes (Dores *et al.* 2002, McClendon *et al.* 2010). Mollereau *et al.* (1999) postulated that N/OFQ and DYN A underwent a coordinated inversion of the ‘message’ and ‘address’ domains, which rendered selective ligands for KOR and NOP. According to this theory, DYN A will exert its activity through a direct interaction of the N-terminal domain with the receptor, whereas it is not the case for N/OFQ. Mollereau *et al.* (1999) established that the biological activity of N/OFQ resides in the positively charged residues located at the C-terminus, instead of in the hydrophobic N-terminal domain. Therefore, the analysis of the pharmacological profile of NOPs in fishes supports the hypothesis of separate ‘message’–‘address’ domains for the opioid and N/OFQ peptides.

Gonzalez-Nunez *et al.* (2003) reported that one of the N/OFQ peptides from zebrafish, drNOC, is highly

similar to mDYN A, and presents the classical ‘opioid address’ (Tyr-Gly-Gly-Phe) at its N-terminus. We hypothesized that drNOC might bind to opioid receptors with high affinity, and that drNOP will recognize and bind opioid ligands with high affinity too. Besides, it has been reported that white sturgeon PNOC-derived peptides are able to bind to the three mammalian opioid receptors (μ , δ , and κ), as well as to mammalian NOP (Danielson *et al.* 2001). Besides, competition-binding assays performed with the rough-skinned newt NOP have revealed that amphibian NOP binds PDYN-derived peptides with broad affinity, although no significant binding was found for other opiate drugs (Walthers *et al.* 2005). The latter result is consistent with the fact that highly-selective ligands for the mammalian opioid receptors (e.g. ([D-Ala², N-MePhe⁴, Gly⁵-ol]enkephalin) – DAMGO, D-Penicillamine(2,5)-enkephalin – DPDPE or [(5 α ,7 α ,8 β)-(+)–N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide] -U69,593-) failed to show effective displacement on amphibians and teleosts, such as zebrafish (Gonzalez-Nunez *et al.* 2006). However, less selective ligands, as Bre or Nx, are able to recognize and displace [³H]nociceptin binding on drNOP, which clearly differs from the published reports for amphibian NOPs (Walthers *et al.* 2005, Stevens *et al.* 2007).

In conclusion, our results clearly show that drNOP recognizes and binds N/OFQ as well as opioid ligands, showing a preference for the κ -selective agents. Hence, drNOP might be a potential evolutionary link between NOP and KOR, and will help to bridge the gap between opiate and NOP pharmacology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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