Cloning, expression and pharmacological characterization of a vasopressin-related receptor in an annelid, the leech *Theromyzon tessulatum*

A Levoye^{*1}, B Mouillac^{*2}, G Rivière^{1,3}, D Vieau^{1,3}, M Salzet¹ and C Breton^{1,3}

¹Laboratoire de Neuroimmunologie des Annelides, UMR 8017 CNRS, SN3, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France

²U469 INSERM, CCIPE, 141 rue de la Cardonille, 34094 Montpellier Cedex 5, France

³Laboratoire de Neuroendocrinologie du Développement, UPRES-EA 2701, SN4, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France

(Requests for offprints should be addressed to C Breton; Email: Christophe.Breton@univ-lille1.fr)

A Levoye is now at Institut Cochin, UMR 8104 CNRS, U567 INSERM, Université Paris V, Département de Biologie Cellulaire, 22 rue Méchain, 75014 Paris, France

*(A Levoye and B Mouillac contributed equally to this work)

Abstract

In annelids, it has been established that arginine-vasopressin (AVP)/oxytocin (OT) superfamily peptides are involved in the maintenance of water and electrolyte homeostasis as well as reproduction. At present, there is little information on their receptors. In this study, we report the characterization of a 1.7 kb cDNA for an AVP-related receptor from the leech *Theromyzon tessulatum*. The open reading frame encodes a 435-amino-acid transmembrane protein that displays seven segments of hydrophobic amino acids, typical of G-protein-coupled receptors. The overall predicted protein exhibits about 30% amino-acid identities to other invertebrate, as well as vertebrate, AVP/OT receptor family members, and displays conserved characteristic features belonging to the AVP/OT receptor superfamily. RT-PCR expression experiments showed that mRNA is expressed in the genital tract, the ovary and the brain. The receptor expression is stage specific, showing a weak expression

after the two first blood meals, increasing dramatically after the last blood meal during the period of sexual maturation and disappearing after egg laying. Thus, the leech AVP-related receptor may mediate reproductive functions. When expressed in COS-7 cells, the receptor binds ligands with the following rank order of potency: AVP = Arg-vasotocin > Arg-conopressin > mesotocin = OT = Lys-conopressin = isotocin > annetocin. This shows an AVP-like pharmacological profile. The transfected receptor mediates AVP-induced accumulation of inositol phosphates, indicating that the leech AVP-related receptor is functional. This study describes the characterization of a novel AVP/OT superfamily receptor in annelids, which are considered the most distant group of coelomate metazoans possessing a functional AVP/OT-related endocrine system.

Journal of Endocrinology (2005) **184**, 277–289

Introduction

The arginine-vasopressin (AVP)/oxytocin (OT) neuroendocrine system is widely distributed in the animal kingdom. In vertebrates, more than 10 related peptides and over 30 related receptors belonging to the G-protein-coupled receptor (GPCR) family have been described from cyclostomes to mammals (Acher 1993, Acher *et al.* 1999). Neurohypophyseal peptides and their receptors can be regarded as having two distinct lineages with divergent physiological activity: the vasotocin-vasopressin line concerned with water and electrolyte balance, and the isotocin-mesotocin-oxytocin

line concerned with reproduction (Darlison & Richter 1999).

In invertebrates, data are scarce. Seven AVP/OT-related nonapeptides have been isolated in different phyla. By the relationships of the precursor primary structure and physiological actions, these peptides may be associated with either AVP or OT lineages, whereas they often control both osmoregulation and reproductive functions (Hoyle 1999). In insects, one brain AVP-like diuretic hormone has been isolated in *Locusta migratoria*. This nonapeptide acts as a neurohormone on Malpighian tubules to enhance water excretion (Proux *et al.* 1987). Although several putative

AVP/OT-related receptors are detected *in silico* in *Drosophila melanogaster* genome databases, no insect receptor of this superfamily has been fully characterized.

In molluscs, a structurally AVP-related peptide termed 'Lys-conopressin' was purified and cloned from the pond snail *Lymnaea stagnalis*. This nonapeptide functions as a neurotransmitter/neuromodulator in both the brain and the male copulatory organs, acting as a vertebrate OT-like peptide in reproduction (Van Kesteren *et al.* 1995a). In the cephalopod *Octopus vulgaris*, two structurally OT-related peptides termed 'cephalotocin' and 'octopressin' have been purified; they act as multifunctional neurohormones that control reproduction, homeostasis and feeding (Takuwa-Kuroda *et al.* 2003). At present, only three functional mollusc AVP/OT receptors have been reported from the *L. stagnalis* genital tract, the so-called conopressin receptors (LSCPR1 and LSCPR2) (Van Kesteren *et al.* 1995b, 2003) and from *O. vulgaris* (CTR) (Kanda *et al.* 2003). In the nematode *Caenorhabditis elegans*, several putative AVP/OT-related receptors are also detected *in silico* in the genome database but without any pharmacological evidence of AVP/OT receptor lineages.

In annelids, which are considered the most distant group of coelomate metazoans possessing a functional AVP/OT-related endocrine system, immunoreactivities with antibodies raised against vertebrate peptides were first detected in the brain of several species of leech, including *Theromyzon tessulatum* (Malecha 1983). Then, an AVP-related peptide that exhibits identical sequence with mollusc Lys-conopressin was purified from *Erpobdella octoculata*. In leeches, this peptide mainly exerts a potent diuretic effect via pulsatory contractions and bladder-shaking movements of the nephridia as well as regulation of reproduction (Fujino *et al.* 1999). In the lumbricid earthworm *Eisenia foetida*, an OT-related peptide termed 'annetocin' has also been characterized (Oumi *et al.* 1994, Satake *et al.* 1999). In annelids, annetocin induces egg laying, which is accompanied by secretion of mucus from the clitellum (Ukena *et al.* 1995). However, little information is currently available on OT/AVP-related receptors in this phylum. This study describes the cloning and pharmacological characterization of an AVP-related receptor in an annelid, the leech *Theromyzon tessulatum*.

Materials and Methods

Animals, dissections and surgical procedures

Rynchobdellid leeches of the species *T. tessulatum* were reared under laboratory conditions, as previously described (Malecha 1983). Their life cycle is subdivided into stages defined by taking as indicators the three blood meals (stage 0, from hatching until the first blood meal, and stages 1–3, after respectively the first, second and third blood meals). Despite high blood volume absorption, after the first and the second meals, total body water content is low and

undergoes little change. These stages are characterized by important diuretic mechanisms. Stage 3 is subdivided into substages (A–F) defined by the degree of sexual maturation and reproductive functions. In short, soon after the third and last meal corresponding to stage 3A, spermatogenesis and ovogenesis occur during stages B and C–D respectively. These stages are characterized by male and female genital tract maturation as well as intake of water at stage 3B, followed by water retention until the end of the life cycle. After egg laying and hatching maternal care that correspond to stage 3E, the animals die, representing stage F. After anaesthesia with chloretone, the animals were pinned flat, ventral side up, in leech Ringer's solution. For RNA extraction, excised tissues were immediately frozen in liquid nitrogen and stored at -70°C before use.

RNA preparation, RT-PCR and rapid amplification of cDNA ends (RACE) cloning

Based on leech physiological data, receptor cloning was performed with enriched central nervous system and genital tract tissues isolated from 3A–D stage animals. Total cellular RNA was extracted with Trizol reagent (Gibco BRL, Strasbourg, France), according to the manufacturer's instructions. An amount of 3 μg RNA was converted into single-stranded cDNA with 200 U MMLV reverse transcriptase, primed with 3 μg random primers (Life Technologies, Cergy Pontoise, France) in a 50 μl solution containing 50 mM KCl, 50 mM Tris-HCl, 10 mM MgCl_2 (pH 8.3) and 2 mM each of dNTP and 20 U RNasin. The reaction mix was incubated for 1 h at 37°C . Alignment of AVP/OT superfamily receptors allowed us to design two degenerate oligonucleotides, forward TGCTGG(GT)CGCCITTC(TT)(GC)TI(GT)(CT)ICAGATGTGG and reverse (GT)A(GT)CCA(GC)GGTTTC(AG)TGCAGCTGTTTCAG, directed to transmembrane domain (TM) 6 and TM 7 conserved regions respectively. The first-strand synthesis reaction (1:6) was amplified for 35 cycles with 1 U Taq Polymerase (Eurogentec, Liège, Belgium) and 100 pmol of each forward and reverse primer, as previously described (Breton *et al.* 1995). The cycling parameters were: 94°C for 90 s, 42°C for 90 s and 72°C for 120 s. Negative control RT-PCR reactions were performed by omitting reverse transcriptase or RNA from the reaction mixture. After amplification, the samples were separated on 2% agarose gel and revealed by ethidium bromide staining. PCR products of the expected size were subcloned into pGEM-T easy vector (Promega) and sequenced with an automated DNA sequencer (ABI PRISM 377 DNA sequencer; Applied Biosystems, Courtaboeuf, France) and the ABI PRISM Original Drhodamine Terminator Cycle Sequencing Kit (Applied Biosystems). Among the mixture of amplified cDNAs, one fragment displaying greatest sequence homologies with AVP/OT receptor was used to design forward- and reverse-specific primers. In order to

obtain a complete sequence, 3'- and 5'-RACE were performed with the SMART RACE cDNA amplification kit (Clontech), as described by the manufacturer.

RT-PCR expression analysis

Male and female stage-specific animal tissue total RNA was extracted and used as a template for semiquantitative RT-PCR assay. The method, indicated above, has been previously described and validated (Breton *et al.* 1995). To exclude the possibility that a positive signal was due to contamination with genomic DNA during RNA preparation, the PCR was also performed without preincubation with reverse transcriptase or with ribonuclease-free deoxyribonuclease I-treated total RNA, showing similar results. Primers specific for leech AVP-related receptor and actin were used to amplify tissue cDNA. For the receptor amplification, a sense oligonucleotide 5'-GTT GCTATGTACGCGTCGTCTTACA-3' and an antisense oligonucleotide 5'-CCTTCACCGCTTCGTTAT TCTCGCA-3' were designed to amplify a 359 bp cDNA fragment (corresponding to residues 133–254). As control for amplification, a sense oligonucleotide 5'-CCCTGAAG AACATCCAGTCC-3' and an antisense oligonucleotide 5'-CGAGAGATGATGAAGCAGCA-3' were designed to amplify a 419 bp actin mRNA (residues 59–199). The *T. tessulatum* actin cDNA sequence was kindly provided by Dr Christophe Lefebvre. It was cloned in our laboratory and served as control of amplification (the sequence is available at the GenBank Database, accession No. CK640381). The cycling parameters were as follows: 94 °C for 90 s, 60 °C for 90 s and 72 °C for 120 s. Amplification of the receptor and actin cDNA was carried out for 35 and 22 cycles respectively. PCR amplification products were sequenced to verify the specificity of the amplification. All experiments were performed in triplicate with similar results.

Preparation of expression plasmid and transient transfection of COS7 cells

A sense oligonucleotide 5'-CGGGATCCCGGTATA TCAGCACATCACTATTTACGACGTGA-3' located 72 bp upstream of the ATG codon and an antisense oligonucleotide 5'-CCCAAGCTTGTACAACAAAGTA GTTTAATTTAGTGCAGAAT-3' centred 30 bp downstream of the stop codon were designed to amplify the full-length cDNA encoding the leech AVP-related receptor. At the 5'-end, the primers contained a recognition sequence for the restriction endonucleases EcoRI and BamHI respectively. Oligo(dT)-primed cDNA was synthesized with SuperScript Plus transcriptase and amplified for 30 cycles, using 2 U of proofreading pfu Taq, as described by the manufacturer (Promega). The cDNA fragment was digested with restriction enzymes and ligated into a eukaryotic expression vector already used to sub-

clone rat and human AVP and OT receptors (Mouillac *et al.* 1995). The entire PCR product was sequenced twice in both strands to verify absence of amplification errors, thus confirming the sequence obtained by RACE procedures. Expression plasmid DNA was prepared for transfections by the Plasmid Maxi Kit (Qiagen) procedure. The receptor was transiently expressed in COS-7 cells by electroporation, as previously described (Mouillac *et al.* 1995, Breton *et al.* 2001). Briefly, cells were suspended in electroporation buffer (10^7 cells per 0.3 ml) and incubated with plasmid DNA (20 µg carrier DNA and 2 µg expression vector containing the cDNA insert) for 10 min at room temperature before being pulsed (280 V, 950 µF, GeneZapper system, Kodak Scientific Imaging). After electroporation, cells were plated in Petri dishes and grown in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker, Cambrex Bio Science, Emerainville, France) supplemented with 10% fetal calf serum, 1 MEM non-essential amino acids (Invitrogen), 500 units/ml penicillin and streptomycin each, and 0.25 µg/ml amphotericin B in an environment containing 95% air and 5% CO₂ at 37 °C. Transfected cells were harvested 48 h after electroporation, and membranes were prepared. Cells were washed twice in PBS without Ca²⁺ and Mg²⁺, Polytron-homogenized in lysis buffer (15 mM Tris-HCl (pH 7.4), 2 mM MgCl₂ and 0.3 mM EDTA) and centrifuged at 800 *g* for 5 min at 4 °C. Supernatants were recovered and centrifuged at 44 000 *g* for 20 min at 4 °C. Pellets were washed in Buffer A (50 mM Tris-HCl (pH 7.4) and 5 mM MgCl₂) and centrifuged at 44 000 *g* for 20 min at 4 °C. Membranes were suspended in a small volume of Buffer A, and protein content was determined by the Bradford method (BioRad), using bovine serum albumin (BSA) as the standard. Aliquots of membranes were used immediately for binding assays or stored at -80 °C.

Radioligand binding assays

The peptides Arg-vasopressin, Arg-vasotocin, Arg-conopressin (conopressin G), Lys-conopressin (conopressin S), OT, mesotocin and isotocin were obtained from Bachem, Voisins-le-Bretonneux, France. Annetocin was synthesized by Drs Maurice Manning and Ling L Cheng (Medical College of Ohio, Toledo, OH, USA) and characterized by HPLC and MALDI mass spectrometry. [³H]AVP (60–80 Ci/mmol) was purchased from NEN-PerkinElmer, Courtaboeuf, France. The leech AVP-related receptor binding assays were performed at 30 °C, using [³H]-AVP as the radioligand and 15–20 µg membrane proteins in standard radioligand saturation and competition binding assays, as previously described (Mouillac *et al.* 1995, Breton *et al.* 2001). Briefly, membranes were incubated in Buffer A supplemented with 1 mg/ml BSA (binding buffer) and with radiolabelled and displacing ligands for 30 min. Affinities (*K_d*) for

[³H]AVP (concentrations 0.1–20 nM) were directly determined in saturation experiments. Affinities (K_i) for the unlabelled ligands were determined by competition experiments using [³H]AVP (1–2 nM) as the radioligand. The concentration of the unlabelled ligands varied from 1 pM to 10 μ M. In saturation and competition experiments, non-specific binding was determined by adding 10 μ M unlabelled AVP. Bound and free radioactivities were separated by passing over Whatman GF/C filters presoaked in 10 mg/ml BSA solution for 3–4 h. The ligand binding data were analysed by non-linear least-squares regression by the computer program Ligand (Elsevier-Biosoft, Cambridge, UK). Unless otherwise indicated, all assays were performed in triplicate on at least three separate batches of electroporated cells.

Inositol phosphate assays

The accumulation of inositol phosphates was determined as described earlier (Mouillac *et al.* 1995, Breton *et al.* 2001). Briefly, COS-7 cells transfected with the leech AVP-related receptor were plated and grown in 12-well clusters for 24 h in Dulbecco's modified Eagle's supplemented medium and then labelled with L-*myo*-[1,2-³H]inositol (65 Ci/mmol; PerkinElmer) at a final concentration of 1 μ Ci/ml in a serum-free, inositol-free medium (Invitrogen). Cells were washed twice in PBS medium, equilibrated at 37 °C in PBS medium for 1 h, and then incubated for 20 min in PBS medium supplemented with 10 mM LiCl. COS-7 cells were then stimulated for 20 min with increasing concentrations of AVP (from 10^{-12} to 10^{-5} M) or with a maximal concentration of other natural peptide hormones (10^{-5} M). The reaction was stopped by adding ice-cold perchloric acid 5%. After neutralizing of the samples, total inositol phosphates were extracted and purified on anion-exchange chromatography columns (Dowex AG 1-X8, formate form, 200–400 mesh; Bio-Rad). For each sample, a fraction containing total inositol phosphates was collected and counted. An EC_{50} constant corresponding to concentrations of AVP leading to half-maximum accumulation of inositol phosphates was calculated by non-linear regression analysis with a sigmoidal dose–response equation (Prism4, GraphPad Software, Macintosh, GraphPad Software Inc., San Diego, USA). All assays were performed at least in triplicate unless otherwise indicated from three separate batches of electroporated COS-7 cells.

Results

Cloning of the leech AVP-related receptor

With degenerate primers, a 126-bp cDNA fragment has been amplified from *T. tessulatum* brain- and genital tract-enriched RNA. This sequence exhibited a maxi-

mum 68% identity with the human V1b receptor amino-acid sequence as well as characteristic features belonging to the AVP/OT receptor superfamily. Using 5'- and 3'-specific primers and RACE strategy, we were able to clone about 1700 bp of the leech AVP-related receptor. This includes 216 bp of 5'-untranslated and 123 bp of 3'-untranslated sequence. Taken together, the unique 3' poly(dT) primer used for RACE technique and the presence of a canonical polyadenylation signal suggest that the complete 3'-end has been cloned. The cDNA contains an open reading frame (ORF) of 1305 bp encoding a 435-amino-acid protein with a predicted molecular mass of 50.3 kDa (Fig. 1). The ORF is preceded by an in-frame stop codon at position –186, indicating that the coding region is complete at the 5'-end. Although an in-frame ATG sequence is also present at position –126, the ATG at position 1 is suggested to be the translation initiation site since it best fits both the GCC(A/G)CCATGG Kozak consensus sequence and the predicted peptide signal position. Hydrophobicity analysis of the deduced amino-acid sequence indicates the presence of seven putative transmembrane-spanning regions that are characteristic of GPCR (Fig. 2). As indicated in Fig. 1, this sequence displays several other characteristics of GPCR. Although no potential N-linked glycosylation (Asn-X-Ser/Thr) sites were present, several putative phosphorylation sites were predicted. These sites include 10 serine, 4 threonine and 3 tyrosine phosphorylation sites, such as consensus sequences (i) for phosphorylation by protein kinase C (Thr/Ser-X-Arg/Lys) in the second (positions 159–161) and third (positions 286–288, 321–323 and 348–350) intracellular loops, (ii) for protein kinase A (Arg-X-Ser/Thr) (positions 354–356), (iii) for casein kinase II (Thr/Ser-X-X-Asp/Glu) (positions 349–352) and (iv) for cAMP-dependent protein kinase (Arg/Lys-Arg/Lys-X-Ser/Thr) (positions 259–262) in the third intracellular loop.

Structure of the leech AVP-related receptor

At the amino-acid level, the entire ORF exhibited about 30% identities with other invertebrate and vertebrate AVP/OT receptors. The N-terminal extracellular domain shows hardly any sequence homology. However, surprisingly, sequences including TM and extracellular loops exhibit the closest similarity with vertebrate AVP/OT receptors. Among these regions known to be crucial for ligand binding, sequences comparison analysis reveals over 65% amino-acid identities with vertebrate AVP as well as vasotocin receptors, but it displays only 40% identities with the three mollusc receptors. As shown in Fig. 3, the third intracellular loop is considerably longer than the vertebrate as well as invertebrate receptors described so far, containing 55 amino-acid residues more than mollusc receptors (Van Kesteren *et al.* 1995b, 1996, Kanda *et al.* 2003). In addition, a cysteine doublet after TM 7 that may be modified by palmitoylation and may thereby anchor the

act ggg cat aaa atg ttc **aat aaa** ttt tta ttc cat ttc (a) n 1431

cytoplasmic tail in the lipid bilayer is absent as well as the C-terminal domain corresponding to the cytoplasmic tail. Further analyses also reveal the presence of the most

Journal of Endocrinology (2005) **184**, 277–289

Figure 2 Hydropathicity profile of the leech AVP-related receptor. Numbers below the figure indicate amino-acid residue positions in the leech AVP-related receptor. The seven hydrophobic regions that represent putative transmembrane domains (TM) are shown in black. Putative transmembrane helices are numbered by the convention used with G-protein-coupled receptor family members.

In Fig. 3, amino-acid residues that have been suggested to contribute to the ligand-binding domain are indicated by an asterisk (Howl & Wheatley 1996, Barberis *et al.* 1998). For example, this sequence displays a unique stretch of seven amino acids (Phe-Asn-Ile-Leu-Pro-Gln-Leu) at the end of TM 2 similar to the one found in mollusc receptors. The differences that are observed with vertebrate receptors involve only conserved substitutions (thus, Gln-Val is replaced by Asn-Ile). In the first intracellular loop, where all vertebrate receptors have Gly-Pro-Asp, this receptor has Asn instead of Pro. In the conserved region Asp-Cys-Trp-Ala of the second extracellular loop, Asp is replaced by Asn. Gln residues located in positions 1 (TM 2), 2 (TM 3), 3 (TM 4) and 4 (TM 6) are highly conserved in all AVP/OT receptors and are thought to be important for the agonist-binding pocket. Moreover, in the first and the second extracellular loops, two cysteine residues are conserved, suggesting that the two loops may be linked together by a disulphide bridge and form a putative ligand-binding pocket crucial for neurohypophyseal peptide affinity (Mouillac *et al.* 1995, Peter *et al.* 1995, Barberis *et al.* 1998). Therefore, and because of its amino-acid identities with the AVP/OT receptor superfamily, the predicted protein was named 'leech AVP-related receptor'.

Expression of the leech AVP-related receptor

To investigate the developmental expression of the leech AVP-related receptor, total RNA was extracted from leeches at different developmental stages defined on the basis of feeding pattern and reproductive activity (Malecha *et al.* 1986). Because leech AVP-related receptor mRNA is hardly detectable by Northern blot analysis, due to the low number of animals available, we performed semi-quantitative RT-PCR analysis. As shown in Fig. 4A, a faint signal was observed after the first (stage 1) and second (stage 2) blood meals. However, soon after the third and last blood meal (stage 3A), receptor mRNA level increased dramatically and remained high during most of the time occupied by spermatogenesis (stage 3B) and ovogenesis (stage 3C). A high expression level was still maintained thereafter during the final stage of ovogenesis (stage 3D). In contrast, the transcript gradually disappeared after egg laying (stage 3E) and returned to a barely detectable level when the animal died (stage 3F). These findings correlate well with the maturation of reproductive organs as well as reproduction processes, with an important increase of receptor expression. We have thus performed RT-PCR experiments using total RNA (stage 3C) from anterior, medium and posterior parts of animals and from ovary, testis (female and male genital tract) and brain. As shown in Fig. 4B, our analysis revealed that receptor transcripts were present in all tissues in different abundance. The medium part containing most of the mature female reproductive tract (ovary and oviduct), the posterior part and the dissected female genital tract showed the more intense signal, suggesting that this receptor may mediate reproductive functions. A weak PCR signal was also identified in the anterior part that does not contain genital organs and in the brain, suggesting that this receptor may have additional functions. This receptor is probably involved in the central regulation of reproduction.

Pharmacological properties of the leech AVP-related receptor expressed in COS-7 cells

Because the leech AVP-related receptor possesses some of the hallmark residues of the AVP/OT family of GPCRs as well as some conserved residues involved in the binding of the neurohypophyseal hormones to their respective receptors, we investigated its pharmacological properties by transient expression in the heterologous COS-7 cell system. Thus, the receptor cDNA was subcloned into a

Figure 3 Alignment and comparison of the amino-acid sequences of the leech AVP-related receptor and receptors of the AVP/OT family. The amino-acid sequence of the *T. tessulatum* receptor (leech) is aligned with those of the *Lymnaea stagnalis* Lys-conopressin receptors LSCPR1 and 2, the *Octopus vulgaris* cephalotocin receptor CTR, the chicken AVT (AVT-Chic) receptor and the human (Hum) V1a and OT receptors. Alignment was carried out with ClustalW. Amino-acid residues that are identical in all receptor proteins are shown in black boxes. Asterisks indicate amino-acid residues that are highly conserved in the AVP/OT receptor superfamily, but not in other G-protein-coupled receptors, and that have been suggested to participate and to be important in receptor-ligand interaction. Bars indicate the seven putative transmembrane domains (TM 1–7).

Leech MLALAFVGNLKVI GLLYSTRYCNSYGRNNANINGKNTFSKNHKRTDAE
LSCPR1 MDLELNLNTNGFMLMPPTKRAVIQIASNEKVG YDVSQPVNAT TDLNT TRMNA TNSTEVS YFHGVDE
LSCPR2 MRPPTS VF IANVS SVKDP TAASPEINLADWRI GQDNA TNKTLTG RDE
CTR MRYITTHPNEISTQIWN NFSSTEIWSNFSAAKNETQPIRRNQ-
AVT-chic MKNFS FPMQDSTHQTESP PPHRLLSLTNKS DPVGRPERDE
Vla-hum MRLSAGPDAGPS GNS SPWWPLATGAGNTS REAEALGEGNG PPRDV RNE
OT-hum MEGALAA NW SAE AANA SAA PPGAEGNRTAGFPRRNE

TM1 **TM2**
Leech -IVRFETPRRFGNENFGPANNFLVPKAYSGSRRLR-LTRMNYMI AHITLTFMFVAQFNILPOLIWDITTEFRYGNDAICR
LSCPR1 DLLKTEIAVQATILYMTLFGNGIVLLVLR-LRRQK-LTRMQWFI AHIAFADIFVGFENILPOLISDVITIVFHGDEFTCR
LSCPR2 SLARTEILVQSI ILALAIIGNSCVLTALA--RRGKAASRMHLFIHLSIADLLVAVENILPOLIWDITTEFRYSGDLICR
CTR DLANAEVITLAVVIIITVIGNSIVLITLF-ORRKK-LTRMHLFIHLSVTDLFVAFENNPQLIWDITTEFLGLDILICR
AVT-chic QLAQVEIAVLGVIFLTASVGNFILILVLW-LRRKK-LSRMYVFMHLSIADLVVAFQVLPOLIWDITTEFRYSGDLICR
Vla-hum ELAKTEIAVLAVTFAVAVLGNSVLLALH--RTPRKTSRMHLFIHLSLADLAVAFQVLPQMCWDITTEFRYSGDLICR
OT-hum ALARVEIVAVLCLILLLALSGNACVLLALRTTRQKHS--RLFFFMKHLSIADLVVAFQVLPOLIWDITTEFRYSGDLICR
* ***** ***** ***

TM3 **TM4**
Leech SVTYIQLVAMYASSYIIIVATGMDRYRAICQFFNSIKWSARS SNSL-VTIAWGLSMFFSVPTIIFSYRLD--DRNEMNC
LSCPR1 LATPEPDGMQAYITWVFVANYVI-PFLLLAFCYGRICHVVMMSVAAKESAAYSSMRNGCTESSRPIKMRISFHRNRDN
LSCPR2 YIKFNOVYVMYLSYVYMLVMTADVRYRAVCHPLGAFNTSTRTPLYCMIVSAYVISGVLSLPQPIIFKYREKSHGSGDYEC
CTR LVTYIQSVAMYASSYVLVATAIDRYFAICHP LSSHKTARVHVM-VFIAWMLSFLFSTPQLFIWSMQFSNIGL--TC
AVT-chic IIKYIQLLGMFASTYMI VVMTVDRYQAVCYPMVTFQKKRALW-NIPICTSWSISLISLSPQVFI FSKIEISPG--IFEC
Vla-hum VVKHIOVFGMFASAMMLVVMTADRYIAVCHPLKTQQPARRS-RLMIAAAWVLSFVLS TPQYVFVFSMI EVNNVTKARDC
OT-hum LVKYLQVFGMFASVYLLLSIDRLAICQPLRLS--RRRTDRLAVLATWLGCLVASAPQVHIFSLREVADGVF--DC
** * * * *

TM5
Leech WAVEPDAITVRIVYVLSALTIIYAI-EVLILLVIYTRLCRAVWLSFCENNEAVKVKNNRKKISLTSYSSSFHITHTFES
LSCPR1 LATPEPDGMQAYITWVFVANYVI-PFLLLAFCYGRICHVVMMSVAAKESAAYSSMRNGCTESSRPIKMRISFHRNRDN
LSCPR2 WGRFEPPTILNIIYITSFTFAVYIV-PLAILI FAYVSICCTIWRKYKSAENERKHM LNSGSSDSSLGNRNIYSNHVTHSALF
CTR QATPEPPTILKHYITWLTVAIWI LPTIALTLFYGM MCFAVWKRGRSTLGSSRTRNRSFLTNRVSTRIGQSHLQAGFSE
AVT-chic WAEFIQPNQPRAYVTWLLVVI-FFIPSTILITCQVKICKIIRNIYVKQNEYQVTNQKQVLP SRASVSN-----
Vla-hum WATEIQPNQSRAYVTWMTGGI-FVAPVILGTCYGFICYNIWCNVRGKTA SRQSKGAEQGVAFQKGFLLAP-----
OT-hum WAVEFIQPNQPKAYITWITLAV-YIPEVIVLATCYGLISFKIQWNLRLK TAAAAAAEAPEGAAAGDGGRVALA-----
** * ***

Leech ILRRKNTAKHLQGNKNADDEHDYERENQLEDVMKIEKVEKRTIKMKLLKSSFTQTSVDTIMS IKTASSSKRNFRYSN
LSCPR1 TN-----ATLTLSDRHDASAVTSSDSKK
LSCPR2 RH-----RGVIERRNRLVQRCRPAPMAAP
CTR ED-----MEGQ
AVT-chic -----
Vla-hum -----CVSVSKS-
OT-hum -----RVSVKLI

TM6 **TM7**
Leech NSKVKNRNSRAKLKTIKLTIIINVSFIICWGPFFVSCWAAAFDPGAPY---EGSTMTHITVLASLNSCNNEFIYFVRN-
LSCPR1 PRGHORGVS KSKNKTIKITLTVVLCYLFCWAPFFVQVWMSAFDDDSGI---EHPVTVICMLIASLNSCTNEMIYLA FSG
LSCPR2 RAHSLRGSRAKLKTVKITEFVVIYAVYVCWSPFFELSDWLYDEQEQEH---NHAVVIMVLLASLNSCCNEMIYLA FSG
CTR SVNYNRGISRAKVRSAITLSV VACCFICWSPFFVQVWMAAWDENAPY---SGAIYITLILSLNSCTNEMIYMI FSV
AVT-chic -----CISKAMIKTVKMTIIVTVVAYVICWSPFFELQLWSVWFPSGIT---EGSAFTIILGLNLSCTNEMIYMYFCG
Vla-hum -----ISRAKIRTVKMTFIVITAYIVCWAPFFELQWMSVWDPM SVWTESENPTIITLALGLNSCCNEMIYMFSG
OT-hum -----SKAKIRTVKMTFIIIVLAFIVCWTPFFVQVWMSVWDANAPK---EASAFIIVMLASLNSCCNEMIYMLFTG
* *

Leech -----
LSCPR1 RTCQRNHNANRNTSRSWGSPSTHTVSGGDSHEFKTRSSFI DLSHARMNARSSSPRFSEMNKLNH SKC-----
LSCPR2 NLI RHCMPMRPNSRNCCCLDRRCRRRQFYRRRSSNNNHDDTHSPLSPS DERIHHRRDGMLDMEIRELIADGINKRK
CTR FQHRAKTSRFVNDDETTSVTVLSRNDIRLMSMKKLEQTARN-----
AVT-chic HIPYCTNKQLENTSAQEDSVVTGSIHLVD RPEENNSTCA-----
Vla-hum HLLQDCVQSPFCQNMKEKFNKEDTDSMSRRQTFYSNNRSPTNSTGMWKDSPKSSKSIKFI PVST-----
OT-hum HLFHELVRFLCCSASYLKGRRLGETSASKNSSSSFVLSHRS SSQRSCSQPSTA-----

Leech -----
LSCPR1 -----
LSCPR2 QPYIKSAPDVTVSKTVTTKTGTPISSTQLVKRESLHKTA VLVQNGRHGLDWT HPLPNLV
CTR -----
AVT-chic -----
Vla-hum -----
OT-hum -----

Figure 4 Expression analysis of the leech AVP-related receptor. (A) RT-PCR analysis of the leech transcript expression at different developmental stages. RT-PCR was performed with either no RNA (negative control (CT), lane 1) or 3 µg total RNA from different stages 1, 2 and 3A–F, as indicated above (lanes 2–9) (upper panel). (B) RT-PCR analysis of leech transcript expression in various sections and organs at stages 3A–D. Lane 1 corresponds to the negative control, in which no RNA was added. Lanes 2–9 represent the amplification signal from the different tissues indicated above (upper panel). For A and B, aliquots of cDNA obtained by reverse transcription were amplified by PCR using the primer pairs described in the Materials and Methods section. As a cDNA control, PCR amplification was also performed with a primer pair specific for *T. tessulatum* actin (lower panel). The sizes of the amplified products are indicated by arrows from number of base pairs on the right. The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized by UV illumination.

eukaryotic expression vector, and cells were transfected by electroporation. The natural peptide ligand for the leech AVP-related receptor being not known in the *T. tessulatum* species, the affinity of various peptide hormones of the AVP/OT family was measured in standard binding assays. AVP, which is available as a tritiated compound, [^3H]AVP, was first tested in a saturation-binding experiment. As shown in Fig. 5, the mammalian peptide hormone bound to the COS-7 cell membranes expressing the receptor with a very high affinity. Indeed, as illustrated in a representative Scatchard plot experiment, an apparent dissociation constant (K_d) for [^3H]AVP of 0.36 nM and a maximal binding capacity of 303 fmol/mg of membrane proteins were calculated respectively. Surprisingly, the invertebrate leech AVP-related receptor binds AVP with an affinity equivalent to the one described for human or rat receptors. In addition, using [^3H]AVP as the radioligand, competition experiments were carried out to determine

Figure 5 Binding of [^3H]AVP to the leech AVP-related receptor: Scatchard representation. COS-7 cells were transfected with the leech AVP-related receptor cDNA and cultured, and membranes were prepared. The protein content of each membrane preparation was determined, and binding of [^3H]AVP was performed as indicated in the Materials and Methods section. The figure shows the Scatchard plot of a representative [^3H]AVP binding experiment. This figure is representative of at least three independent experiments done on three different batches of electroporated cells. In the experiment depicted here, K_d for [^3H]AVP was 0.36 nM, B_{max} (maximum binding capacity) was 0.3 pmol/mg of membrane proteins and the Hill coefficient was 1.01, indicating a single class of binding sites.

the pharmacological activities of the leech AVP-related receptor toward other peptides belonging to the super-family. A series of natural peptide hormones isolated from other vertebrates and invertebrates was tested. As shown in Fig. 6, most of the ligands used in the study were able to displace [^3H]AVP binding. Calculated inhibition constants (K_i) are summarized in Table 1. Peptides containing an arginine residue at position 8, such as Arg-vasotocin or Arg-conopressin, displayed a relatively high affinity, like AVP, in the 1–10 nM range. Peptides, such as OT, mesotocin, isotocin or Lys-conopressin were at least 100 times less efficient than AVP, having a K_i in the 200–400 nM range. Finally, annetocin, isolated from the earthworm *Eisenia foetida*, was almost inactive, having a K_i above µM concentrations. In conclusion, the rank order of potency was AVP = Arg-vasotocin > Arg-conopressin > mesotocin = OT = Lys-conopressin = isotocin > annetocin. Taking all binding studies together, the leech AVP-related receptor displays a pharmacological profile more closely related to mammalian AVP receptors than the other invertebrate receptors described so far.

Functionality of the leech AVP-related receptor expressed in COS-7 cells

To determine whether the leech AVP-related receptor expressed in COS-7 cells is able to couple to G protein and phospholipase C/calcium signalling pathways, as most AVP/OT members of this receptor family do, we

Figure 6 Pharmacological profile of the leech AVP-related receptor: binding of different peptide hormones. COS-7 cells were transfected with the leech AVP-related receptor cDNA and cultured, and membranes were prepared. Binding of the different peptide hormones was performed as described in the Materials and Methods section. For each peptide used in the study, Arg-vasotocin, Arg-conopressin, OT, mesotocin, isotocin, Lys-conopressin and annetocin, a representative competition binding curve is shown. Each curve is representative of at least three independent experiments done on three different batches of electroporated cells. For each peptide, experimental values are indicated, and each displacement curve is best fitted by the equation $B = NS \cdot (B_{\max} - NS) / (1 + ([L]/IC_{50}))$, where B is specific binding, NS is non-specific binding, $B_{\max}$ is the maximal binding capacity and [L] is the concentration of the displacing agent. In this equation, the Hill coefficient is set to 1.

stimulated cells with different agonist ligands and measured the consecutive accumulation of inositol phosphates. As illustrated in Fig. 7A, the mammalian hormone AVP induced a concentration-dependent accumulation of inositol phosphates (eightfold above the basal value with a maximal 10^{-5} M concentration). The EC_{50} for AVP, calculated from the dose-response curve, was 3.13 ± 0.9 nM ($n=3$), a value in agreement with the affinity of the ligand calculated in saturation-binding assays (0.44 nM). This result demonstrated that the leech AVP-related receptor, transfected in a mammalian cell system, is able to couple to mammalian Gq protein and inositol phosphate/calcium signalling and thus is a functional receptor. In addition, as depicted in Fig. 7B, all natural vertebrate and invertebrate peptide hormones of the AVP/OT superfamily for which affinities have been determined were able to stimulate the leech AVP-related receptor. Indeed, Arg-vasotocin (AVT) and Arg-conopressin, when used at 10^{-5} M, were almost as potent as AVP. Mesotocin, oxy-

tocin, Lys-conopressin, isotocin or annetocin, although less potent than AVP, AVT or Arg-conopressin, all stimulated a significant accumulation of inositol phosphates. In terms of efficiency, the rank order of the different peptides seems to be in perfect agreement with the one defined in binding assays, except for isotocin. In conclusion, the leech AVP-related receptor is functional and can be activated by vertebrate and invertebrate AVP-related hormones.

Discussion

In the present paper, we reported, for the first time, the molecular cloning, expression and pharmacological profile of an AVP-related receptor in an annelid, the leech *T. tessulatum*.

A large body of evidence strongly suggests that this primary sequence, so-called leech AVP-related receptor, belongs to the AVP/OT receptor superfamily. First, following database searches, this sequence exclusively

Table 1 Affinity of natural peptides hormones of the leech AVP-related receptor. All values are expressed as the means ± s.e. calculated from at least three independent experiments done on three different batches of electroporated cells. The numbers of independent determinations are given in parentheses.

	Sequence	Inhibition constant (K_i in nM)
Peptide		
Arg-vasopressin*	CYFQNCPRG-NH ₂	0.44 ± 0.06(3)
Arg-vasotocin	CYIQNCPRG-NH ₂	2.77 ± 0.45(3)
Arg-conopressin	CIIRNCPRG-NH ₂	10.7 ± 2.1 (4)
Mesotocin	CYIQNCPIG-NH ₂	282 ± 61 (3)
Oxytocin	CYIQNCPLG-NH ₂	296 ± 119 (3)
Lys-conopressin	CFIRNCPKG-NH ₂	331 ± 110 (4)
Isotocin	CYISNCPIG-NH ₂	444 ± 187 (3)
Annetocin	CFVRNCPTG-NH ₂	4258 ± 963 (4)

*Affinity (K_d) for [³H]AVP was directly determined in saturation experiments. Affinity (K_i) for other ligands was determined in competition binding by displacement of [³H]AVP. Binding of the natural peptide hormones was performed as described in the Materials and Methods. Affinities (K_i values) were determined with the equation $K_i = (IC_{50} \times K_d / [L] + K_d)$, where K_d is the affinity of [³H]AVP for the leech AVP-related receptor, [L] is the concentration of labelled AVP and IC_{50} is the concentration of peptide able to displace 50% of the maximal [³H]AVP binding capacity.

exhibits the highest sequence homologies with the AVP/OT receptor family. Secondly, the leech receptor sequence possesses some of the hallmark residues of the AVP/OT receptor family (Peter *et al.* 1995, Howl & Wheatley 1996, Barberis *et al.* 1998). In addition, RT-PCR genomic analysis revealed the presence of a unique 82 bp intron located between TM 6 and 7 (data not shown). The position of the intron is conserved in all members of the AVP/OT superfamily characterized so far (Hoyle 1999). Finally, the longer third intracellular loop, compared with the vertebrate receptor counterpart, appears to be a characteristic of invertebrate receptor within this AVP/OT superfamily (Van Kesteren *et al.* 1995b, 1996, Kanda *et al.* 2003).

Analysis of the transcript expression also shows that this receptor belongs to the AVP/OT superfamily. We showed that the receptor mRNA level is low during the first and second stages, whereas the expression increases dramatically during the third stage corresponding to the reproductive organs maturation of the adult animal. Although the exact function of the leech AVP-related receptor is unclear, these findings indicate that this receptor plays an important role in the animal's physiology with special reference to peripheral and central reproductive events in which AVP/OT-related peptides are involved (Fujino *et al.* 1999). Our results are in good agreement with the fact that Lys-conopressin and annetocin, when injected in leeches and earthworms, induce rapid egg-laying-like behaviour accompanied by much mucus secretion. Furthermore, leeches in the non-breeding season responded to peptides less conspicuously than those in the breeding season, suggesting that the involvement of peptides in the control of reproductive events is periodical (Ukena *et al.* 1995, Satake *et al.* 1999). These findings also correlate well with the pond snail LSCPR functions that

induce muscle contractions of the vas deferens, thus stimulating the transport of both eggs and semen during copulation and inhibiting the central neuroendocrine cells that coordinate female reproductive behaviour (Van Kesteren *et al.* 1995b, 1996). Moreover, in line with known AVP/OT-related peptide functions, we cannot rule out that the leech AVP-related receptor is expressed in other organs involved in osmoregulation (Salzet *et al.* 1993, Fujino *et al.* 1999).

Finally, the pharmacological characterization convincingly demonstrated that the leech receptor belongs to the AVP/OT receptor family. Interestingly, among various nonapeptides of the AVP/OT superfamily, the leech AVP-related receptor can distinguish ligands having an Arg residue at position 8 from those having another residue at this position. Indeed, ligands with an Arg residue (AVP, Arg-vasotocin or Arg-conopressin) displayed a nanomolar affinity equivalent to that determined for these agonists to vertebrate receptors, whereas the affinity for OT-related ligands (OT, mesotocin or isotocin), Lys-conopressin and annetocin is much lower.

As far as AVP is concerned, the leech AVP-related receptor presents a mammalian AVP-like pharmacological profile (Thibonnier *et al.* 1998). Strikingly, both TM and extracellular loop sequences of the leech AVP-related receptor found to play an important role in the binding of these ligands are more closely related to those of vertebrates than of invertebrates. Among these regions, conserved Gln residues in TM 2, 3, 4 and 6, as well as a Lys residue in TM 3, are crucial (Mouillac *et al.* 1995, Thibonnier *et al.* 2000). These residues are present in the leech AVP-related receptor (Gln108, -131, -185 and -395) except for the Lys in TM 3. A Glu residue in the first extracellular loop (Glu115) is in a position equivalent to that of the Asp/Tyr/Phe residues in the V2, V1a and

Figure 7 The leech AVP-related receptor is a functional G protein-coupled receptor. Myo-[³H]inositol-prelabelled COS-7 cells transfected with the leech AVP-related receptor were first incubated for 20 min at 37 °C in PBS medium supplemented with 10 mM LiCl. The cells were then stimulated for 20 min with increasing concentrations of AVP (from 10⁻¹² to 10⁻⁵ M) or with a maximal concentration (10⁻⁵ M) for other natural peptide hormones. After stopping the reaction with 5% perchloric acid and neutralization of the samples, total inositol phosphates were extracted, counted and expressed as d.p.m./10⁵ cells. For each condition tested, a mean value ± S.E.M. was calculated from at least three different experiments, each done in triplicate. Data were analysed with the software GraphPad Prism, as indicated in the Materials and Methods section. (A) AVP-induced accumulation of inositol phosphates: the dose-response curve presented here was calculated and fitted from three separate experiments. Mean values ± S.E.M. are shown. (B) Comparison of the efficiency of the different peptide hormones: the effect of AVP is compared with that of vasotocin, Arg-conopressin, mesotocin, OT, Lys-conopressin, isotocin or annetocin. Mean values ± S.E.M. are shown, calculated from at least three different determinations.

OT receptors respectively, which have been defined as crucial determinants for binding selective analogues (Chini *et al.* 1995, Ufer *et al.* 1995). The Arg residue at position 8 in the AVP molecule has been demonstrated to interact directly with these residues in the first extracellular loop.

AVP in the leech AVP-related receptor could interact with the Glu115 in a similar way (ionic bond between Glu and Arg8) as in the V2 receptor (presence of a negative charge; Asp residue), explaining such a high affinity for this hormone. An aromatic cluster in TM 6 (Trp388, Phe391, Phe392), involved in discriminating between agonists and antagonists, is also present (Phalipou *et al.* 1999, Cotte *et al.* 2000). In the N-terminal part of the AVP/OT receptors, a conserved Arg residue (for example, Arg46 in the human V1a receptor) has been demonstrated to be necessary for agonist binding (Hawtin *et al.* 2002). Such an Arg residue (Arg44) is also present in the leech receptor. Altogether, these residues could define a binding pocket adapted to AVP.

It is interesting to note that Arg-conopressin and Lys-conopressin, which both display a positively charged residue at position 8 (Arg and Lys respectively), do not bind equivalently the leech AVP-related receptor (10⁻⁷ vs 331 nM). Presumably, the guanidinium group of Arg is better for interacting with Glu115. Moreover, one could also explain this significant difference by the presence in these two peptides of a variable residue at position 2. Indeed, Ile is found in the Arg-conopressin and Phe is present in the Lys-conopressin. According to the three-dimensional models developed for AVP receptors (Mouillac *et al.* 1995, Fanelli *et al.* 1999), this part of the peptide ligand should be embedded deeply in the receptor TM cleft. It would have to be demonstrated that Ile is better docked into this binding pocket than Phe. Thus, further structure/function analysis of leech functional domains should provide valuable information for understanding the molecular determinants responsible for the ligand-binding pocket.

Expression of the cDNA in COS-7 cells and measurement of AVP-induced accumulation of inositol phosphates confirmed that the AVP-related receptor is fully functional. The increase of AVP as well as other AVP/OT-related peptide hormones in inositol phosphate turnover suggests the coupling of the leech AVP-related receptor to the phospholipase C/IP3/intracellular calcium signalling pathway, as is the case for most mammalian receptors of this family.

To date, the leech AVP-related receptor is the only invertebrate receptor of the AVP/OT family able to bind AVP. It is noteworthy that there are only a few peptide-binding GPCR that have been shown to have high pharmacological conservation over such a long evolutionary distance (Darlison & Richter 1999, Hoyle 1999). Thus, why the leech AVP-related receptor displays such a high affinity for AVP or vasotocin, and whether this property is related to the parasitism and/or aquatic physiology of this particular annelid, are still open questions. However, the leech receptor's resemblance to vertebrate AVP and vasotocin receptors does not seem to be solely related to the parasitic existence of *T. tessulatum*, because AVP-related receptors with similar

features are present in other non-parasitic annelids (data not shown).

As described in molluscs (Van Kesteren *et al.* 1995b, 1996, Kanda *et al.* 2003), we speculate that there are at least two different receptors also present in annelids that might fulfill more than one physiological function. This hypothesis is supported by several arguments. First, annetocin, F3-annetocin and Lys-conopressin elicited equivalent potency for both egg-laying behaviour and reduction of body weight in other leeches (Fujino *et al.* 1999, Satake *et al.* 1999). Secondly, injection of Lys-conopressin exerts a potent diuretic effect in stage 2 *T. tessulatum*, leading to a severe loss of mass, while the expression of leech receptor is barely detectable (Malecha *et al.* 1986, Salzter *et al.* 1993). Thirdly, at least two different PCR products encoding AVP-related receptor isoforms have been characterized in several annelids, including *T. tessulatum* (data not shown).

In conclusion, we have cloned a novel receptor of the AVP/OT receptor superfamily from *T. tessulatum* that might mediate reproductive as well as mucus excretion functions during the period of sexual maturation. Pharmacological and functional experiments showed that this receptor displays a strikingly mammalian AVP-like profile. Taken together, our data provide additional evidence for the conservation of the AVP/OT neuroendocrine system during the course of evolution.

Acknowledgements

We thank Stéphanie Ruault for technical assistance, Drs Maurice Manning and Ling L Cheng (Medical College of Ohio, Toledo, Ohio) for the generous gift of annetocin, and Drs Claude Cocquerelle and Béatrice Bocquet-Muchembled for sequence analysis (Université de Lille, France). This study was supported partly by the Centre National de la Recherche Scientifique (CNRS), the Fond Européen Développement Etat Région (FEDER), the regional Nord-Pas de Calais council and the Institut National de la Santé et de la Recherche Médicale (INSERM). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Acher R 1993 Neurohypophysial peptide systems: processing machinery, hydroosmotic regulation, adaptation and evolution. *Regulatory Peptides* **45** 1–13.
 Acher R, Chauvet J, Chauvet MT & Rouille Y 1999 Unique evolution of neurohypophysial hormones in cartilaginous fishes: possible implications for urea-based osmoregulation. *Journal of Experimental Zoology* **284** 475–484.
 Barberis C, Mouillac B & Durroux T 1998 Structural bases of vasopressin/oxytocin receptor function. *Journal of Endocrinology* **156** 223–229.

Breton C, Pechoux D, Morel G & Zingg HH 1995 Oxytocin receptor messenger ribonucleic acid: characterization, regulation, and cellular localization in the rat pituitary gland. *Endocrinology* **136** 2928–2936.
 Breton C, Chellil H, Kabbaj-Benmansour M, Carnazzi E, Seyer R, Phalipou S, Morin D, Durroux T, Zingg HH, Barberis C & Mouillac B 2001 Direct identification of human oxytocin receptor-binding domains using a photoactivatable cyclic peptide antagonist: comparison with the human V1a vasopressin receptor. *Journal of Biological Chemistry* **276** 26931–26941.
 Chini B, Mouillac B, Ala Y, Balestre MN, Trumpp-Kallmeyer S, Hoflack J, Elands J, Hibert M, Manning M, Jard S & Barberis C 1995 Tyr115 is the key residue for determining agonist selectivity in the V1a vasopressin receptor. *EMBO Journal* **14** 2176–2182.
 Cotte N, Balestre MN, Aumelas A, Mahe E, Phalipou S, Morin D, Hibert M, Manning M, Durroux T, Barberis C & Mouillac B 2000 Conserved aromatic residues in the transmembrane region VI of the V1a vasopressin receptor differentiate agonist vs. antagonist ligand binding. *European Journal of Biochemistry* **267** 4253–4263.
 Darlison MG & Richter D 1999 Multiple genes for neuropeptides and their receptors: co-evolution and physiology. *Trends in Neuroscience* **22** 81–88.
 Fanelli F, Barbier P, Zanchetta D, De Benedetti PG & Chini B 1999 Activation mechanism of human oxytocin receptor: a combined study of experimental and computer-simulated mutagenesis. *Molecular Pharmacology* **56** 214–225.
 Fujino Y, Nagahama T, Oumi T, Ukena K, Morishita F, Furukawa Y, Matsushima O, Ando M, Takahama H, Satake H, Minakata H & Nomoto K 1999 Possible functions of oxytocin/vasopressin-superfamily peptides in annelids with special reference to reproduction and osmoregulation. *Journal of Experimental Zoology* **284** 401–406.
 Hawtin SR, Wesley VJ, Parslow RA, Simms J, Miles A, McEwan K & Wheatley M 2002 A single residue (arg46) located within the N-terminus of the V1a vasopressin receptor is critical for binding vasopressin but not peptide or nonpeptide antagonists. *Molecular Endocrinology* **16** 600–609.
 Howl J & Wheatley M 1996 Molecular recognition of peptide and non-peptide ligands by the extracellular domains of neurohypophysial hormone receptors. *Biochemical Journal* **317** 577–582.
 Hoyle CH 1999 Neuropeptide families and their receptors: evolutionary perspectives. *Brain Research* **848** 1–25.
 Kanda A, Takuwa-Kuroda K, Iwakoshi-Ukena E, Furukawa Y, Matsushima O & Minakata H 2003 Cloning of *Octopus* cephalotocin receptor, a member of the oxytocin/vasopressin superfamily. *Journal of Endocrinology* **179** 281–291.
 Malecha J 1983 L'osmorégulation chez l'hirudinée rhynchobdelle *Theromyzon tessulatum* (O.F.M.). Localisation expérimentale de la zone sécrétrice d'un facteur de régulation de la balance hydrique. *General and Comparative Endocrinology* **49** 344–351.
 Malecha J, Tramu G, Cardon C & Verger-Bocquet M 1986 Evidence of apparent vasopressin and oxytocin peptides in the brain of the leech *Rhynchobdelle Theromyzon tessulatum* (O.F.M.). *General and Comparative Endocrinology* **64** 13–20.
 Mouillac B, Chini B, Balestre MN, Elands J, Trumpp-Kallmeyer S, Hoflack J, Hibert M, Jard S & Barberis C 1995 The binding site of neuropeptide vasopressin V1a receptor. Evidence for a major localization within transmembrane regions. *Journal of Biological Chemistry* **270** 25771–25777.
 Oumi T, Ukena K, Matsushima O, Ikeda T, Fujita T, Minakata H & Nomoto K 1994 Annetocin: an oxytocin-related peptide isolated from the earthworm, *Eisenia foetida*. *Biochemical and Biophysical Research Communications* **198** 393–399.

- Peter J, Burbach H, Adan RA, Lolait SJ, Van Leeuwen FW, Mezey E, Palkovits M & Barberis C 1995 Molecular neurobiology and pharmacology of the vasopressin/oxytocin receptor family. *Cellular and Molecular Neurobiology* **15** 573–595.
- Phalipou S, Seyer R, Cotte N, Breton C, Barberis C, Hibert M & Mouillac B 1999 Docking of linear peptide antagonists into the human V(1a) vasopressin receptor. Identification of binding domains by photoaffinity labeling. *Journal of Biological Chemistry* **274** 23316–23327.
- Proux JP, Miller CA, Li JP, Carney RL, Girardie A, Delaage M & Schooley DA 1987 Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. *Biochemical and Biophysical Research Communications* **149** 180–186.
- Salzet M, Bulet P, Van Dorselaer A & Malecha J 1993 Isolation, structural characterization and biological function of a lysine-conopressin in the central nervous system of the pharyngobdellid leech *Erpobdella octoculata*. *European Journal of Biochemistry* **217** 897–903.
- Satake H, Takuwa K, Minakata H & Matsushima O 1999 Evidence for conservation of the vasopressin/oxytocin superfamily in Annelida. *Journal of Biological Chemistry* **274** 5605–5611.
- Takuwa-Kuroda K, Iwakoshi-Ukena E, Kanda A & Minakata H 2003 *Octopus*, which owns the most advanced brain in invertebrates, has two members of vasopressin/oxytocin superfamily as in vertebrates. *Regulatory Peptides* **115** 139–149.
- Thibonnier M, Conarty DM, Preston JA, Auzan C, Wilkins PL, Berti-Mattera LN & Mattera R 1998 Molecular pharmacology of human vasopressin receptors. *Advances in Experimental Medicine and Biology* **449** 251–276.
- Thibonnier M, Coles P, Conarty DM, Plesnisher CL & Shoham M 2000 A molecular model of agonist and nonpeptide antagonist binding to the human V(1) vascular vasopressin receptor. *Journal of Pharmacology and Experimental Therapeutics* **294** 195–203.
- Ufer E, Postina R, Gorbulev V & Fahrenholz F 1995 An extracellular residue determines the agonist specificity of V2 vasopressin receptors. *FEBS Letters* **362** 19–23.
- Ukena K, Oumi T, Matsushima O, Ikeda T, Fujita T, Minakata H & Nomoto K 1995 Effects of anetocin, an oxytocin-related peptide isolated from the earthworm *Eisenia foetida*, and some putative neurotransmitters on gut motility of the earthworm. *Journal of Experimental Zoology* **272** 184–193.
- Van Kesteren RE, Smit AB, De Lange RP, Van Kits KS, Van Golen FA, Van Der Schors RC, De With ND, Burke JF & Geraerts WPM 1995a Structural and functional evolution of the vasopressin/oxytocin superfamily: vasopressin-related conopressin is the only member present in *Lymnaea*, and is involved in the control of sexual behavior. *Journal of Neuroscience* **15** 5989–5998.
- Van Kesteren RE, Tensen CP, Smit AB, Van Minnen J, Van Soest PF, Kits KS, Meyerhof W, Richter D, Van Heerikhuizen H, Vreugdenhil E & Geraerts WPM 1995b A novel G protein-coupled receptor mediating both vasopressin- and oxytocin-like functions of Lys-conopressin in *Lymnaea stagnalis*. *Neuron* **15** 897–908.
- Van Kesteren RE, Tensen CP, Smit AB, Van Minnen J, Kolakowski LF, Meyerhof JW, Richter D, Van Heerikhuizen H, Vreugdenhil E & Geraerts WPM 1996 Co-evolution of ligand-receptor pairs in the vasopressin/oxytocin superfamily of bioactive peptides. *Journal of Biological Chemistry* **271** 3619–3626.

Received in final form 27 September 2004

Accepted 30 September 2004

Made available online as an

Accepted Preprint 6 October 2004