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Functional characterisation of a 5-HT_2 receptor cDNA [1] cloned from *Lymnaea stagnalis*

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Abstract

A G-protein-coupled receptor (5-HT_{2Lym}) resembling members of the 5-HT_2 receptor subfamily was cloned from the mollusc *Lymnaea stagnalis*. Serotonin induces a concentration-dependent increase in intracellular inositol phospates in HEK293 cells expressing this receptor (EC_{50} = 114 nM). 5-HT_{2Lym} differs from mammalian 5-HT_2 receptors by the presence of a large amino-terminal region. This large domain appears to preclude an adequate level of expression of 5-HT_{2Lym} in HEK293. Therefore, we constructed a cDNA encoding an amino-terminally truncated receptor (ΔN-5-HT_{2Lym}) that appeared to be much better expressed in HEK293 cells. ΔN-5-HT_{2Lym}-expressing cells exhibit a serotonin-induced stimulation of phosphatidylinositol bisphosphate hydrolysis (EC_{50} = 11.4 nM) and a high-affinity binding of the 5-HT_2-selective antagonist [^{3}H]mesulergine (K_d = 4 nM). Inhibition of this binding by several 5-HT_2 antagonists and agonists revealed a pharmacological profile most closely resembling those of $5HT_{2Dro}$, 5-HT_{2B} and 5-HT_{2C}.

Keywords: 5-HT (5-hydroxtryptamine, serotonin); 5-HT_2 receptor; G-protein-coupled receptor; (*Lymnaea stagnalis*); (Mollusc)

1. Introduction

Serotonin (5-HT) is a major neurotransmitter in both vertebrate and invertebrate species which is involved in a wide variety of physiological and behavioural processes (reviewed in Kravitz, 1988; Bicker and Menzel, 1989; Wilkinson and Dourish, 1991). Recent molecular studies in mammals have revealed the presence of at least 14 different serotonin receptors, all but one belonging to the class of G-protein-coupled receptors (reviewed in Hoyer et al., 1994). It is thought that this large number of serotonin receptor subtypes, all of which are expressed in a cell-specific manner, contributes to the complexity of the effects of serotonin.

In several invertebrate species, the role of serotonin is well studied. It plays a role in learning, feeding, locomotion, circadian rhythm and defense behaviour. In certain molluscan species, the effects of serotonin have even been studied at the level of single identified neurons. This was possible since the central nervous system (CNS) of these molluscs contains a relatively small number of neurons, some of which are large, easily identifiable and accessible. Particularly at the electrophysiological level, detailed knowledge has been acquired on the short-term effects of serotonin on molluscan neurons (reviewed in Gerschenfeld and Paupardin-Tritz, 1974; Rózsa, 1984). These studies suggest the existence of at least 6 different serotonin receptor subtypes in molluscan neurons. More recently, Walcourt-Ambakederemo and co-workers (Walcourt-Ambakederemo and Winlow, 1994a,b, 1995) were able to

Abbreviations: 5-HT, 5-hydroxytryptamine; HEK, human embryonic kidney; PCR, polymerase chain reaction; bp, basepair(s); TM, transmembrane (region(s)); RACE, rapid amplification of cDNA ends; SSC, standard saline citrate; PIP_2, phosphatidyl inositol bisphosphate; IP, inositol phosphates; DMEM, Dulbecco's modified Eagle's medium; mCPP, *m*-chlorophenylpiperazine; DOB, 5-bromo-3,4-di-methoxyphenylisopropylamine; CNS, central nervous system

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identify 5-HT_1-, 5-HT_2- and 5-HT_3-like receptor subtypes in a single cell type in the CNS of the pond snail *Lymnaea stagnalis*.

The molecular cloning of molluscan receptors and their localisation in identified neurons will allow us to study their signal transduction at the level of single, identified cells in great detail. The availability of the relevant cDNAs will make it possible to design highly specific molecular tools that can be used to manipulate the expression and/or function of the pertinent receptors.

In addition, elucidating the molecular structure of invertebrate receptors will contribute to a better insight in the evolutionary conservation of receptor subtype structure. Subsequent pharmacological characterisation of cloned and expressed invertebrate receptors, and the comparison of their characteristics with those of species homologues, may lead to a better understanding of structure-function relationships of receptor subtypes.

As yet, the cloning of 3 molluscan serotonin receptor genes have been reported. One cDNA was isolated from the CNS of *Lymnaea stagnalis* that encodes a receptor displaying some characteristics of mammalian $5HT_1$-like receptors (Sugamori et al., 1993). Recently, cDNAs encoding 2 homologous serotonin receptor subtypes were cloned from *Aplysia*. Both receptors appeared to be related to the 5-HT_{dro1} and mammalian 5-HT_6 receptors (Li et al., 1995).

Here, we present the structure of a second *Lymnaea* serotonin receptor gene, called 5-HT_{2Lym}. This is the first cloned gene encoding a molluscan serotonin receptor which belongs to the subfamily of 5-HT_2 receptors and, likewise, it shows highest sequence identity with the recently cloned *Drosophila* 5-HT_2 receptor gene (Colas et al., 1995) and with the members of the mammalian 5-HT_2 receptor subfamily (Hoyer et al., 1994). It appeared that the very large amino terminus of 5-HT_{2Lym} was deleterious to high level expression of the receptor in mammalian cell lines. Therefore, we constructed a mutant receptor (ΔN-5-HT_{2Lym}), which was found to be much better expressed in HEK293 cells than the full-length receptor. This manuscript describes the functional properties of both 5-HT_{2Lym} and ΔN-5-HT_{2Lym} expressed in HEK293 cells.

2. Materials and methods

2.1. Animals

Adult *L. stagnalis* (shell height 28–34 mm) were bred in the laboratory under standard conditions (Van der Steen et al., 1969).

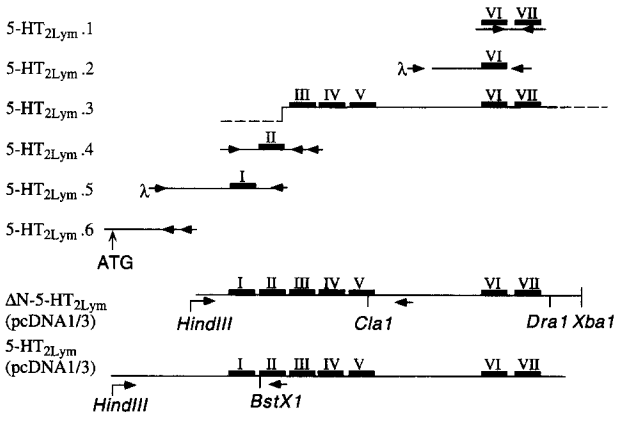

Fig. 1. Cloning of 5-HT_{2Lym} and ΔN-5-HT_{2Lym}. 5-HT_{2Lym}.1: cDNA fragment obtained by PCR with degenerate oligonucleotides on cDNA of CNS of *L. stagnalis*. Arrows represent PCR primers. 5-HT_{2Lym}.2: cDNA fragment present in CNS cDNA library, 5-HT_{2Lym}.3: genomic fragment subcloned from genomic library of *L. stagnalis*. 5-HT_{2Lym}.4: cDNA fragment obtained by '5′ anchored PRC', 5-HT_{2Lym}.5: cDNA fragment present in CNS cDNA library, 5-HT_{2Lym}.6: cDNA fragment obtained by '5′ anchored PRC' which contains the putative start codon. The 5′ part of ΔN-5-HT_{2Lym} was constructed by means of PCR amplification on CNS cDNA with a sense oligonucleotide primer containing a *Hin*dIII restriction site; this fragment was combined with a *Cla*I-*Dra*I genomic subclone and cloned *Hin*dIII-*Xba*I into pcDNA1 and pcDNA3. In order to construct the full-length 5-HT_{2Lym}, the region encoding the complete amino terminus and TM1 was amplified using a sense primer containing a *Hin*dIIIrestriction site. Then the *Hin*dIII-*Bst*XI of ΔN-5-HT_{2Lym} was exchanged for the *Hin*dIII-*Bst*XI fragment that contains the start codon, thus leading to a full-length receptor cDNA. Boxes numbered by I–VII represent putative TM regions.

2.2. PCR

PCR on *Lymnaea* cDNA and analysis of PCR products has been described in detail by Tensen and co-workers (Tensen et al., 1994). The following strongly degenerate oligonucleotides were used: TM6-sense, 5′-GGGAATTC-G(G,T)I(A,G)(C,T)ITT(C,T)I(C,T)I(A,C,T)TITG(C,T)TG-GI(C,T)TA(A,T,G)A(A,T)(A,T,G)A(C,G,T)IGG(A,G)T-3′ and TM7-antisense, 5′-CCGGATCCTTIA(A,G)(A,G)-(A,C)AI(C,G)I(A,G)TA(A,T,G)A(A,T)(A,T,G)A(C,G,T)-IGG(A,G)T-3′ (I = inosine). These oligonucleotides correspond to particularly highly conserved amino acids in transmembrane (TM) regions 6 and 7 present in G-protein-coupled bioamine receptors (Probst et al., 1992).

2.3. Cloning of 5-HT_{2Lym} and ΔN-5-HT_{2Lym}

Oligonucleotides TM6-sense and TM7-antisense were used in a PCR on *Lymnaea* CNS cDNA. The resulting fragments were cloned and sequenced and yielded (a.o.)

Fig. 2. Nucleotide and deduced amino-acid sequence of 5-HT_{2Lym} cDNA. Putative TM regions are boxed, circles (●) represent consensus sites for N-linked glycosylation, open (□) or closed (■) squares represent consensus phosphorylation sites for protein kinase C or protein kinase A, respectively. The arrow (↑) indicates the position of the truncation in ΔN-5-HT_{2Lym} and the asterisk (*) indicates the position of the intron-exon boundary.

clone 5-HT_{2Lym}.1 (Fig. 1). Next, an oligonucleotide specific for 5-HT_{2Lym}.1 (based on bp 2360–2378; Fig. 2) was used in a PCR-based screening of *Lymnaea* CNS-specific cDNA libraries (Gibbons et al., 1991). This resulted in the isolation and sequencing of 5-HT_{2Lym}.2 (Fig. 1). Subsequently, 5-HT_{2Lym}.2 was labeled with [α-^{32}P]dATP (prime-a-gene kit, Promega) and used as a probe to screen a *Lymnaea* lambda-EMBL3 genomic library (Bogerd et al., 1991) under stringent conditions (65°C, 0.1 × SSC). DNA of positive plaques was subcloned and sequenced, resulting in the isolation and characterisation of 5-HT_{2Lym}.3 (Fig. 1). Screening methods, DNA phage isolation and DNA sequence analysis were performed according to standard protocols (Sambrook et al., 1989). Next, '5′ anchored PCR' was performed according to Ausubel et al. (1994). Poly(A)$^+$ RNA from *Lymnaea* CNS was isolated from total brain RNA with oligo(dT) Dynabeads (Dynal, Oslo) and reverse-transcribed into cDNA with random hexamers and Superscript reverse transcriptase (BRL). 5′ RACE was performed using 2 nested primers (based on bp 1183–1201 and 1115–1133; Fig. 2) and resulted in the isolation of 5-HT_{2Lym}.4. Sequence information of this clone was then used to generate an oligonucleotide (based on bp 982–1001; Fig. 2) that was used in a PCR-based screening of cDNA libraries (see above), yielding clone 5-HT_{2Lym}.5. Finally, a second round of '5′ anchored PCR' was performed, using 2 nested primers (based on bp 502–519 and 308–331; Fig. 2). This yielded 5-HT_{2Lym}.6, a cDNA clone comprising the putative initiation codon (Fig. 1).

The sequence of all PCR-generated clones was determined by analysis of at least 5 independent clones each in order to identify possible (point) mutations due to PCR artefacts. To minimize such PCR artefacts, the proofreading polymerase Ultma (Perkin Elmer) was used.

Using 5-HT_{2Lym}.3 and with the aid of sequence information of 5-HT_{2Lym}.5 and 5-HT_{2Lym}.6, we constructed 2 new clones (Fig. 1). One encoding a full-length receptor (5-HT_{2Lym}), the other encoding a truncated receptor (ΔN-5-HT_{2Lym}), having an amino-terminal deletion of 220 amino acids, thus resulting in a receptor with a first extracellular domain of 60 amino acids. This latter clone was constructed as follows. The 5′ part was amplified from *Lymnaea* genomic DNA using 2 oligonucleotides (based on bp 661–678 and 1620–1640; Fig. 2). The sense primer contained a *Hin*dIII restriction site, so the PCR fragment could be cut with *Hin*dIII and *Cla*I and ligated with a *Cla*I-*Xba*I restriction fragment of clone 5-HT_{2Lym}.3 (Fig. 1) into the *Hin*dIII and *Xba*I sites of pcDNA3 (Invitrogen). The final construct, pcDNA3-ΔN-5-HT_{2Lym}, was sequenced to make sure that no mutations had been introduced as a consequence of PCR artefacts. The wild-type 5-HT_{2Lym} clone was constructed in a similar way. The 5′ part was generated by PCR (using primers based on bp 1–25 and 1112–1138; Fig. 2) and provided at its 5′ end with a *Hin*dIII site. This fragment was cut with *Hin*dIII and *Bst*XI (Fig. 1) and ligated into pcDNA3-ΔN-5-HT_{2Lym} digested with *Hin*dIII and *Bst*XI. The final construct, pcDNA3-5-HT_{2Lym}, was sequenced.

2.4. Transfection

HEK293 cells were transfected with pcDNA3-5-HT_{2Lym} or pcDNA3-ΔN5-HT_{2Lym} using the calcium phosphate method as described in Stam et al. (1992). Transfected cells were exposed to geneticin (G418) in a concentration of 800 μg/ml 24 h after transfection. In the case of ΔN-5-HT_{2Lym}, membrane fractions of resistant colonies were tested for the ability to bind [^{3}H]mesulergine (85 Ci/mmol, Amersham). Resistant colonies transfected with pcDNA3-5-HT_{2Lym} were tested for their ability to hydrolyze PIP_2 upon stimulation with serotonin. Two cell lines were isolated which expressed 4 and 7 pmol ΔN-5-HT_{2Lym}/mg membrane protein. The latter was used for radioligand-binding studies and measurements of agonist-induced IP formation. Two cell lines expressing 5-HT_{2Lym} were isolated based on the fact that they showed a 4 times higher serotonin-induced IP production as control cells.

2.5. Measurements of [^{3}H]inositol phosphates formation

Cells stably expressing 5-HT_{2Lym} or ΔN-5-HT_{2Lym} were grown in 24-well plates. 1 μCi [^{3}H]*myo*-inositol (18 Ci/mmol, Amersham) was added to these cells per ml medium, and 24 h later cells were washed twice with DMEM (GIBCO-BRL) and incubated with DMEM containing LiCl (10^{-2} M), pargyline (10^{-6} M) and paroxetin (10^{-6} M) for 10 min at 37°C. Serotonin was added and after 60 min at 37°C under 5% CO_2 the medium was aspirated and the cells were lysed by adding ice-cold chloroform : methanol (1 : 2). Cells were incubated on ice for 10 min, sonicated and transferred to a glass tube. After adding H_2O : chloroform (1 : 1), phases were separated by centrifugation (5 min at 3000 rpm) and 2.5 ml H_2O and 0.5 ml Dowex AG 1 × 8 anion exchange resin were added to the aqueous phase. Resin was washed with H_2O 3 times and IP were eluted with 0.1 M formate acid, 1 M ammonium formate. All numerical values given in this paper represent means of at least 3 independent experiments, in which each measurement is performed in triplicate.

2.6. Receptor-binding studies

Cells stably expressing 5-HT_{2Lym} or ΔN-5-HT_{2Lym} were harvested in ice-cold 50 mM Tris-HCl, pH 7.4 and centrifuged for 20 min at 26000 × g. Cell pellets were lysed in 5 mM Tris-HCl, pH 7.4, centrifuged again (20 min at 26000 × g) and membranes were resuspended in assay buffer (4 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.7). Protein concentration was measured according to Bradford (1976). For the determination of [^{3}H]mesulergine concentration binding isotherms, ±20 μg membrane protein was resuspended in 0.4 ml assay buffer and incubated with increas-

ing concentrations of [^{3}H]mesulergine (85 Ci/mmol, Amersham) in a total volume of 0.5 ml for 60 min at 25°C. Unlabeled mesulergine (1 μM) was added to determine non-specific binding. In order to determine the affinity of various compounds for ΔN-5-HT$_{2Lym}$, increasing concentrations of the compounds (10^{-5}–10^{-10} M) were incubated with 10 μg of membrane protein from HEK293 cells stably expressing ΔN-5-HT$_{2Lym}$ and 4 nM [^{3}H]mesulergine in a total volume of 0.5 ml for 60 min at 25°C. Incubations were stopped by vacuum filtration over Whatman GF/B filters, which were rinsed 3 times with ice-cold 50 mM Tris-HCl, pH 7.4. All values mentioned in this paper represent means of at least 3 independent experiments, in which each measurement is performed in triplicate.

3. Results

3.1. Isolation and structure of the gene encoding 5-HT$_{2Lym}$

We employed the PCR technique to clone cDNAs encoding neurotransmitter receptors that are expressed in the CNS of *L. stagnalis*. Using degenerated oligonucleotides corresponding to conserved amino-acid motifs in TM regions 6 and 7 of a large number of G-protein-coupled receptors, several cDNA fragments were amplified which encode putative G-protein-coupled receptors. The isolation of the total coding sequence corresponding to one of these fragments is described at length in Materials and methods and is schematically illustrated in Fig. 1. Conceptual translation of this nucleotide sequence reveals an amino-acid sequence showing all characteristics of a G-protein-coupled receptor (Fig. 2). A computer search of the SwissProt database revealed high similarity with a 5-HT$_2$ receptor that was recently cloned from *Drosophila* (Colas et al., 1995; 57% identity in TM regions) and with the members of the subfamily of vertebrate 5-HT$_2$ receptors (reviewed in Hoyer et al., 1994; Baxter et al., 1995; 46–49% identity in TM regions). Therefore, the *Lymnaea* receptor was putatively designated 5-HT$_{2Lym}$. Fig. 3 shows the alignment of the predicted amino-acid sequences of the members of the 5-HT$_2$ receptor subfamily. Sequence analysis of a partial genomic clone (5-HT$_{2Lym}$.3; Fig. 1) revealed the presence of an intron upstream from the region encoding

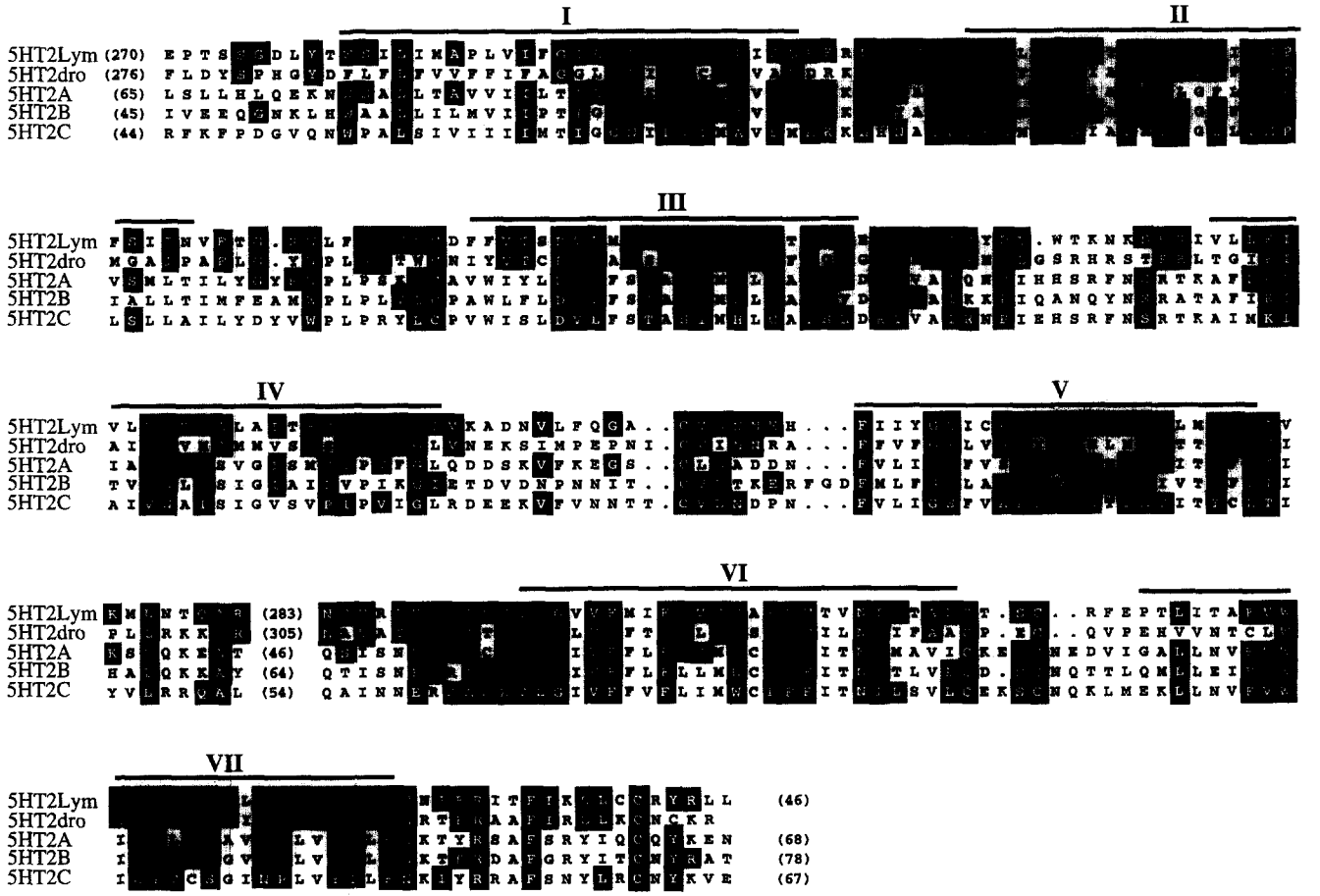

Fig. 3. Alignment of the deduced amino-acid sequence of 5-HT$_{2Lym}$ with the other members of the 5HT$_2$ receptor subfamily: human 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, and 5-HT$_{2Dro}$. Identical residues are highlighted in reverse contrast; horizontal lines represent the putative TM regions (numbered by Roman numerals). Gaps, indicated by dashes, have been introduced to optimize the alignment. Parts of the amino terminus and the 3rd intracellular loop of 5-HT$_{2Lym}$ have been omitted.

TM3. This intron is located at a position where all known 5-HT_2-receptor-encoding genes are interrupted by an intron, thereby underlining that the newly cloned *Lymnaea* receptor belongs to the subfamily of 5-HT_2 receptors. In order to study the organisation of the remaining part of the gene encoding 5-HT_{2Lym}, PCR experiments were performed on both cDNA and genomic DNA with sets of primers along the entire coding region of 5-HT_{2Lym} (data not shown). From these studies, we were able to conclude that just a single intron is present in the coding region of the 5-HT_{2Lym} gene.

Hydropathy analysis of 5-HT_{2Lym} indicates the presence of 7 hydrophobic domains which are thought to represent TM domains. There is no indication for an 8th hydrophobic domain preceding TM1, as was found in the 5-HT_{2C} receptor (Yu et al., 1991) and in the *Drosophila* serotonin receptors (Saudou et al., 1992), nor for a hydrophobic region which might function as a signal peptide, as has been reported for the 5-HT_{2Dro} receptor (Colas et al., 1995). A further structural comparison (Fig. 3) of 5-HT_{2Lym} with other 5-HT_2 receptors shows that the *Lymnaea* receptor contains a very large amino-terminal, extracellular region (in general, this region is around 60 amino acids in length, in 5-HT_{2Lym} it measures 280 amino acids) and a large 3rd intracellular loop (in general, this loop is 70–80 amino acids, in 5-HT_{2Lym} it is 305 amino acids). In this respect, the structure of 5-HT_{2Lym} resembles that of the 5-HT_{2Dro} receptor (Colas et al., 1995).

3.2. Signal transduction of 5-HT_{2Lym}

The sequence similarity between 5-HT_{2Lym} and the members of the vertebrate 5-HT_2-receptor family suggests the existence of a functional similarity. To test this assumption, we investigated whether serotonin was able to elicit an increase in intracellular inositol phosphates (IP) in HEK293 cells stably transfected with 5-HT_{2Lym}. Indeed, such cells show a concentration-dependent increase of PIP_2 hydrolysis upon stimulation with serotonin (Fig. 4). Maximal response was 4-fold over basal levels and the EC_{50} for serotonin was 113.9 ± 4.8 nM. Non-transfected HEK293 cells did not show any change in IP concentration in response to serotonin (data not shown). In addition, cells expressing 5-HT_{2Lym} did not show any serotonin-induced changes in cAMP levels (data not shown).

3.3. Pharmacology of ΔN-5-HT_{2Lym}

We then performed binding experiments using several labeled serotonergic radioligands ([^{3}H]5-HT, [^{3}H]mesulergine, [^{125}I]aminoketanserin). It appeared not to be possible to obtain any appreciable binding of either of these ligands (data not shown). Because it has been shown that truncation of the amino-terminal regions of 5-HTdro2A and 5-HTdro2B could strongly improve the expression levels of these receptors (Saudou et al., 1992), we presumed that the long amino-terminal region of 5-HT_{2Lym} might impair high expression levels of this receptor. Therefore, we constructed a mutant receptor (ΔN-5-HT_{2Lym}) in which the first 220 amino acids were deleted, thus leaving an amino terminus of a size comparable to the amino terminus of mammalian 5-HT_2 receptors (± 60 amino acids). Indeed, cells stably transfected with this cDNA do express high levels of receptor protein as monitored by specific binding of [^{3}H]mesulergine. Fig. 5 shows the saturation binding isotherm of [^{3}H]mesulergine binding to ΔN-5-HT_{2Lym} ($K_d = 4.1 \pm 1.6$ nM, $B_{max} = 7$ pmol/mg protein).

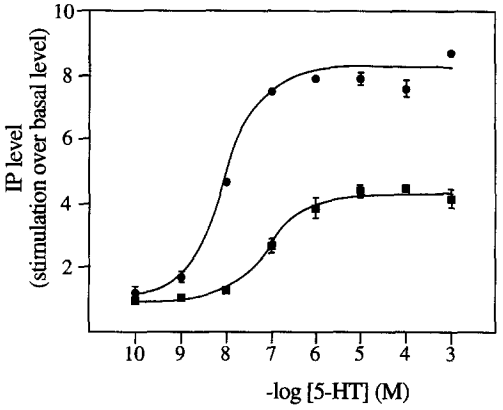

Fig. 4. Representative concentration-effect curves for the production of intracellular inositolphosphates by 5-HT in HEK293 cells stably transfected with 5-HT_{2Lym} (■) or ΔN-5-HT_{2Lym} (●). Each point represents the mean ± S.E.M. of triplicate determinations.

As mentioned above, radioligand binding to 5-HT_{2Lym} was below the level of detection, whereas high-affinity binding of [^{3}H]mesulergine to the truncated receptor was easily measurable. As a consequence, we could only establish a pharmacological profile for ΔN-5-HT_{2Lym}. Membranes of HEK293 cells stably expressing ΔN-5-HT_{2Lym} ($B_{max} = 7$ pmol/mg protein) were used to determine

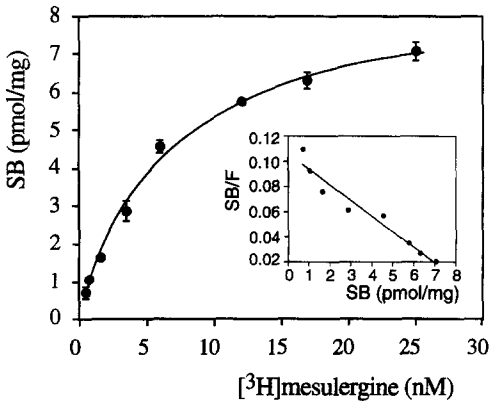

Fig. 5. Representative saturation-binding isotherm of [^{3}H]mesulergine binding to ΔN-5-HT_{2Lym}. SB, specific binding, as calculated by subtraction of non-specific binding from total binding (pmol/mg protein). Each point represents the mean ± S.E.M. triplicate determinations. Inset: Scatchard plot of the same data. F, free ligand.

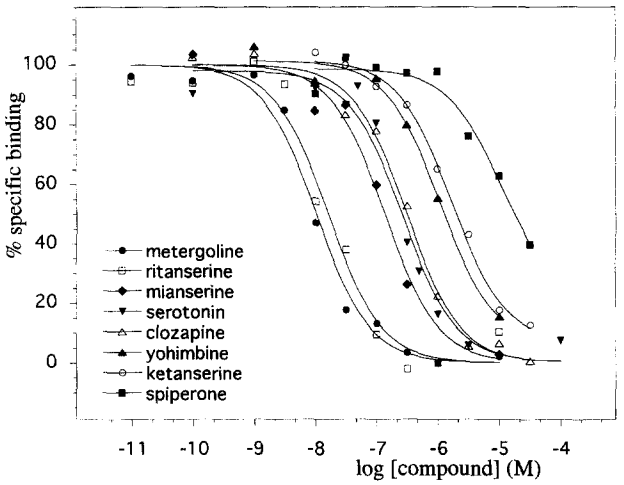

Fig. 6. Representative inhibition-binding curves of selected compounds, depicted as % of specific binding of [^{3}H]mesulergine to ΔN-5-HT$_{2Lym}$.

the ability of several established 5-HT$_2$ receptor (ant)agonists to compete for [^{3}H]mesulergine binding (Fig. 6). We observed the following rank order of potencies. For drugs with affinities in the low nanomolar range, metergoline > ritanserin > mianserine > methysergide. For drugs with affinities in the high nanomolar range, clozapine > *m*-chloro-phenyl-piperazine (mCPP) > 5-HT > yohimbine > 5-bromo-3,4-di-methoxy-phenyl-isopropylamine (DOB); and, for drugs with affinities in the micromolar range, ketanserin > xylamidine > spiperone > cisapride = pipamperone. K_i values obtained for the various compounds are presented in Table 1.

Table 1
Pharmacological profile of ΔN-5-HT$_{2Lym}$

	K_i (M)	pK_i	n_H
Agonists			
mCPP	$1.2 \pm 0.2 \cdot 10^{-7}$	6.9	0.90±0.08
5-HT	$1.3 \pm 0.2 \cdot 10^{-7}$	6.9	0.81±0.18
DOB	$7.2 \pm 1.8 \cdot 10^{-7}$	6.1	1.08±0.08
Antagonists			
Metergoline	$6.4 \pm 2.3 \cdot 10^{-9}$	8.2	1.27±0.18
Ritanserin	$7.6 \pm 1.8 \cdot 10^{-9}$	8.1	0.95±0.20
Methysergide	$9.7 \pm 0.5 \cdot 10^{-9}$	8.0	1.05±0.06
Mianserine	$7.2 \pm 1.1 \cdot 10^{-8}$	7.1	0.93±0.07
Clozapine	$9.9 \pm 2.0 \cdot 10^{-8}$	7.0	0.90±0.03
Yohombine	$5.1 \pm 0.7 \cdot 10^{-7}$	6.3	0.98±0.02
Ketanserine	$1.9 \pm 0.2 \cdot 10^{-6}$	5.7	0.73±0.14
Xylamidine	$5.5 \pm 0.1 \cdot 10^{-6}$	5.3	1.04±0.14
Spiperone	$9.4 \pm 0.1 \cdot 10^{-6}$	5.0	1.19±0.08
Cisapride	$> 10^{-5}$	< 5	
Pipamperone	$> 10^{-5}$	< 5	

Dissociation constants (K_i / pK_i) and Hill coefficients (n_H) of various serotonergic agonists and antagonits as determined by competition binding with [^{3}H]mesulergine. mCPP, *m*-chlorophenylpiperazine; 5-HT, 5-hydroxytryptamine; DOB, 5-bromo-3,4-dimethoxyphenylisopropylamine. Values represent the mean±S.E.M. of at least 3 independent experiments, performed in triplicate.

3.4. Further characteristics of 5-HT$_{2Lym}$ and ΔN-5-HT$_{2Lym}$

We then examined whether truncation of the amino terminus was of influence for the coupling of the receptor to phospholipase C (Fig. 4). The maximal serotonin-induced increase in IP (8-fold over basal levels) was substantially higher in cells expressing ΔN-5-HT$_{2Lym}$ than in cells expressing 5-HT$_{2Lym}$. In addition, the EC$_{50}$ for serotonin of ΔN-5-HT$_{2Lym}$ (11.4 ± 1.7 nM) was 10 times lower than the value for 5-HT$_{2Lym}$. In order to test if the lack of measurable binding of [^{3}H]mesulergine to 5-HT$_{2Lym}$ was due to a low expression level of the receptor, or to an inablility of mesulergine to bind to the receptor, we examined the ability of mesulergine to inhibit the serotonin-induced increase in IP. Cells were preincubated with increasing concentrations of mesulergine for 15 min and stimulated with a concentration of serotonin which is 10 times the EC$_{50}$ (10^{-6} M and 10^{-7} M for 5-HT$_{2Lym}$ and ΔN-5-HT$_{2Lym}$, respectively). Fig. 7 shows that mesulergine can decrease the IP formation in a concentration-dependent manner and that the K_i, as calculated from the IC$_{50}$, is similar in cells expressing 5-HT$_{2Lym}$ and in cells expressing ΔN-5-HT$_{2Lym}$ (2.9 ± 1.4 and 4.95 ± 2.6 nM, respectively).

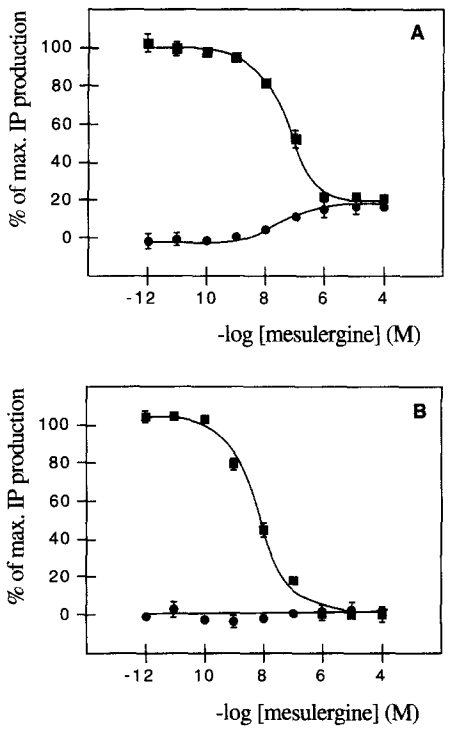

Fig. 7. Representative dose-effect curves for the effect of mesulergine on the production of intracellular inositolphosphates in HEK293 cells, stably transfected with (A) ΔN-5-HT$_{2Lym}$ or (B) 5-HT$_{2Lym}$. Circles (●) represent the effect of mesulergine on basal levels of inositol phosphates; squares (■) represent the effect of mesulergine on 5-HT-stimulated levels of inositol phosphates. Cells were stimulated with a concentration of 5-HT which is 10 times the EC$_{50}$ (10^{-6} and 10^{-7} M for 5-HT$_{2Lym}$ and ΔN-5-HT$_{2Lym}$, respectively).

Furthermore, it was noticed that mesulergine could not completely antagonize the IP production in cells expressing ΔN-5-HT_{2Lym}, while it could do so in cells expressing 5-HT_{2Lym} (Fig. 7). Apparently, mesulergine can act as a partial agonist in cells expressing ΔN-5-HT_{2Lym} (EC_{50} = 20.6 ± 8.3 nM) whereas it lacks this activity in cells expressing 5-HT_{2Lym}. No effects of mesulergine on the basal IP levels were observed, indicating that the receptor was not precoupled to PLC.

4. Discussion

We have cloned a gene encoding a G-protein-coupled receptor (5-HT_{2Lym}) from *L. stagnalis*, which shows high sequence similarity to 5-HT_2 receptors cloned from *Drosophila* (57% identity in TM regions) and mammals (46–49% identity in TM regions). The *Lymnaea* receptor contains several conserved amino acids and amino-acid motifs that have been shown to be of crucial importance for the functioning of mammalian 5-HT_2 receptors. Two highly conserved Asp residues (corresponding to Asp-325 and Asp-359 in 5-HT_{2Lym}) have been shown to play a role in allosteric activation of G-proteins and in high-affinity binding of agonists, respectively, in the rat 5-HT_{2A} receptor (Wang et al., 1993). At the border of TM3 and the 2nd intracellular loop, still another conserved Asp residue is found within the sequence Asp-Arg-Tyr. This Asp is present in most G-protein-coupled receptors and is thought to be important for maintaining the 3-dimensional structure of G-protein-coupled receptors, rather than playing a direct role in G-protein-coupling or agonist binding (Wang et al., 1993). At the corresponding position in 5-HT_{2Lym}, a conserved substitution, Glu-376, is found. Two amino acids, Ala-242 in the rat 5-HT_{2A} receptor (Kao et al., 1992) and Phe-340 in the human 5-HT_{2A} receptor (Choudary et al., 1993) that have been reported to play a role in mesulergine binding, are conserved in 5-HT_{2Lym} (Ala-445 and Phe-780). The large amino-terminal region (280 amino acids) and the large 3rd intracellular loop (305 amino acids) of 5-HT_{2Lym} are not present in the mammalian 5-HT_2 receptors. Such features are, however, also present in 5-HT_{2Dro} and thus might represent properties of invertebrate (5-HT_2) receptors. Although the sizes of these domains are very similar in the *Drosophila* and the *Lymnaea* 5-HT_2 receptor, they have no sequence similarity which makes it unlikely that they will serve a specific function. Within the the amino-terminal domain of 5-HT_{2Lym} 14 putative N-linked glycosylation sites can be recognized, 6 of which are still present in ΔN-5-HT_{2Lym}. Several consensus sites for phosphorylation by protein kinase A and/or protein kinase C are present in the intracellular loops of 5-HT_{2Lym} as well as a Ser/Thr-rich repeat which is also seen in the *Drosophila* 5-HT_2 receptor.

The genomic organisation of the genes of the 5-HT_2 receptor subfamily is characterised by the existence of multiple introns within the coding region (Foquet et al., 1992; Stam et al., 1992, 1994; Chen et al., 1992; Yang et al., 1992; Schmuck et al., 1994; Colas et al., 1995). DNA sequence analysis of genomic clones and PCR experiments on genomic and cDNA revealed the presence of only a single intron in the 5-HT_{2Lym} gene. Interestingly, this intron (indicated by an asterix in Fig. 2) is located at a position where all other 5-HT_2-receptor genes also contain an intron, thereby underlining the evolutionary relationship of 5-HT_{2Lym} with the vertebrate members of the 5-HT_2 receptor family as well as with the 5-HT_{2Dro} receptor.

Like all members of the 5-HT_2 receptor subfamily, 5-HT_{2Lym} can stimulate the formation of IP in stably transfected HEK293 cells (EC_{50} = 112 nM). Apparently, the expressed snail receptors are capable to couple functionally to human G-proteins, probably of the $G_{q/11}$ type. Consistently, the *Lymnaea* $G_{\alpha q}$ subunit that was recently cloned by us (Knol et al., 1995) exhibits a high degree of amino-acid sequence conservation with mammalian $G_{\alpha q}$ subunits (80% identity).

We noticed that HEK293 cells that stably expressed 5-HT_{2Lym} could not reach high receptor densities. Based on the observations of Saudou et al. (1992), we hypothesised that the very long amino-terminal region of the protein might impede high expression levels. Saudou and co-workers have shown that in the 5-HTdro2A and 5-HTdro2B (now suggested to be called 5-HT_{1ADro} and 5-HT_{1BDro}; Colas et al., 1995) truncation of the amino-terminal region increased receptor expression 75- and 450-fold, respectively, without changing receptor function (Saudou et al., 1992). We furthermore noticed that 5-HT_{2Lym} has no amino-terminal hydrophobic sequence which could function as a signal peptide. Such a sequence improves the expression of other G-protein-coupled receptors with long amino termini (e.g. many peptide receptors and the 5-HT_{2Dro}). To test whether truncation of the amino terminus of 5-HT_{2Lym} would increase its expression, we constructed a deletion mutant (ΔN-5-HT_{2Lym}) in which the first 660 bp of the coding region were deleted. Because the amino-terminal region of G-protein-coupled receptors recognising bioamines is involved in receptor trafficking and probably plays no role in ligand binding (Buck et al., 1991; Saudou et al., 1992; Colas et al., 1996), such a deletion mutant can be useful to study the pharmacological properties of 5-HT_{2Lym}.

Our functional studies with cells stably expressing ΔN-5-HT_{2Lym} or 5-HT_{2Lym} clearly indicate that truncation of the amino-terminal domain of 5-HT_{2Lym} strongly enhances expression of the receptor. These indications are 3-fold. Firstly, we have found that cells expressing ΔN-5-HT_{2Lym} show binding of [^{3}H]mesulergine (Fig. 5), whereas in cells expressing the full-length receptor no appreciable binding of this ligand could be detected. Inhibition of the binding of [^{3}H]mesulergine to ΔN-5-HT_{2Lym} was used to determine the affinity of several serotonergic drugs for 5-HT_{2Lym}. From these studies, it can be concluded that

5-HT_{2Lym} reveals a pharmacological profile which is clearly related to the vertebrate 5-HT_{2B} and 5-HT_{2C} receptors and, to a lesser extent, to the 5-HT_{2A} receptor (Table 1). The clearest indications for classifying 5-HT_{2Lym} as a 5-$HT_{2B/2C}$-like receptor, are the low, micromolar affinity of ketanserin, spiperone and cisapride; antagonists which interact with the vertebrate 5-HT_{2A} receptor in the nanomalor range and with the 5-HT_{2B} and 5-HT_{2C} receptors in the micromolar range. As yet, yohimbine is the only drug known that can discriminate between the 5-HT_{2B} and 5-HT_{2C} receptors, since it shows a higher affinity for 5-HT_{2B} than for 5-HT_{2C} receptors. The affinity of yohimbine for 5-HT_{2Lym} is equal to that of the human 5-HT_{2B} receptor (Bonhaus et al., 1995), indicating a closer relationship between 5-HT_{2Lym} and the 5-HT_{2B} receptors than with the 5-HT_{2C} receptors. When the pharmacology of 5-HT_{2Lym} is compared to that of 5-HT_{2Dro}, which shows a 5-HT_{2B}-like pharmacology, the largest difference is observed in the affinity of ketanserin (K_i = 1.9 mM vs. 63 nM) and spiperone (K_i = 9.4 mM vs. 50 nM).

A second indication that truncation of the amino terminus increases the receptor expression level, came from the observation that cells expressing ΔN-5-HT_{2Lym} showed an increased capacity to hydrolyse PIP_2 in response to serotonin as compared to cells expressing the wild-type receptor. This was reflected in both a 10-fold decrease in EC_{50} as well as in a 2-fold increased maximal response (Fig. 4). These differences seem to be a direct consequence of the different levels of expression of both the full-length and the truncated receptor. In a number of cases, it has now been well documented that both the potency and the intrinsic activity of an agonist increases with increasing receptor number (Boddeke et al., 1992; Whaley et al., 1994; MacEwan et al., 1995).

A third indication for the different receptor densities in cells expressing either 5-HT_{2Lym} or ΔN-5-HT_{2Lym} was obtained when we studied the effect of mesulergine on PIP_2 hydrolysis. It appeared that mesulergine acts as a partial agonist in cells expressing ΔN-5-HT_{2Lym}, whereas it is a full antagonist in cells expressing 5-HT_{2Lym} (Fig. 7). It has been suggested that receptor overexpression can induce partial agonism of compounds that otherwise do not show such features (reviewed in Hoyer and Boddeke, 1993). The K_i values for the antagonistic effects of mesulergine are similar for 5-HT_{2Lym} or ΔN-5-HT_{2Lym} (2.9 and 4.9 nM, respectively). This indicates that, at least as far as mesulergine binding is concerned, the amino-terminal region of the receptor indeed does not influence its ligand binding properties. Recent studies (Colas et al., 1996) indeed show that truncation of the amino terminus of 5-HT_{2Dro} only marginally influences binding properties of the receptor. Furthermore, the K_i values correspond well to the K_d value found for [^{3}H]mesulergine binding to ΔN-5-HT_{2Lym} (4.1 nM).

At present, we do not know whether 5-HT_{2Lym} is the only 5-HT_2-like receptor in *Lymnaea*. A putative 5-HT_{2A}-like receptor was pharmacologically characterized on identified neurons in the CNS of *L. stagnalis* (Walcourt-Ambakederemo and Winlow, 1994b). Unfortunately, the characterisation of this receptor subtype was carried out using rather high concentrations of pharmaca and no dose-response relationships were presented. Therefore, it cannot be concluded whether or not this 5-HT_{2A}-like receptor is different from 5-HT_{2Lym}.

PCR and in situ hybridisation experiments that will be published elsewhere (Gerhardt et al., in preparation) have indicated that only few neurons in the CNS of *Lymnaea* express 5-HT_{2Lym} and that higher levels of expression can be found in the heart, oesophagus, salivary gland and spermoviduct. Such an expression pattern resembles that of the vertebrate 5-HT_{2B} receptor (which shows a wide peripheral tissue distribution) rather than that of the 5-HT_{2A} and 5-HT_{2C} receptors (which are primarily expressed in the CNS). Knowlegde on the localisation of transcripts encoding 5-HT_{2Lym} in the CNS and periphery of *Lymnaea*, combined with the possibility to block receptor function using specific antagonists, will be helpful in elucidating the function(s) of 5-HT_{2Lym} in *Lymnaea*.

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