Molecular cloning and characterization of an invertebrate homologue of a neuropeptide Y receptor

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Abstract

Neuropeptide Y is an abundant and physiologically important peptide in vertebrates having effects on food intake, sexual behaviour, blood pressure and circadian rhythms. Neuropeptide Y homologues have been found in invertebrates, where they are very likely to play similar, important roles. Although five neuropeptide Y-receptor subtypes have been identified in mammals, none has been reported from invertebrates. Here we describe the cloning of a neuropeptide Y-receptor from the brain of the snail *Lymnaea stagnalis*. The identity of the receptor was deduced by expressing the neuropeptide Y-receptor-encoding cDNA in Chinese Hamster Ovary cells, which were subsequently challenged with size-fractionated *Lymnaea* brain extracts. An active peptide, selected on the basis of its ability to induce changes in cAMP levels, was purified to homogeneity, analysed by mass spectrometry and amino acid sequence determination, and turned out to be a *Lymnaea* homologue of neuropeptide Y.

Introduction

Neuropeptides in vertebrates and invertebrates play key roles as signalling molecules in the regulation of physiology, behaviour and development. Their receptors generally belong to the class of G-protein-coupled receptors (GPCRs), a superfamily of proteins characterized by a remarkable degree of functional and structural similarity (Probst *et al.*, 1992). The vertebrate neuropeptide Y family of peptides includes neuropeptide Y (NPY), which is expressed in the central and peripheral nervous system, peptide YY (PYY) and pancreatic polypeptide (PP), which are gut endocrine peptides (see Larhammar, 1996a for review). These peptides display a wide array of biological activities that are thought to be mediated through pharmacologically distinct GPCRs. To date, five members of the human NPY receptor family have been cloned (see Larhammar, 1996b; Blomqvist & Herzog, 1997 for a review). Of these, three (designated Y1, Y2 and Y5) have similar affinities for NPY and PYY (Herzog *et al.*, 1992; Larhammar *et al.*, 1992; Gerald *et al.*, 1995; Rose *et al.*, 1995; Gehlert *et al.*, 1996; Gerald

et al., 1996), the fourth (Y4) is selective for PP (Bard *et al.*, 1995; Lundell *et al.*, 1995). In addition, a sequence encoding a fifth, non-functional member (designated y6) has been reported (Gregor *et al.*, 1996; Matsumoto *et al.*, 1996).

In the mollusk *Lymnaea stagnalis*, a number of individual neurons have been identified with defined functions, e.g. control of heart rate, egg laying or feeding behaviour (Geraerts *et al.*, 1991). These neurons provide excellent model systems to unravel basic aspects of neuropeptidergic transmission. Recently, we cloned several cDNAs encoding G-protein α subunits and one β subunit from the central nervous system (CNS) of *Lymnaea* (Knol *et al.*, 1992; Knol *et al.*, 1994; Knol *et al.*, 1995a, 1995b). The deduced proteins are highly homologous to their mammalian counterparts, suggesting that the molecular mechanisms underlying G-protein-mediated signal transduction in these invertebrate neurons are similar to mechanisms operative in the mammalian brain.

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As a further step towards the identification of proteins involved in G-protein-mediated signal transduction pathways in *Lymnaea*, we isolated cDNAs encoding neuronal GPCRs using PCR amplification of cDNA synthesized from mRNA isolated from *Lymnaea* brains, in combination with sets of degenerate oligonucleotides corresponding to conserved domains of identified vertebrate neuropeptide receptors. This resulted in the isolation of several cDNAs encoding putative neuropeptide receptors. Initial results showed that the addition of a crude peptide extract isolated from *Lymnaea* brains reduced forskolin-stimulated cAMP levels when added to CHO cells transfected with the *Lymnaea* 'orphan' receptor GRL105. This assay was then used to purify the ligand to homogeneity. Protein sequence and mass spectroscopic analysis showed that the peptide had considerable homology to vertebrate NPYs. We therefore conclude that GRL105 represents the first NPY-receptor isolated from an invertebrate.

Materials and methods

Animals

Adult *Lymnaea stagnalis* (shell height 28–34 mm) were bred in the laboratory under standard conditions.

Molecular procedures

Standard procedures were performed as described by Sambrook *et al.* (1989). Restriction enzymes were purchased from Boehringer Mannheim (Almere, the Netherlands).

Isolation of cDNA clones

Total RNA isolated from *Lymnaea* CNSs was converted into first strand cDNA using oligo(dT) and reverse transcriptase, as described previously (Tensen *et al.*, 1994). Two primers corresponding to DNA sequences within TMIII and TMVI of GPCRs (Libert *et al.*, 1989; Probst *et al.*, 1992), sense: 5' CCGGATCCG(CT)(GC)AT-(CT)(GA)(GC)GCIT(GT)GAC(CA)G(GC)TA-3'; antisense 5'-AC-GAATTCGG(GC)(CA)ICCA(GA)CAGAI(GC)(GA)(CT)(GA)AA-3'; with I = Inosine), were used in a PCR following conditions described earlier (Tensen *et al.*, 1994). Multiple PCR products were generated, and those of the expected size (400–800 bps) were isolated, cloned and sequenced as described (Tensen *et al.*, 1994). Oligonucleotides based on one of the obtained sequences were used to screen and isolate the corresponding full length cDNA clone from a λ ZAP II *Lymnaea* CNS cDNA library. The cDNA insert from this λ clone was *in vivo* excised as a pBluescript SK-phagemid (pBS-GRL105) and sequenced on both strands using the dideoxy chain termination method (Sanger *et al.*, 1977) with denatured double stranded DNA as a template, using the Sequenase DNA sequencing kit (United States Biochemicals, Cleveland, OH, USA). Automated sequencing was also performed with an ABI 373 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Expression of GRL105 and second messenger assays

The complete open reading frame of pBS-GRL105 was amplified by PCR (25 cycles) using 100 ng of pBS-GRL105, a thermostable enzyme with proof-reading activity (Pfu, Stratagene, La Jolla, CA, USA), and two primers based on the cDNA sequence. Sense: 5'-CCGACCTAAGCTTCCGCCATGGCCGTTCTGCTCGAGCA-3' [corresponding to a region comprising the start codon with the nucleotides surrounding the start ATG (underlined) modified to an optimal consensus sequence for translation initiation (Kozak, 1987)]. Antisense: 5'-GGCTCTAGACCCACGTCAAGAGAACTC-

GTCCATCA-3' (corresponding to a region localized 200 nts downstream of the predicted stop codon). The resulting cDNA (containing a *Hind*III restriction site at the 5' end and a *Xba*I restriction site at the 3' end, as engineered in the PCR primers) was digested with *Hind*III and *Xba*I, and cloned into the *Hind*III and *Xba*I sites of plasmid pcDNA I/neo (Invitrogen, San Diego, CA, USA). After sequence confirmation, this plasmid was transfected into CHO-K1 cells (ATCC CCL 61) using lipofectin following the manufacturers instructions (Life Technologies, Gaithersburg, MD, USA). Stably transfected cells were selected by continuous selection with the neomycin analogue G418 (400 μ g/mL) in DMEM supplemented with 10% foetal calf serum and 1% glutamine.

cAMP was assayed in whole cells (2×10^5 cells/0.2 mL reaction volume) and quantified as described by Leurs *et al.* (1994). Intracellular calcium was assayed as described previously (Cox *et al.*, 1997) and quantified using equations described by Grynkiewicz *et al.* (1985).

Extraction and HPLC purification

Lymnaea CNSs were dissected, collected on dry ice and stored at -60°C until use. CNSs were rapidly thawed at room temperature, sonicated in 1 M acetic acid over ice, centrifuged at 10 000 *g* for 5 min and the resulting supernatant was applied to a C18 solid phase extraction column (Supelclean, Supelco, Bellefonte, PA, USA). Bound material was eluted with 60% CH_3CN and 7 mM trifluoroacetic acid (TFA), and directly analysed by high-performance gel permeation chromatography (HPGPC) by application to Protein-Pak columns I-125 and I-300 connected in series (Waters Associates, Milford, CA, USA). The running buffer was 7 mM TFA in 30% CH_3CN . Obtained fractions were lyophilized, redissolved in distilled water and aliquots were tested in the bioassays. The combined fractions 18–20, obtained after HPGPC, were subjected to reversed phase HPLC (rpHPLC). First a Nucleosil C18 column (Maherey-Nagel, Germany; 250×4.6 mm) was used, applying a discontinuous linear gradient of water and CH_3CN in 7.5 mM TFA (10 min 0% CH_3CN , then from 0 to 60% CH_3CN over 60 min). After lyophilization, bioactive fractions were rechromatographed on a similar column (Nucleosil C18; 250×2.1 mm) applying a discontinuous linear gradient of water and CH_3CN in 0.05% HCl (10 min 0% CH_3CN , then from 0 to 60% CH_3CN over 60 min). In the last run, lyophilized bioactive fractions were rechromatographed using the same column and elution system, but using a more shallow gradient (in 10 min to 12% CH_3CN , then from 12 to 24% CH_3CN over 40 min).

Amino acid sequence determination

Amino acid sequences were determined with a pulse liquid automatic sequencer (Applied Biosystems, Foster City, CA, USA, model 477) and an on-line rpHPLC system (Applied Biosystems, Foster City, CA, USA, model 120A) to detect phenylthiohydantoin amino acids using sequencing programs recommended by the manufacturer.

Peptide synthesis

LyNPY and lyNPY analogues were synthesized on an Applied Biosystems (Foster City, CA, USA, model 432A) peptide synthesizer by Fmoc and tBOC chemistry, following procedures recommended by the manufacturer.

Mass determination

The precise molecular masses of peptides were obtained using a Quattro-BQ triple quadrupole mass spectrometer (Micromass, UK).

The mass spectrometer was equipped with an electrospray atmospheric pressure ionization source. The peptide was dissolved in 20 μ L of 50:50 acetonitril/water plus 1% formic acid, and the resulting solution was injected via a 10 μ L loop into the electrospray source. The flow rate was 4 μ L per min and the mobile phase was 50:50 acetonitril/water plus 1% formic acid. Data were acquired over a suitable mass range and several 10 s continuum scans were accumulated.

Results

Isolation of cDNAs encoding neuropeptide receptors in the brain of Lymnaea stagnalis

A combination of two degenerate oligonucleotide primers corresponding to amino acid sequence motifs conserved in TMIII and TMVI of many GPCRs was used to perform PCR reactions on *Lymnaea stagnalis* CNS cDNA. Sequence analysis of cloned PCR products showed that at least three of the fragments were derived from cDNA sequences encoding different GPCRs. Based on the sequence of one of these clones, a specific oligonucleotide was synthesized which was used in a PCR-based screening of a *Lymnaea* CNS-specific λ ZAP II cDNA library (Tensen *et al.*, 1994) in order to isolate an individual λ clone encoding the full-length cDNA sequence. Sequence analysis of this clone revealed a cDNA insert of 3.0 kb containing an open reading frame of 1008 bp. This sequence has been deposited in the GenBank database under accession number X82088. We propose that translation initiation takes place at the ATG at position 776–778, because the sequence context of this ATG is in good agreement with the consensus sequence for eukaryotic translation initiation sites (CCA/GCCATGG; Kozak, 1987) and stops are found in all three reading frames preceding this ATG. Conceptual translation of the open reading frame following the proposed initiation codon predicts a protein of 336 amino acids (designated GRL105; see Fig. 1), which shows defining characteristics of GPCRs. These include seven hydrophobic, putative transmembrane-spanning domains, as well as amino acid residues highly conserved among GPCRs. A search of the GenBank (Pearson & Lipman, 1988) showed that this putative GPCR has the highest homology to mammalian NPY receptors (Herzog *et al.*, 1992; Krause *et al.*, 1992; Larhammar *et al.*, 1992; Gerald *et al.*, 1995; Rose *et al.*, 1995; Gerald *et al.*, 1996; see Fig. 1).

Identification of the endogenous ligand for neuropeptide receptor GRL105

Although the *Lymnaea* receptor shows significant homology to vertebrate NPY-receptors (e.g. 28.1% to Y1, 31.1% to Y2, 28.0% to Y4, 28.5% to Y5 and 31.5% to y6 when comparing the transmembrane regions and interconnecting sequences), this itself is not sufficient to propose *a priori* that GRL105 is indeed a *Lymnaea* homologue of a vertebrate NPY-receptor. To identify the endogenous ligand for this putative *Lymnaea* NPY-receptor, the complete open reading frame of the GRL105 cDNA was cloned into a mammalian cell expression vector (pcDNA I/neo) and then stably transfected into CHO-K1 cells. Several clones were selected for further experiments. We used a biological characteristic of GPCRs, modulation of second messenger levels at the cytoplasmic face of the plasma membrane upon binding of a specific extracellular signalling molecule, as a bioassay for the identification of the endogenous ligand for GRL105. Because the receptor is expressed

in the CNS, we presumed that its endogenous ligand(s) would also be present there. Therefore, the neuropeptide content of 550 snail brains was purified using HPGPC (Fig. 2A1). Subsequently, these HPGPC fractions were tested for their ability to alter cyclic AMP (cAMP) levels in 10 stably transfected cell lines. None of the HPGPC fractions was able to stimulate an increase in cAMP levels directly. However, the pooled fractions 17–21 reduced forskolin-stimulated increase in cAMP levels to almost basal levels (Fig. 2A2) in one of the 10 cell lines tested (designated 105KCHO6). When these fractions were tested separately on 105KCHO6, the ability to reduce forskolin-stimulated cAMP levels was found to reside in fractions 18–20 (Fig. 2A3). Active material was further purified using three rounds of reversed phase HPLC (Fig. 2B–D), applying the inhibition of forskolin-stimulated cAMP increase in 105KCHO6 as a bioassay. By the use of mass spectroscopy, it was shown that both positive fractions from the final HPLC run (see Fig. 2D) contained a single peptide with either a M_w of 4249 Da (fraction 39) or 4681 Da (fraction 41). Partial protein sequencing revealed that the two peptides were identical, except for the four N-terminal amino acids that were absent in the 4249 Da peptide. Since the mass difference between both peptides was accounted for by the observed difference of four amino acids, we assume that the peptide in fraction 39 is an N-terminally truncated form of the fraction 41 peptide. For complete sequence analysis of this latter peptide, a further 500 *Lymnaea* brains were successfully processed in a similar way, thus generating the sequence of 38 amino acid residues. The calculated mass of these residues is 4535 Da, thus leaving a mass difference of 146 Da between the mass of the peptide in fraction 41 and the mass of the first 38 residues. As the mass of an amidated phenylalanine is precisely 146 Da, this is a strong indication that the fraction 41 peptide ends in a Phe residue. To really prove this assumption, we have cloned the cDNA encoding the precursor of the pertinent peptide (Tensen *et al.*, unpublished). The sequence predicted from this cDNA is fully consistent with our protein sequencing data, but continues at position 39, with a Phe followed by the sequence Gly-Lys-Arg being the common signal for processing and subsequent amidation (Bradbury & Smyth, 1991). The peptide sequence thus obtained has a clear homology to vertebrate, as well as invertebrate NPYs (Fig. 3), and therefore is designated *Lymnaea* NPY (lyNPY).

These data strongly suggest that lyNPY is the ligand for the receptor GRL105. To confirm this, lyNPY was synthesized chemically and tested for its ability to reduce forskolin-stimulated cAMP levels in 105KCHO6. Indeed, synthetic lyNPY showed potent activity (see dose–effect curve in Fig. 4A), with an EC_{50} of $6.0 \pm 1.5 \times 10^{-10}$ M, confirming the identity of lyNPY as natural ligand for the putative NPY receptor GRL105. No effect on cAMP accumulation was found in untransfected control cell lines (not shown). We also examined the effects of lyNPY on intracellular Ca^{2+} levels. In Fig. 4B, we show that stimulation of 105KCHO6 cells with lyNPY results in an increase in intracellular calcium. This dose-dependent rise in intracellular calcium is maximal at 10 nM lyNPY. Experiments with synthetic lyNPY analogues consisting of residues 3–39 and 10–39 showed that the 3–39 form can elicit a Ca^{2+} response in GRL105-transfected CHO cells, although a much higher concentration (10^{-6} M) is required for a maximal response. The 10–39 form has only a minimal effect at a concentration of 10^{-6} M, while lower concentrations were completely inactive. Several other peptides (human NPY, human PP, VIP, FMRFamide, CCK, substance P, substance K and elodisin) were tested on 105KCHO6, but were unable to elicit any calcium response even at concentrations as high as 1 μ M (not shown).

GRL 105	MSVL--LEQD AYDSSSNVTM EDLLATLNGT ENYEAWRSOW TVFAEN--KW	46
Y1	M-----NS T-LFSQ--VE NHSVHSNFSE KNAQLLAFEN DDCHLPL-AM	39
Y2	MGPIGAEADE NQTVEEMKVE QYGPQTTPRG ELVPDPEPEL IDSTKLI-EV	49
Y4	M-----NT SHLLAL--LL PKSPQGENRS KPLGTPYNFS EHCQDSV-DV	40
Y5	MSFYSKQDYN MDLELDEYYN KTLATENNTA ATRNSDFPVW DDYKSSVDDL	50
Y6	M----- EVSL NHPASNTTST KNNNSAFFYF ESCQPPS-PA	34
	* * *	
TMII		
GRL 105	QTIVITLYAV VIIFGFFANL -LVVVVIIRY KQLHTVTNIF ICYLAIADVA	96
Y1	IFTLALAYGA VIILGVSGNL -ALIIILKQ KEMRNVNINL IVNLSFSDLL	89
Y2	QVVLILAYCS IILLGVIGNS -LVIHVVIKF KSMRTVTNFF IANLAVADLL	99
Y4	MVFIVTSYSI ETVVGVGLNL -CLMCVTVRQ KEKANVTNLL IANLAFSDFL	90
Y5	QYFLIGLYTF VSLLGFMGNL -LILMALMKK RNQKTTVNFL IGNLAFSDIL	100
Y6	LLLLCIAYTV VLIVGLFGNL SLIIIFKKQ RKAQNFTSIL IANLSLSDTL	85
	* * * * *	
TMIII		
GRL 105	LCVFNLPQL HYQLSNNWMF GRVLCYVALP TFGVPLFSSS LSILMIAVDR	146
Y1	VAIMCLPFTF VYTLMDHWVF GEAMCKLNPF VQCVSITVSI FSLVLIAVER	139
Y2	VNTLCPLFTL TYTLMGEWKM GPVLCILVPY AQGLAVQVST ITLTVIALDR	149
Y4	MCLLCQPLTA VYTMDYWIF GETLCKMSAF IQCMSVTVSI LSLVLVALER	140
Y5	VVLFCSPFTL TSVLLDQWMF GKVMCHIMPF LQCVSVLVST LILISIAIVR	150
Y6	VVCMCIHFTI IYTLMDHWIF GDTMCRITSY VQSVSISVSI FSLVFTAVER	135
	* * * * *	
TMIV		
GRL 105	YMLIVYPFKK RMTNMQAII VVVIICITVA LSTPLIVYLD YV---EFVHP	193
Y1	HQLIINPRGW RPNNRHAYVG IAVIWLAVA SSLPFLIYQV MTDEPFQNV	187
Y2	HRCIVYHLES KISKRISFLI IGLAWGISAL LASPLAIFRE YSLI-EIIPD	198
Y4	HQLIINPTGW KPSISQAYLG IVLIWVIACV LSLPFLANSI LENVFHKNHS	190
Y5	YHMIKHPISN NLTAHNGYFL IATVWTLGFA ICSPLPVFHS LV---ELQET	197
Y6	YQLIVNPRGW KPSVTHAYWG ITLIWLFSLL LSPFFLSYH LTDEPFRLNS	185
	* * * * *	
TMV		
GRL 105	LMRE---QKA YCAEQWPSFL D---KQIYSV SIFLLQFVIP ICLTSFFYKH	240
Y1	L---DAYKDKY VCFDQFPSPS H---RLSYTT LLLVLQYFGP LCFIFICYFK	236
Y2	F-----EIV ACTEKWPGE E KSIYGTVYSL SLLILYVLP LGIISFSYTR	244
Y4	KALEFLADKV VCTESWPLAH H---RTIYTT FLLLQYCLP LGFILVCYAR	239
Y5	FGSALLSSRY LCVESWPSDS Y---RIAFIT SLLLVQYILP LVCLTVSHTS	247
Y6	LPTDLYTHQV ACVENWPSKK D---RLLETT SLFLLQYFVP LGFILICYLK	233
	* * * * *	
Y5	VC.....100 amino acid residues.....CF	351
TMVI		
GRL 105	ICH-VLRNRP V-----KK HDTRRNQRTN RILIAVLTFT TVCWLPLNLF	282
Y1	IYI-RLKRRN NMMDKMRDNK YRSSETKRIN IMLLSIVVAF AVCWLPLTIF	286
Y2	IWS-KLKNHV SPGAANDHYH QRRQKTTK-- -MLVCVVVF AVSWLPLHAF	291
Y4	IYR-RLQRQG RVFHKGTYS LAGHMKQVN VVLVVMVAF AVLWLPLHVF	288
Y5	EIKPEENS DV HELRVKRSVT RIKKRSRVF YRLTILILVF AVSWMPLHLF	401
Y6	IVI-CLRRRN AKVDKKKENE GRLNENKRIN TMLISIVVTF GACWLPRISS	281
	* * * * *	
TMVII		
GRL 105	ALTAEFSSHV VKGKYFTFID LMLKVFAMSS ACINPFLYGW LNDRFKKELG	332
Y1	NTVFDWNHQI IATCNHNLFF LLCHLTAMIS TCVNPIFYGF LKNKFQDLQ	336
Y2	QLAVDIDSQV LDLKEYKLIF TVFHIIAMCS TFANPLLYGW MNSNYRKAFL	341
Y4	NSLEDWHHEA IPICHGNLIF LVCHLLAMAS TCVNPIFYGF LNTNFKKEIK	338
Y5	HVVTDFNDNL ISNRHFKLVY CICHLLGMS CCLNPILYGF LNNGIKADLV	451
Y6	MSSLTGIMRC	290
	* * * * *	
GRL 105	KLFGYRLC	336
Y1	FFNFCDFRSR DDDYETIAMS TMHTDVSKTS LKQASPVAFK KINNDDNEKI	384
Y2	SAFRCEQLDA IHSESVTFK AKKNLEVRKN SGPNSDFTEA TNV	381
Y4	ALVLTCCQSAP LEESEHPLS TVHTEVSKGS LRLSGRSNPI	375
Y5	SLIHCLHM	455
	*	

Fig. 1. Sequence comparison of GRL105 with the amino acid sequences of cloned human NPY-receptors. Gaps introduced to obtain optimal alignment are indicated by dashes. Asterisks mark the positions where at least two of the aligned sequences are identical to GRL105. Bars numbered TMI to TMVII indicate the positions of the transmembrane regions. Sequences are taken from Herzog *et al.* (1992) (Y1), Lundell *et al.* (1995) (Y4), Gerald *et al.* (1995) (Y2), Gerald *et al.* (1996) (Y5) and Matsumoto *et al.* (1996) (y6).

FIG. 2. Purification of the endogenous ligand for GRL105. All assays were performed on a CHO cell line stably transfected with GRL105 cDNA (designated 105KCHO6). The top half of each panel gives the chromatogram for the HPLC purification indicated. (A1) HPGPC fractionation of an extract of 550 *Lymnaea* brains, (B1) rpHPLC fractionation of the combined fractions 18–20 from (A), (C1) rpHPLC fractionation of the combined fractions 36 and 37 from (B), (D1) rpHPLC fractionation of the combined fractions 50 and 51 from (C). The bottom half of each panel shows the effect of the indicated HPLC fractions on the forskolin-induced cAMP levels in 105KCHO6 cells (average of three determinations). The cAMP level obtained after stimulation by 10 μ M forskolin was taken as 100% [B = Basal cAMP level, F = forskolin-stimulated cAMP level (on average 22 pmol cAMP/well)]. cAMP assays as shown in (A2), (A3), (B2), (C2) and (D2) were performed using 2, 2, 4 and 20 CNS equivalents for each determination, respectively.

Discussion

Isolation of a cDNA encoding a neuropeptide receptor in the brain of *Lymnaea stagnalis* and identification of the endogenous ligands for this receptor

We have described a PCR-based cloning of a putative neuropeptide receptor-encoding cDNA from the CNS of *Lymnaea stagnalis*. Based on a sequence comparison with cloned receptors, the translated amino acid sequence (called GRL105) revealed the highest sequence identity with mammalian neuropeptide Y-receptors. In order to identify the endogenous ligand for this putative *Lymnaea* homologue of an NPY-receptor, we stably expressed the GRL105 cDNA in CHO cells. These transfected cells were challenged with partially purified

Lymnaea brain extracts, and inhibition of forskolin-stimulated cAMP accumulation was monitored. Finally, bioactive peptides were purified to homogeneity by successive rounds of rpHPLC. Using this approach, we were able to isolate two endogenous ligands for the *Lymnaea* neuropeptide receptor. Both peptides displayed considerable homology with the family of NPY and NPY-related peptides, and differed only in the N-terminal part. Most likely, one peptide is an N-terminally truncated form of the other, lacking the first four amino acids. We assume that this truncation is due to a specific degradation of the complete peptide, as a result of extraction and purification steps. The truncated form, tested for its potency in reducing forskolin-stimulated cAMP levels in 105KCHO6 cells, has only about 10% of the activity of the full length peptide (not shown).

lyNPY	TEAMLTPPERPEEFKPNPNELRKYLKALNEYAIVGRPRF-NH ₂
NPF <i>Moniezia</i>	PDKDFIVNPSDLV-LDNKAALRDYLRQINEYFAIIGRPRF-NH ₂
NPF <i>Helix</i>	STQMLSPPERPREFRHPNELRQYVKELNEYAIVGRTRF-NH ₂
NPY <i>Aplysia</i>	DNSEMLAPPPEEETSAQQLRQYLAALNEYYSIMGRPRF-NH ₂
NPY <i>Torpedo</i>	YPSKPDNPGEGAPAEEDLAKYYSALRHYINLITRQRY-NH ₂
NPY <i>Goldfish</i>	YPTKPDNPGEGAPAEELAKYYSALRHYINLITRQRY-NH ₂
NPY <i>Frog</i>	YPSKPDNPGEDAPAEEDMAKYYSALRHYINLITRQRY-NH ₂
NPY <i>Chicken</i>	YPSKPDSPGEGAPAEEDMARYYSALRHYINLITRQRY-NH ₂
NPY <i>Rat</i>	YPSKPDNPGEDAPAEEDMARYYSALRHYINLITRQRY-NH ₂
NPY <i>Human</i>	YPSKPDNPGEDAPAEEDMARYYSALRHYINLITRQRY-NH ₂
PMY <i>Lamprey</i>	MPPKPDNPSPDASPEELSKYMLAVRNVINLITRQRY-NH ₂
PYY <i>Salmon</i>	YPPKPDNPGEDAPPEELAKYYSALRHYINLITRQRY-NH ₂
PP <i>Bullfrog</i>	APSEPHHPGDDATPDQLAQYYSDDLQYITFITRPRF-NH ₂
PP <i>Guinea Pig</i>	APLEPVYPGDDATPDQMAQYAAEMRRVINMLTRPRF-NH ₂
PP <i>Turkey</i>	GPSQPTYPGDDAPVEDLIRFYDNLQOYLNVTTRHRY-NH ₂
PYY <i>Rat</i>	YPAKPEAPGEGDASPEELSRYYASLRHYINLITRQRY-NH ₂
PP <i>Sheep</i>	ASLEPEYPGDNATPEQMAQYAAELTRRYINMLTRPRY-NH ₂

FIG. 3. Amino acid sequence of lyNPY and comparison with related peptide sequences. Amino acids are given by their single-letter codes. The amino acid sequence of lyNPY is given at the top of the figure and is compared with invertebrate NPY and NPF sequences, with vertebrate NPY sequences and vertebrate NPY-related sequences. Residues identical to the corresponding ones in lyNPY are highlighted in reversed contrast, residues that are evolutionary related to lyNPY are shaded grey. The dash in the *Moniezia* NPF sequence was introduced to allow for optimal alignment. Sequences were taken from Larhammar *et al.* (1993).

FIG. 4. Dose-effect curves for synthetic lyNPY and lyNPY analogues. All assays were performed in the cell line 105KCHO6. (A) Inhibition of forskolin-induced cAMP production by different concentrations of lyNPY. The cAMP level is expressed as a percentage of the level obtained after stimulation by 10 μ M forskolin (corresponding to 24 pmol cAMP/well). Shown is a representative experiment out of three performed. (B) Effect of various concentrations of lyNPY on the intracellular Ca²⁺ concentration. The data are given as the increase in Ca²⁺ concentration and are the mean of three determinations $\pm$ SD. (C) Effect of lyNPY analogues on the intracellular Ca²⁺ concentration. The data are given as the increase in Ca²⁺ concentration and are the mean of three determinations $\pm$ SD.

The ligand for neuropeptide receptor GRL105 is a Lymnaea homologue of NPY

In vertebrates, NPY is the most abundant neuropeptide in the brain, and is known to exert a vast number of peripheral and central effects, including the regulation of blood pressure, heart rate, respiration, sexual behaviour, psychomotor activities, food intake and circadian

rhythm (Colmers & Bleakman, 1994; Grundemar & Håkanson, 1994; Larhammar, 1996a). Vertebrate NPYs (and PPs) are characterized by the presence of two peptide domains and a number of specific amino acids that are important for creating a typical three-dimensional structure of these peptides. The N-terminal part forms a polyproline helix, in which the two prolines at positions 5 and 8 seem essential

since they have been conserved (see Fig. 3). The proline at position 2 that is found in most vertebrate NPYs and PPs is likely to be of lesser importance since it is lacking in sheep PP (see Fig. 3); a corresponding proline is also absent from all invertebrate NPY-like peptides. The polypyrrolone helix is folded onto an amphipathic helix formed by the carboxy terminal 20 amino acids and interacts directly with three residues at positions 20, 24 and 27 in this carboxy-terminal region (Glover *et al.*, 1983; Glover *et al.*, 1985; Allen *et al.*, 1987). The five residues mentioned above, which are essential for the three-dimensional structure, are conserved between lyNPY, Torpedo NPY, Goldfish NPY and human NPY, suggesting that lyNPY can adopt a similar tertiary structure. A further comparison of lyNPY, other invertebrate NPY homologues, and vertebrate NPYs and PPs (Fig. 3) shows that in particular the last four residues, including the carboxy-terminal amidation, have been strongly conserved during evolution. Interestingly, this region has been shown to be essential for the biological activity of vertebrate NPYs (Lundberg *et al.*, 1990).

The Lymnaea neuro peptide receptor GRL105 is a molluscan homologue of an NPY-like-receptor

In humans, NPY acts on at least six different receptor subtypes (i.e. Y1, Y2, Y3, Y4, Y5 and a peripheral Y2-like subtype; Blomqvist & Herzog, 1997) of which the Y1, Y2, Y4 and Y5 receptors (Lundell *et al.*, 1995; Herzog *et al.*, 1992; Larhammar *et al.*, 1992; Gerald *et al.*, 1995; Rose *et al.*, 1995; Gerald *et al.*, 1996) have been cloned. The Y1 and Y2 receptors were originally distinguished on the basis of their ability to bind specific NPY subfragments: Y1 binds only the complete NPY, while Y2 also binds carboxy terminal fragments. Recently, N-terminally truncated forms of NPY were also shown to be able to inhibit forskolin-induced cAMP accumulation in HEK 293 cells (Gerald *et al.*, 1995) and Ca^{2+} mobilization in CHO cells (Rose *et al.*, 1995) transfected with the human Y2 receptor. The pharmacological profile of the Y5 receptor most resembles that of the Y1 receptor. However, the Y5 receptor can be fully activated with porcine NPY (3–36) and human PYY (3–36), analogues exerting low or no activity on cloned Y1 receptors. In addition, the Y5 receptor differs from all other NPY-receptors as it exhibits an extremely long intracellular loop between TMV and VI, and has a short C-terminal region.

We propose that GRL105 should be classified as a molluscan homologue of an NPY-like receptor based on the following: (i) the endogenous ligand for GRL105 is a *Lymnaea* counterpart of NPY; (ii) the amino acid sequence of GRL105 is most closely related to the sequence of mammalian NPY-receptors; and (iii) the signal transduction pathways and threshold concentration for activation of GRL105 expressed in CHO cells are similar to those of heterologously expressed mammalian NPY-receptors. A further classification of GRL105 as belonging to a particular subtype on the basis of its amino acid sequence seems inept, as GRL105 shares almost equal sequence identity to all NPY-receptor subtypes. The observation that N-terminally truncated forms of lyNPY have impaired or no biological activity, however, indicates that the *Lymnaea* receptor is not a Y2 subtype.

The physiological role for NPY-like peptides in invertebrate species is largely unknown, although they have been implicated in the regulation of motor functions during egg laying in the seaslug, *Aplysia californica* (Rajpara *et al.*, 1992). In addition, a receptor has been cloned from the fruitfly *Drosophila melanogaster* (Li *et al.*, 1992) exhibiting an EC_{50} of 0.3 μ M for human PYY. However, the sequence identity of this supposed *Drosophila* NPY receptor to mammalian NPY receptors is no greater than to other mammalian GPCRs

(Larhammar, 1996b). Also, an endogenous ligand for this fruitfly receptor has never been identified. In *Lymnaea*, the isolation of lyNPY and the cloning of a cDNA encoding its cognate receptor will now permit for the first time the detailed mapping of those cells that produce the ligand and those neurons that express the receptor. In addition, the advantages of the *Lymnaea* CNS as a model system, i.e. the ability to map individual neurons and determine their function in relation to control of physiological processes, as well as the possibility to reconstitute functional neuronal networks from individual neurons *in vitro* (Syed *et al.*, 1990), will now enable us to unravel the role of NPY and its cognate receptor in neuronal communication.

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Abbreviations

CNS	central nervous system
G protein	guanine, nucleotide binding regulatory protein
GPCR	G-protein-coupled receptor
HPGPC	high-performance gel permeation chromatography
NPY	neuropeptide Y
PP	pancreatic polypeptide
PYY	peptide YY
rpHPLC	reverse phase high-pressure liquid chromatography
TFA	trifluoroacetic acid

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