The pancreatic polypeptide family includes neuropeptide Y (NPY), one of the most abundant neuropeptides in the mammalian nervous system, as well as peptide YY (PYY) and pancreatic polypeptide (PP). This peptide family is involved in numerous physiological processes such as memory, pain, blood pressure, appetite, anxiety, and circadian rhythm. Of the multiple Y-type receptors proposed for PP family members, only the Y1 subtype was cloned previously. We now report the isolation of a human Y2 (hhY2) receptor cDNA by expression cloning from a human hippocampal cDNA library, using a 125I-PYY binding assay. hhY2 cDNA encodes a predicted protein of 381 amino acids with low amino acid identity to the human Y1 receptor (31% overall; 41% transmembrane). 125I-PYY binding to transiently expressed hY2 receptors was saturable (pKd = 10.17) and displaceable by human PP family members in rank order: Y2 (pKd = 9.47) > NPY (pKd = 9.27) >> PP (pKd < 6) and by peptide analogs: NPY2-36 (pKd = 8.80) > NPY13-36 (pKd = 8.55) > C2-NPY (pKd = 8.54) > NPY 26-36 (pKd = 6.51) > [Leu31,Pro34]NPY (pKd = 6.23). Human Y2 receptor (cAMP) and increased intracellular [Ca2+] in hY2 transfected 293 cells.

Expression Cloning and Pharmacological Characterization of a Human Hippocampal Neuropeptide Y/Peptide YY Y2 Receptor Subtype*

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The pancreatic polypeptide family includes neuropeptide Y (NPY), one of the most abundant neuropeptides in the mammalian nervous system, as well as peptide YY (PYY) and pancreatic polypeptide (PP). This peptide family is involved in numerous physiological processes such as memory, pain, blood pressure, appetite, anxiety, and circadian rhythm. Of the multiple Y-type receptors proposed for PP family members, only the Y1 subtype was cloned previously. We now report the isolation of a human Y2 (hhY2) receptor cDNA by expression cloning from a human hippocampal cDNA library, using a 125I-PYY binding assay. hhY2 cDNA encodes a predicted protein of 381 amino acids with low amino acid identity to the human Y1 receptor (31% overall; 41% transmembrane). 125I-PYY binding to transiently expressed hY2 receptors was saturable (pKd = 10.17) and displaceable by human PP family members in rank order: Y2 (pKd = 9.47) > NPY (pKd = 9.27) >> PP (pKd < 6) and by peptide analogs: NPY2-36 (pKd = 8.80) > NPY13-36 (pKd = 8.55) > C2-NPY (pKd = 8.54) > NPY 26-36 (pKd = 6.51) > [Leu31,Pro34]NPY (pKd = 6.23). Human Y2 receptor (cAMP) and increased intracellular [Ca2+] in hY2 transfected 293 cells.

The pancreatic polypeptide family includes neuropeptide Y (NPY),1 peptide YY (PYY), and pancreatic polypeptide (PP), all of which are 36 amino acid peptides characterized by an NH2-terminal proline polypeptide helix and a COOH-terminal α-helix brought together by a hairpin loop (1). NPY functions primarily as a neurotransmitter and is widely distributed throughout the central and peripheral nervous system, with additional local functions as a circulating hormone with increasing levels postprandially; small amounts are also found in central and peripheral neurons (1, 2, 4). PYY is known to regulate intestinal secretion and motility, as well as emesis (5, 6). PYY and NPY act similarly in a majority of physiological models (e.g. to stimulate feeding and increase blood pressure), but exceptions have been noted (1). PP is localized primarily in endocrine cells of pancreatic islets and exerts regulatory effects on gastrointestinal processes such as pancreatic exocrine secretion, gall bladder contraction and gastric emptying (7, 8).

NPY and related family members are proposed to activate at least five receptor subtypes (1, 2): 1) Y1 binds NPY, PYY, and [Leu31,Pro34]NPY > PP and COOH-terminal fragments; 2) Y2 binds NPY, PYY, and COOH-terminal fragments > PP and [Leu31,Pro34]NPY; 3) Y3 binds NPY > PYY; 4) the PP receptor binds PYY > [Leu31,Pro34]NPY > NPY; and 5) the putative hypothalamic Y1-like feeding receptor is activated by NPY, PYY, [Leu31,Pro34]NPY, and NPY2-36 > COOH-terminal fragments. Only the Y1 subtype was reported previously to be cloned (9–13). We describe here the expression cloning and pharmacological characterization of a human hippocampal Y2 receptor.

MATERIALS AND METHODS

Cloning and Sequencing—Total RNA was prepared by a modification of the guanidine thiocyanate method (14) from 6 g of human hippocampus. Poly(A)+ RNA was purified with a FastTrack kit (Invitrogen Corp., San Diego, CA). Double-stranded (ds) cDNA was synthesized from 4 μg of poly(A)+ RNA according to Gubler and Hoffman (15), except that ligase was omitted in the second strand cDNA synthesis. After size selection, high molecular weight fractions were ligated in pEXJ.BS (an Okayama and Berg expression vector) cut by BstXI, as described by Aruffo and Seed (16). The ligated DNA was electroporated in Escherichia coli MC 1061 (Gene Pulser, Bio-Rad). The library (2.2 × 106 cfu; 3-kb average insert size) was plated on Petri dishes (ampicillin selection). Only the Y1 subtype was reported previously to be cloned (9–13). We describe here the expression cloning and pharmacological characterization of a human hippocampal Y2 receptor.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) U36269.

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1 The abbreviations used are: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; ds, double-stranded; kb, kilobase pair(s); DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PCR, polymerase chain reaction; hhY2, human hippocampal Y2; MTN, multiple tissue Northern blots; HBS, Hanks’ buffered saline; GPCR, G-protein-coupled receptor.
Human Y2 Receptor

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Fig. 1. Alignment of the deduced amino acid sequence of the human hippocampal Y2 receptor (GenBank™ U36269) with the human Y1 and Y4 receptor sequences. The Pile Up algorithm (Genetics Computer Group) was used for the alignment with the human Y1 receptor (9, 10) and the human Y4 receptor (see accompanying manuscript (46)). Putative transmembrane domains are boxed and numbered I–VII. Residues identical between the Y1, Y2, and Y4 receptors are shaded in gray.

Results and Discussion

In order to clone a human NPY/PYY Y2 receptor subtype, we used an expression cloning strategy in COS-7 cells (23–25). Because the Y2 receptor is described as a presynaptic receptor, it is difficult to locate cell bodies that actually contain this specific mRNA in restricted brain areas. We reasoned that human hippocampus was a good source of mRNA, because it contains both a large number of interneurons and has been shown to carry a particularly dense population of Y2 receptors (26–29). A single clone encoding a Y2 receptor (designated hhY2) was isolated from a human hippocampal cDNA library (see “Materials and Methods”). The isolated clone carries a 4.2-kb cDNA. This cDNA contains an open reading frame between nucleotides 1003 and 2145 that encodes a 381-amino-acid acid protein. The flanking sequence around the putative initiation codon conforms to the Kozak consensus sequence for optimal translation initiation (30, 31). The hydrophobicity plot displayed seven hydrophobic, putative membrane-spanning regions which make the human hippocampal Y2 receptor a member of the G-protein-coupled superfamily. The deduced hhY2 amino acid sequence and an alignment with the human Y1 and Y4 receptors (see accompanying paper by Bard et al. (46)) are shown in Fig. 1. The hhY2 receptor presents features common to most members of the GPCR family (32, 33), including a potential N-linked glycosylation site and multiple potential phosphorylation sites in the putative intracellular loop regions. In addition, it carries transmembrane amino acid residues highly conserved within the neuropeptide receptor family (34). Interestingly, the two amino acid residues “RY” downstream of transmembrane domain 3, found in almost all known GPCR sequences (32), are replaced by “RH” in all three Y1, Y2, and Y4 receptor amino acid sequences (Fig. 1). As seen in Fig. 1, the hhY2 amino acid sequence shows a surprisingly low overall identity of 31% with the Y1 receptor. The alignment scores for the TM domains are 41 and 43% identity with the Y1 and the Y4 receptors, respectively. When compared with other neuropeptide GPCR sequences, the amino acid transmembrane domains identities are very low, ranging from 26% (human bradykinin (2, 35)) to 33% (human neurokinin 1 and PR4, a Y2-like Drosophila receptor (36, 37)) with the notable exception of the orphan sequence MUSGIR score at 43% (38). Using the human Y2 probe, Northern hybridizations reveal a unique

Manufacturer. Nucleotide and peptide sequences analysis were performed with GCG programs (Genetics Computer Group, Madison, WI). Northern and Southern Blots—Multiple tissue Northern blots (brain MTAL, MTN, and MTN IL, colliculus, and brain stem, CA) carrying mRNA purified from various human brain areas or from various peripheral tissues were hybridized at high stringency according to the manufacturer’s specifications. The probe was a 1.15-kb DNA fragment generated by PCR, corresponding to the entire coding region of the human Y2 receptor. The probe was labeled with [32P]dATP and a random-primed DNA labeling kit (specific activity: 2 × 10⁸ cpm/μg; Pharmacia). A Southern blot (Geno-blot, Clontech) containing human genomic DNA cut with five different restriction endonucleases was hybridized at high stringency according to the manufacturer’s specification. The probe, corresponding to the TM1–TM5 coding region, was labeled with [32P]dATP and a random-primed DNA labeling kit (specific activity: 1 × 10⁹ cpm/μg; Pharmacia).

Cdl Culture—Stock plates of COS-7 (African green monkey kidney) and 293 (human embryonic kidney) cells were grown on 150-mm plates in DMEM with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin) at 37°C, 5% CO₂ and split 1:6 with trypsin every 3–4 days.

Receptor Expression—COS-7 cells were transiently transfected with the unmodified hhY2 construct (expression vector containing the hhY2 gene) or with human Y1 receptor (hY1) DNA by a DEAE-dextran method (18) or without plasmid for mock-transfection. For stable expression, plasmid hhY2 was co-transfected with a G418-resistant plasmid (see “Materials and Methods”). The isolated clone carries a 4.2-kb cDNA. This cDNA contains an open reading frame between nucleotides 1003 and 2145 that encodes a 381-amino-acid acid protein. The flanking sequence around the putative initiation codon conforms to the Kozak consensus sequence for optimal translation initiation (30, 31). The hydrophobicity plot displayed seven hydrophobic, putative membrane-spanning regions which make the human hippocampal Y2 receptor a member of the G protein-coupled superfamily. The deduced hhY2 amino acid sequence and an alignment with the human Y1 and Y4 receptors (see accompanying paper by Bard et al. (46)) are shown in Fig. 1. The hhY2 receptor presents features common to most members of the GPCR family (32, 33), including a potential N-linked glycosylation site and multiple potential phosphorylation sites in the putative intracellular loop regions. In addition, it carries transmembrane amino acid residues highly conserved within the neuropeptide receptor family (34). Interestingly, the two amino acid residues “RY” downstream of transmembrane domain 3, found in almost all known GPCR sequences (32), are replaced by “RH” in all three Y1, Y2, and Y4 receptor amino acid sequences (Fig. 1). As seen in Fig. 1, the hhY2 amino acid sequence shows a surprisingly low overall identity of 31% with the Y1 receptor. The alignment scores for the TM domains are 41 and 43% identity with the Y1 and the Y4 receptors, respectively. When compared with other neuropeptide GPCR sequences, the amino acid transmembrane domains identities are very low, ranging from 26% (human bradykinin (2, 35)) to 33% (human neurokinin 1 and PR4, a Y2-like Drosophila receptor (36, 37)) with the notable exception of the orphan sequence MUSGIR score at 43% (38). Using the human Y2 probe, Northern hybridizations reveal a unique
band at 4.3 kb in human brain after a 3-day exposure (Fig. 2A).
This is in good agreement with the 4.2-kb CDNA that we iso-
lated by expression cloning and indicates that our cDNA clone
is full length or nearly full length. The mRNA encoding the
human Y2 receptor subtype is present in significant amounts
in amygdala, corpus callosum, hippocampus, and subthalamic
nucleus. A faint band is detectable in caudate nucleus, hypo-
thalamus, and substantia nigra. No signal could be detected in
thalamus. It should be noted that the Clontech brain MTN blot
does not contain any mRNA from cortex or brain stem. No
detectable signal was observed on Northern blots containing
mRNA extracted from human peripheral tissues after an 8-day
exposure (data not shown). Southern hybridizations to human
genomic DNA followed by high stringency washes (Fig. 2B)
suggest that the human genome contains a single Y2 receptor
gene (single band with EcoRI, HindIII, BamHI, and PstI). The
definite bands at 9 and 12 kb observed with BglII can be explained
by the presence of two BglII restriction sites in the coding
region of the Y2 sequence and are also consistent with a single
Y2 receptor gene.

Characterization of the novel receptor cloned from pool 189
was accomplished using radioligand binding and functional
assays. 125I-PYY (0.06 nM) bound specifically to membranes from
hhY2-transfected COS-7 cells (but not from mock-transfected
cells) at 30 °C. The association curve was biphasic, with
approximately 55% of the specific binding following a rapid
time course (k_{obs} = 1.28 ± 0.02 min⁻¹, t½ = 0.5 min) and 45% fol-
lowing a slower time course (k_{obs} = 0.02 ± 0.00 min⁻¹, t½ = 37 min).
Equilibrium binding composed of both phases was 95%
complete within 120 min and 100% complete within 240 min.
The biphasic time course suggests the possibility of multiple
conformations for the receptor ligand complex. hY1-transfected
COS-7 cell membranes, when studied under the same con-
ditions, yielded a monophasic association curve with k_{obs} = 0.06
± 0.02 min⁻¹, t½ = 12 min, and 100% complete equilibrium
binding within 90 min (n = 3). Subsequent 125I-PYY binding
assays involving both hY1 and hhY2 receptors were conducted
for 120 min. 125I-PYY binding to the transiently expressed
hhY2 receptor was specific and saturable at 125I-PYY concen-
trations ranging from 0.5 pM to 3.0 nM. Binding data were fit to
a one-site model with an apparent K_{D} = 10.17 ± 0.05 (0.067
nM) and B_{max} = 7.7 ± 0.7 pmol/mg membrane protein (n = 5).
The transiently expressed hhY2 receptor binding 125I-PYY with an
apparent K_{D} = 10.19 ± 0.04 (0.065 nM) and B_{max} = 4.0 ± 0.7
pmol/mg membrane protein (n = 9).

hhY2 bound human PP family members in 125I-PYY mem-
brane binding assays in rank order (Table I): PYY > NPY >

| Peptide | hY1 pK_{I} | hY2 pK_{I} | hY2 CAMP pEC_{50} |
|---------|-----------|-----------|------------------|
| NPY, human | 10.06 ± 0.05 | 9.27 ± 0.05 | 9.60 ± 0.17 |
| NPY, porcine | 10.18 ± 0.11 | 9.07 ± 0.07 | 9.86 ± 0.10 |
| NPY, human | 8.61 ± 0.11 | 8.80 ± 0.06 | 9.46 ± 0.13 |
| NPY, porcine | 8.91 ± 0.10 | 8.63 ± 0.06 | 9.14 ± 0.16 |
| NPY, human | 7.16 ± 0.07 | 8.55 ± 0.08 | 8.64 ± 0.15 |
| NPY, porcine | 7.38 ± 0.08 | 8.18 ± 0.11 | 8.75 ± 0.19 |
| NPY, human | 6.67 ± 0.12 | 8.03 ± 0.08 | 8.68 ± 0.20 |
| NPY, porcine | 7.20 ± 0.03 | 8.45 ± 0.05 | 9.17 ± 0.10 |
| NPY, pNP, porcine | <6 | 7.43 ± 0.11 | 8.95 ± 0.10 |
| NPY, human | 9.79 ± 0.09 | 6.23 ± 0.04 | <6.5 |
| NPY, free acid | 6.31 ± 0.11 | <6 |
| PYY, human | 9.77 ± 0.06 | 9.47 ± 0.06 | 9.47 ± 0.15 |
| PYY, porcine | 9.86 ± 0.10 | 9.45 ± 0.07 | 9.65 ± 0.10 |
| PYY, human | 7.34 ± 0.06 | 9.17 ± 0.05 | 8.84 ± 0.35 |
| PYY, porcine | 7.48 ± 0.07 | 8.93 ± 0.05 | 8.95 ± 0.10 |
| [Leu{sup}6,Pro{sup}14]{sup}NPY, human | 9.84 ± 0.13 | <6 |
| [Leu{sup}6,Pro{sup}14]{sup}PP, human | 7.10 ± 0.08 | <6 |
| [Ile{sup}4,Pro{sup}14]{sup}NPY, human | 3.73 ± 0.15 | 7.70 ± 0.07 |

PP. pK_{I} values were in close range for parent compounds and
their COOH-terminal fragments. Noteworthy ligands include
PYY{sup}3–36, a major form of PYY-like immunoreactivity in human
plasma (39), and also C2-NPY, a Cys2 to Cys27 disulfide-stabi-
lized derivative with an 8-amino octanoic linker replacing
NPY{sub}22–36, porcine 7.20

TABLE I
Pharmacological profile of the cloned human hippocampal Y2
receptor and comparison with the cloned human Y1 receptor

IC_{50} values from competitive displacement of 125I-PYY binding to
membranes from COS-7 cells transiently transfected with hY2 (or for
comparison, hY1) were converted to pEC_{50} values according to the
Chang-Prusoff equation, K_{D} = IC_{50}/[L]/K_{D}, and reported as pK_{D} ± S.E. (n ≥ 3). E_{max} values derived from inhibition of forskolin-stimulated cAMP
accumulation in intact 293 cells stably transfected with hY2 are re-
ported as pEC_{50} ± S.E. (n ≥ 3).
receptor family (1, 39; see also Bard et al. (46)). Intracellular free \([\text{Ca}^{2+}]\) was increased by 1 \(\mu\text{M}\) human PYY in 293 cells stably transfected with hY2 and species homologs in a variety of transfected cells. hhY2 closely resembles pharmacologically defined Y2 receptors in a variety of cell and tissue models (1). Y2 receptors were first cloned in 1987 by mammalian peptides (PYY, NPY, C2-NPY, [Pro34]NPY, and PP) at concentrations between 0.03 and 3 \(\mu\text{M}\) with a Y2-like rank order, but functional invertebrate ligands were not identified. As there have been no published reports of an NPY analog in Drosophila, classification of PR4 as Y2-like could reasonably be viewed as tentative.

In summary, we have cloned the gene for a novel human hippocampal Y-type receptor and generated a pharmacological profile using NPY and related peptide family members. hhY2 and hY2 both have a unique pharmacological profile compared to other Y2 receptor subtypes. Although the exact function of these receptors remains unknown, their expression in the brain suggests a role in regulating neuronal function through G protein-coupled receptor signaling pathways.