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Cloning and characterisation of the human 5-HT_{5A} serotonin receptor

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Abstract The human 5-HT_{5A} serotonin receptor has been cloned. As with the mouse and rat 5-HT_{5A} receptors, the gene consists of two coding exons separated by a large intron. The deduced amino acid sequence of the gene reveals a protein of 357 residues which shares 93% (nucleotide) and 84% (amino acid) identity to the cloned mouse 5-HT_{5A} receptor. We have determined the tissue distribution of the receptor by reverse transcriptase-PCR and found expression in all regions of the brain examined with little or no expression in peripheral tissues. The receptor has been transiently expressed in Cos M6 cells and exhibits a pharmacological profile closely resembling the mouse and rat 5-HT_{5A} receptors with high, specific binding for ergotamine and methiothepin.

Key words: G protein coupled receptor; Serotonin; 5-Hydroxytryptamine

1. Introduction

The neurotransmitter 5-hydroxytryptamine (5-HT; serotonin) modulates a wide range of physiological and pathological processes in the central nervous system and periphery, including anxiety, sleep regulation, aggression, feeding and depression [1]. Both pharmacological characterisation and, more recently, molecular cloning of several 5-HT receptor genes has revealed that 5-HT mediates its diverse physiological actions through a multiplicity of receptor subtypes [2]. These receptors belong to at least two different protein superfamilies; ligand- gated ion channel receptors (5-HT_3) and the G protein-coupled 7-transmembrane receptor superfamily (thirteen distinct receptors cloned to date) [1]. In addition, within the G protein-coupled 7-transmembrane receptor superfamily, serotonin exerts its actions through a multiplicity of signal transduction mechanisms; for example, in recombinant systems the 5-HT_{1A} receptor has been demonstrated to couple to inhibition of adenylate cyclase, generation of inositol phosphate, and to an inward rectifying potassium channel [1].

Recently two members of a new subfamily of serotonin receptors has been identified. These have been cloned from mouse and rat and designated 5-HT_{5A} and 5-HT_{5B} [2–5]. The two mouse receptors share 77% amino acid homology [2]. By pharmacological criteria these receptors fall into the 5-HT_1-like class because of their antagonism by methiothepin and agonism by 5-carboxytryptamine [1]. However, there are several reasons why these receptors have been placed in a distinct class. The sequence is not closely related to that of any previously known serotonin receptor, with the best homology being 41% with the *Drosophila* 5HT_{dro2A} receptor [6] and less than 37% to members of the 5-HT_1 family [1]. Additionally the mouse and rat 5-HT_5 genes have an intron between putative transmembrane domain V and VI, in contrast with the 5-HT_1 receptor genes which are intronless [1]. Unlike the 5-HT_1 family of receptors, which all show negative coupling to adenylate cyclase through an inhibitory G protein signal transduction complex [1], expression of 5-HT_{5A} receptors in recombinant mammalian cell lines has failed to demonstrate any G protein coupling [2,3,5], suggesting that these receptors may have a novel signal transduction mechanism. There is a single report of coupling to the rat 5-HT_{5B} receptor to a G protein in COS1 cells, although the nature of the second messenger response is unclear [4]. Similarly there are no known functional correlates of 5-HT_5 receptor activation, although it appears likely that they may perform some functions within the CNS previously attributed to the 5-HT_{1D} family, such as motor control, feeding and anxiety [1,2].

In order to investigate further the role of this novel receptor subfamily we have cloned the human 5-HT_{5A} serotonin receptor. We present an analysis of the pattern of expression of the receptor and an initial pharmacological characterisation.

2. Experimental

2.1. Isolation of genomic clones

A 378 bp fragment of human 5-HT_{5A} was amplified, from genomic DNA, using the polymerase chain reaction (PCR) with degenerate oligonucleotide primers, 5'-GT(G/C)CT(A/G)GTGGC(T/G)GT(G/T)-CTGGT(T/G)ATGCC-3' and 5'-AG(G/A)TAGAAGGC(A/T)CC(A/C/G/T)(A/C)(A/C)GGTGGA(A/G)AA-3', designed from an alignment of the mouse 5-HT_{5A} and 5-HT_{5B} receptor sequences [3]. This fragment of 5-HT_{5A} spans putative transmembrane domains II–V and represents part of exon 1 of the gene. The PCR product was subcloned into pcDNA1 (Invitrogen) and its sequence was found to be 92% identical to mouse 5-HT_{5A} over this region [3]. The rat 5-HT_{5B} cDNA was obtained from Dr. M. Voight [5].

The 378 bp human 5-HT_{5A} fragment and the rat 5-HT_{5B} cDNA were labelled with [α-^{32}P]dCTP (Amersham) by random priming (Stratagene) and used to screen a HepG2 human liver cell line genomic library constructed in λ-FIX (Stratagene). Filter lifts were made onto Hybond-N membrane (Amersham) and hybridisation performed at 60°C for 16 h in 10% dextran sulphate, 1 M NaCl, 1% sodium dodecyl sulphate (SDS). Filters were then washed at 60°C in 1 × SSC, 0.1% SDS. Seven positive clones were detected from 4×10^6 independent plaques. The inserts were characterised by restriction analysis followed by Southern blotting, and small hybridising fragments subcloned into pBluescript (Stratagene).

2.2. Sequencing

Sequence analysis was performed directly on double-stranded plasmid DNA using the Sequenase Version 2.0 DNA sequencing kit [7] (USB). Sequence editing, alignment and comparisons were performed with the Genetics Computer Group software (University of Wisconsin).

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The nucleotide sequences reported in this paper have been submitted to the EMBL database with accession numbers X81411 and X81482.

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2.3. Construction of a full-length clone

Exon 1 was found to lie on a 4.1 kb *Eco*RI–*Hin*dIII restriction fragment and exon 2 on a 1.0 kb *Eco*RI fragment. No single phage was isolated containing both exons. To verify that the two exons represent the same gene, oligonucleotide primers PCR1 (5′-ACTCTCCGCTG-TCATCTCTCTGG-3′), designed from the exon 1 sequence, and PCR2 (5′-TGGCGTGGCGGACCGTGAACAGG-3′), designed from the exon 2 sequence (see Fig. 2), were used to amplify a 293 bp fragment by PCR from human hippocampus cDNA (Clontech). The sequence of this fragment agreed exactly with the genomic sequence obtained previously, confirming the two exons to be the same gene, the gene to be expressed and the precise position of the intron/exon boundary.

In order to join the two exons together, exon 1 was amplified from the genomic fragment by PCR using primers 5′-TGACGAATTCAT-GGATTTACCAGTGAACCTAACC-3′ and 5′-CCCACGTCGACA-GCTTCGGATATGGGTGAG-3′, and the resulting product was digested with *Eco*RI and *Sal*I. Exon 2 was similarly amplified using primers 5′-CTTTTCTCGAGGTGAACGACTCTGCCAAC-3′ and 5′-TGGTGAATTCCCTCAGTGTTGCCTAGAAAAG-3′, and the PCR product digested with *Xho*I and *Eco*RI. The two products were ligated and subcloned into the *Eco*RI site of the expression vector pcDNA4 (a derivation of pcDNA3 (Invitrogen) containing a synthetic intron [8] in the multiple cloning site). The open reading frame was re-sequenced following PCR.

2.4. Cell culture and homogenate preparation

Cos M6 cells maintained at 37°C with 5% CO_2 and 92% humidity, in Dulbecco's modified Eagle's medium, supplemented with 10% foetal calf serum, 1% glutamine and 1% non-essential amino acids (all reagents from Gibco), were transfected with the pcDNA4/5-HT_{5A} expression vector using the DEAE Dextran procedure [9]. Two days post-transfection, cells were detached by incubation in phosphate buffered saline (PBS) containing 5 mM EDTA and harvested by centrifugation at 4500 × *g* for 35 min (Sorvall RC3). The cell pellet was re-suspended in assay buffer (100 mM Tris-HCl, 1 mM EDTA and 1% ascorbic acid; pH 7.7) and counted before homogenisation (Ultraturax). Homogenates were diluted in assay buffer to yield a concentration of 1×10^7 cells/ml.

2.5. Radioligand binding assay

The following conditions were used for radioligand binding: cell homogenate (750 μl), [^{3}H]5-CT (1.0 nM final concentration, 200 μl) and competing drug or assay buffer (50 μl) were incubated at 37°C for 30 min. Incubations were terminated by rapid filtration (Tomtec; 48 well harvester) through Printed Filtermat B filters pre-soaked in 0.1% PEI (polyethyleneimine) for 1 h. The filters were washed four times with 1 ml ice-cold assay buffer. Retained radioactivity was measured (1204 Betaplate BS solid scintillation counter) after melting the dried filters into solid scintillation wax. Under these conditions, specific [^{3}H]5-CT binding (defined by inclusion of 10 μM 5-CT) represented greater than 90% of total binding. Competition and ligand binding data were analysed by using non-linear curve fitting techniques (RS1, Radlig).

2.6. Drugs used

5-Hydroxytryptamine creatinine sulphate (Sigma), methysergide hydrogen-maleate (Sandoz), yohimbine hydrochloride (Sigma), ergotamine tartrate (Sigma), (−)propranolol-HCl (Sigma), RU 24969 (Roussel Uclaf), 8-hydroxy-dipropyl-aminotetralin (RBI), metergoline (Farmitalia), ketanserin (Sigma) and [^{3}H]5-carboxyamidotryptamine (NEN-DuPont). 5-Carboxyamidotryptamine maleate, methiothepin maleate, zacopride and sumatriptan were synthesised by the Chemistry Research Department at Glaxo Research & Development. All drugs used were initially dissolved in a mixture of pure DMSO and 1 M HCl (5:1). Further dilution's were performed in assay buffer.

2.7. Tissue distribution

Reverse transcriptase-PCR (RT-PCR) was performed using the GeneAmp RNA PCR kit (Perkin-Elmer) on 100 ng of human poly(A)$^+$

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                                                                    1
-64   cccctaagtcctcttgaacacccctttctgcaagtaccccagggcggtctcctgacccagagATGGATTTACCAGTGAACCTAACCTCCTTTTCCCTCT
                                                                    M  D  L  P  V  N  L  T  S  F  S  L   12

36    CCACCCCCTCCCCTTTGGAGACCAACCACAGCCTCGGCAAAGACGACCTGCGCCCCAGCTCGCCCCTGCTCTCGGTCTTCGGAGTGCTTATTCTCACCT
      S  T  P  S  P  L  E  T  N  H  S  L  G  K  D  D  L  R  P  S  S  P  L  L  S  V  F  G  V  L  I  L  T   45
                                                                                                TM I
135   TGCTGGGCTTTCTGGTGGCGGCGACGTTCGCCTGGAACCTGCTGGTGCTGGCGACCATCCTCCGTGTACGCACCTTCCACCGCGTGCCCCACAACCTGG
      L  L  G  F  L  V  A  A  T  F  A  W  N  L  L  V  L  A  T  I  L  R  V  R  T  F  H  R  V  P  H  N  L   78
                                                                                                      TM II
234   TGGCATCCATGGCCGTCTCGGATGTCCTGGTGGCCGCGCTGGTCATGCCGCTGAGCCTGGTGCATGAGCTGTCCGGGCGCCGCTGGCAGCTAGGTCGGA
      V  A  S  M  A  V  S  D  V  L  V  A  A  L  V  M  P  L  S  L  V  H  E  L  S  G  R  R  W  Q  L  G  R  111

333   GGCTGTGCCAGCTTTGGATCGCGTGCGACGTGCTTTGCTGCACGGCCAGCATCTGGAACGTGACGGCCATAGCCCTGGACCGCTACTGGTCCATCACGC
      R  L  C  Q  L  W  I  A  C  D  V  L  C  C  T  A  S  I  W  N  V  T  A  I  A  L  D  R  Y  W  S  I  T  144
                  TM III                                                          PCR primer 1 ⇒
432   GCCACATGGAATACACGCTCCGCACCCGCAAGTGCGTCTCCAACGTCATGATCGCGCTCACCTGGGCACTCTCCGCTGTCATCTCTCTGGCCCCGCTGC
      R  H  M  E  Y  T  L  R  T  R  K  C  V  S  N  V  M  I  A  L  T  W  A  L  S  A  V  I  S  L  A  P  L  177
                                                TM IV
531   TTTTTGGCTGGGGAGAGACGTACTCTGAGGGCAGCGAGGAGTGCCAGGTAAGCCGCGAGCCTTCCTACGCCGTGTTCTCCACCGTAGGCGCCTTCTACC
      L  F  G  W  G  E  T  Y  S  E  G  S  E  E  C  Q  V  S  R  E  P  S  Y  A  V  S  F  T  V  G  A  F  Y  210
                                                                   TM V
630   TGCCGCTCTGTGTGGTGCTCTTCGTGTACTGGAAGATCTACAAGGCTGCCAAGTTCCGCGTGGGCTCCAGGAAGACCAATAGCGTCTCACCCATATCCG
      L  P  L  C  V  V  L  F  V  Y  W  K  I  Y  K  A  A  K  F  R  V  G  S  R  K  T  N  S  V  S  P  I  S  243
               740
729   AAGCTGTGGAGgtgggtatctcagcaatccttaaaaatgcccgactt.....INTRON >5Kb.....ccaggcctgtcagtgtccaggctc
      E  A  V  E                                                                                        247
                                                                741                    ⇐ PCR primer 2
      agcctaacaggtagactgaggtggctcctcataaagctcctgcttgtcttttacagGTGAAGGACTCTGCCAAACAGCCCCAGATGGTGTTCACGGTCC
                                                                V  K  D  S  A  K  Q  P  Q  M  V  F  T  V   261

784   GCCACGCCACCGTCACCTTCCAGCCAGAAGGGGACACGTGGCGGGAGCAGAAGGAGCAGCGGGCCGCCCTCATGGTGGGCATCCTCATTGGCGTGTTCG
      R  H  A  T  V  T  F  Q  P  E  G  D  T  W  R  E  Q  K  E  Q  R  A  A  L  M  V  G  I  L  I  G  V  F  294
                                                                   TM VI
883   TGCTCTGCTGGATCCCCTTCTTTCTCACCGAGCTCATCAGTCCCCTCTGCTCCTGTGACATCCCCGCCATCTGGAAAAGCATCTTCCTGTGGCTTGGCT
      V  L  C  W  I  P  F  F  L  T  E  L  I  S  P  L  C  S  C  D  I  P  A  I  W  K  S  I  F  L  W  L  G  327
                                                                                          TM VII
981   ACTCCAACTCCTTCTTTAACCCCCTGATCTATACGGCTTTCAACAAGAACTACAACAGCCGCTTCAAGAACTTCTTTTCTAGGCAACACTGAgggagag
      Y  S  N  S  F  F  N  P  L  I  Y  T  A  F  N  K  N  Y  N  S  A  F  K  N  F  F  S  R  Q  H  *        357

1080  ggattgatggaattc
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Fig. 1. Nucleotide and deduced amino acid sequence of the human 5-HT_{5A} gene. Exon sequence is in upper case; intron and flanking sequence in lower case. The seven putative transmembrane domains of the receptor are underlined and numbered I to VII. Three stop codons, one in each frame, upstream of the start codon are in bold type. The position of PCR primers 1 and two (see section 2) are indicated with arrows.

RNA (Clontech) with primers PCR1 and PCR2 (Fig. 1), which span the 5-HT_{5A} intron, amplifying 293 bp from mRNA and >5 kb from genomic DNA. PCR products were separated by agarose gel electrophoresis and blotted onto Hybond-N membrane (Amersham) prior to hybridisation with a ^{32}P-labelled human 5-HT_{5A} gene probe.

3. Results

3.1. Cloning of human 5-HT_{5A} genomic sequences

A 378bp fragment of human 5-HT_{5A}, spanning putative transmembrane domains II–V, together with the rat 5-HT_{5B} cDNA, was radiolabelled and used as a hybridisation probe to screen a human genomic library for 5-HT_5-like sequences. Seven strongly hybridising clones were isolated and characterised by restriction analysis followed by Southern blotting and hybridisation with single exon probes. These studies revealed no clone that contained both exons. However, small hybridising fragments of each λ phage insert were subcloned and sequenced. A single clone contained a 741 bp open reading frame which shows 83% homology to exon 1 of mouse 5-HT_{5A}; a further two clones contained a 333 bp open reading frame with 85% homology to exon 2 of mouse 5-HT_{5A} (see Fig. 1) [3]. The site of the intron/exon boundary is in an identical position to that in the mouse 5-HT_{5A} gene [3]. To confirm that these clones represent two exons of the same gene, PCR primers were designed flanking the site of the putative intron. RT-PCR analysis of human hippocampus cDNA resulted in the amplification of the expected 293 bp RNA-specific fragment, which was sequenced and shown to be identical to the cloned human 5-HT_{5A} gene (data not presented). This confirms the position of the junction between exons 1 and 2. From restriction analysis of the original λ phage the intron appears to be at least 5 kb long. The start codon was assigned according to homology with mouse 5-HT_{5A}. There are three alternative stop codons, one in each frame, 5′ to this start codon (see Fig. 1), suggesting that the assigned ATG is correct.

Table 1
Comparison of the pharmacological profile of the human and mouse 5-HT_{5A} serotonin receptors

	pKi values	
	Human	Mouse
5-HT	6.9 (8)	6.6 (7)
5-CT	7.7 (7)	7.8 (3)
Ergotamine	8.7 (5)	8.4 (2)
Sumatriptan	5.3 (5)	4.8 (2)
Methiothepin	8.9 (3)	7.0 (2)
Methysergide	7.0 (4)	7.2 (5)
Ketanserin	4.7 (3)	4.8 (2)
Metergoline	6.2 (3)	–
Yohimbine	5.3 (3)	6.0 (2)
(-)Propranolol	5.1 (3)	4.7 (2)
RU 24969	6.0 (3)	6.5 (2)
8-OHDPAT	5.6 (5)	5.9 (2)
Clozapine	6.0 (4)	–
Zacopride	4.8 (3)	–
Spiperone	4.2 (3)	–
Pindolol	4.9 (3)	–
Melatonin	< 4 (2)	–
Noradrenaline	< 4 (2)	–

Numbers in parenthesis correspond to the number of independent experiments with each point measured in duplicate. Human studies were performed with [^{3}H]5-CT; mouse studies with [^{125}I]LSD [12].

3.2. Expression and pharmacological characterisation of the human 5-HT_{5A} receptor

The two exons were joined together, using a PCR strategy, to produce a single open reading frame, which was cloned into the expression vector pcDNA4. The ligand binding characteristics of the receptor were investigated with the ligand [^{3}H]5-CT following transient transfection of the 5-HT_{5A} expression vector into Cos M6 cells. Over a range of ligand concentrations (0.2–14 nM), specific binding was observed. Non-specific binding increased linearly with ligand concentration. Scatchard analysis of the specific binding data revealed a single site wth a K_D value of 4.0 nM and B_{max} of 241 fmol/mg (data not shown). A ligand concentration of 1 nM was used in all displacement experiments. The binding affinity constants (pK_i) for the competition curves of various ligands are given in Table 1.

3.3. Tissue distribution analysis of human 5-HT_{5A} receptor expression

The pattern of expression of the human 5-HT_{5A} serotonin receptor was determined by RT-PCR using oligonucleotides PCR1 and PCR2 (Fig. 1). The 293 bp RNA specific product was amplified from every brain tissue examined (amygdala, caudate nucleus, cerebellum, hypothalamus, substantia nigra and thalamus; Fig. 2). A weaker signal was detected in fetal brain. Expression was not seen in any peripheral tissue (aorta, heart, kidney, liver, small intestine, spleen and uterus). This pattern of expression is similar to that seen in mouse and rat [3,4] and suggests a role for 5-HT_{5A} in CNS function.

4. Discussion

We have cloned the human 5-HT_{5A} serotonin receptor from genomic DNA. The gene consists of two exons separated by a large intron positioned in the third intracellular loop of the deduced amino acid sequence. Removal of the intron creates a 1071 bp open reading frame which would encode a 357 amino acid protein, the same length as the rat and mouse 5-HT_{5A} receptors [3,4]. Hydropathy analysis indicates this protein to posess seven hydrophobic transmembrane domains (data not shown), as expected for a G protein-coupled receptor [10]. The cloned human 5-HT_{5A} receptor posesses 93% (nucleotide) and 84% (amino acid) homology with the cloned mouse 5-HT_{5A} receptor, and only 71% (nucleotide) homology to the mouse 5-HT_{5B} receptor [3], providing further evidence that this gene encodes the human 5-HT_{5A} serotonin receptor.

In the course of our studies we isolated five clones from the HepG2 genomic library which contained an identical 182 bp sequence which shows 93% nucleotide identity to part of exon 2 of mouse 5-HT_{5B} (data not shown) [3]. We believe this to represent part of the human 5-HT5B gene; however, despite extensive studies we have been unable to isolate the putative exon 1 of this gene. In addition we have failed to demonstrate expression of this sequence in brain cDNA. These studies indicate that a functional 5-HT_{5B} receptor may not exist in humans; if so, this would represent the first example of a serotonin receptor isolated in other species but not found in man.

We have expressed the human 5-HT_{5A} serotonin receptor in Cos M6 cells and have determined the ligand binding characteristics of this receptor (Table 1). These values show good agreement with affinity constants derived from studies on the cloned rat and mouse 5-HT_{5A} receptors [2,4], with the exception of

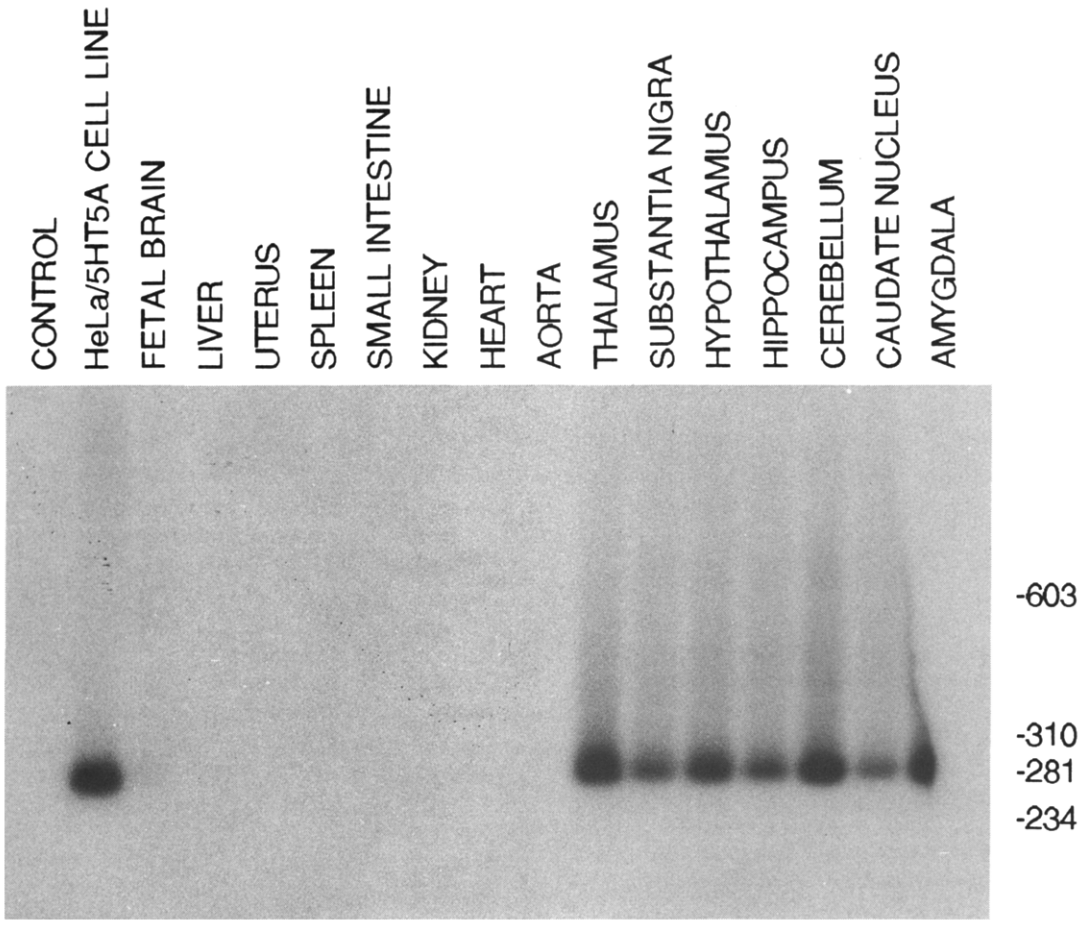

Fig. 2. Reverse transcriptase-PCR analysis of the pattern of expression of RNA encoding the human 5-HT_{5A} receptor (see section 2 for details). DNA fragment sizes are indicated.

methiothepin which shows a 60 fold higher affinity at the human receptor. This represents a novel pharmacology not shared with any other cloned human serotonin receptor [1].

The pattern of expression of the receptor was examined by RT-PCR. The receptor appears to be expressed exclusively in the adult brain with a lower level of expression in fetal brain. This may indicate that the receptor is developmentally, regulated with expression appearing at, or near, birth. We were unable to detect expression in any of the peripheral tissues examined. This pattern of expression is shared with the mouse and rat 5-HT_{5A} receptors [2–4]. We believe the level of expression of this receptor to be low. In previous studies we were unable to isolate the 5-HT_{5A} cDNA from either hippocampal or hypothalamic cDNA libraries.

Expression studies with the mouse and rat 5-HT_{5A} receptors have failed to detect functional coupling of the receptor to any second messenger pathway [2–4]. This may be due to the absence of the appropriate G protein in the cell lines used, or may indicate that this receptor couples to a novel signal transduction mechanism such as ion channels. The construction of a number of stable cell lines constitutively expressing the 5-HT_{5A} receptor will facilitate the identification of the signal transduction mechanism, particularly through expression of the receptor in neuronal cell lines. It remains possible that neuronal cells possess novel G proteins and signal transduction mechanisms, thus cell expression studies, usually performed in epithelial or fibroblast cell lines, may not reveal the true in vivo situation.

The role of this receptor in normal CNS function and its possible involvement in a pathological state also remain unknown. Studies such as immunohistochemistry and in situ hybridisation would allow an identification of the cell types expressing this receptor. However, the identification of a selective ligand, which could be used as a probe in in vitro experiments on various CNS tissues, would allow an examination of functional correlates of receptor activation. Such a ligand could also be used in in vivo behavioural studies to provide a definition of the physiological role of this receptor.

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