Genomic organization and characterization of splice variants of the human histamine H_3 receptor

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In the present paper we report the genomic organization of the human histamine H_3-receptor gene, which consists of four exons spanning 5.5 kb on chromosome 20. Using PCR, six alternative splice variants of the H_3 receptor were cloned from human thalamus. These variants were found to be coexpressed in human brain, but their relative distribution varied in a region-specific manner. These isoforms displayed either a deletion in the putative second transmembrane domain (TM), $H_{3(\Delta TM2,431aa)}$ or a variable deletion in the third intracellular loop (i3), $H_{3(\Delta i3,415aa)}$, $H_{3(\Delta i3,365aa)}$, $H_{3(\Delta i3,329aa)}$ and $H_{3(\Delta TM5+\Delta i3,326aa)}$. In order to determine the biological role of the H_3 receptor variants compared with the 'original' $H_{3(445aa)}$ receptor, three isoforms, namely $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$, were expressed in CHO cells and their pharmacological properties were investigated. Binding studies showed that $H_{3(\Delta TM2,\ 431aa)}$ transiently expressed in CHO cells was unable to bind [^{125}I]iodoproxyfan, whereas both the $H_{3(445aa)}$ and $H_{3(\Delta i3,365aa)}$ receptors displayed a high affinity for [^{125}I]iodoproxyfan [$K_d = 28 \pm 5$ pM ($n = 4$) and 8 ± 1 pM ($n = 5$) respectively]. In addition, $H_{3(\Delta i3,365aa)}$ possessed the same pharmacological profile as the $H_{3(445aa)}$ receptor. However, in CHO cells expressing $H_{3(\Delta i3,365aa)}$, H_3 agonists did not inhibit forskolin-induced cAMP production, stimulate [^{35}S]guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTP[S]) binding or stimulate intracellular Ca^{2+} mobilization. Therefore the 80-amino-acid sequence located at the C-terminal portion of i3 plays an essential role in H_3 agonist-mediated signal transduction. The existence of multiple H_3 isoforms with different signal transduction capabilities suggests that H_3-mediated biological functions might be tightly regulated through alternative splicing mechanisms.

Key words: alternative splicing, histamine H_3-receptor gene, G-protein-coupled receptor, third intracellular loop.

INTRODUCTION

Histamine exerts a wide range of biological functions, including neurotransmission, inflammation, smooth muscle contraction, dilation of capillaries and gastric acid secretion [1]. These effects are mediated through three G-protein-coupled receptor subtypes: H_1, H_2 and H_3 [1]. The H_3 receptor subtype has been identified in both the central nervous system (CNS) and the peripheral nervous system as a presynaptic receptor controlling the release of histamine [2] and the release of several neurotransmitters (for review see [1]). The CNS effect of the H_3 antagonists make them valuable candidates for the treatment of obesity, epilepsy and age-related memory disorders, such as Alzheimer's disease and attention-deficit hyperactivity disorders [3]. In peripheral organs, the negative feedback mechanism on sensory C fibres and the resultant anti-inflammatory effects of H_3 receptor agonists suggest a potential application in the treatment of asthma, migraine, cardiac disorders and neurogenic airway inflammation (for review see [3]).

The human and rat H_3 receptors have recently been cloned and their pharmacological profiles are well established [4,5]. However, apparent H_3 receptor heterogeneity in binding and functional studies has been described by several authors, which suggested the existence of more than one H_3-receptor subtype [6–8]. This hypothesis was reinforced by the recent cloning of two guinea pig H_3-receptor variants generated by alternative splicing [9]. In addition, a GenBank® database survey using the human H_3-receptor sequence as bait allowed the identification of five rat H_3-receptor variants (Genbank® accession number AB015646). Therefore in the present study it was interesting to determine whether H_3 genetic variants exist in humans and, if so, whether this may account for H_3-receptor binding and/or functional heterogeneity.

In the present study, six H_3-receptor variants were isolated by reverse transcriptase-PCR (RT-PCR) from human thalamus. The comparison of H_3-receptor cDNAs with the genomic sequence extracted from the GenBank® database revealed the presence of three introns in the coding region and further demonstrated that the H_3 variants were produced from alternative splicing. RT-PCR experiments were performed in order to assess the relative distribution of the H_3-mRNA variants in several CNS regions. Finally, the pharmacological and functional properties of two H_3 variants, one lacking the C-terminal region of the second transmembrane domain (TM) and another presenting a truncation within the third intracellular loop (i3) were compared with the 'original' H_3 receptor.

EXPERIMENTAL

Cloning and PCR analysis of human H_3-receptor cDNA isoforms

The human H_3-receptor coding sequence was isolated by RT-PCR from human thalamus mRNA (ClonTech Laboratories, Palo Alto, CA, U.S.A.) using the 5′ forward primer (5′-GAATTCCCACCATGGAGCGCGCGCCGCCCGACGGG-

Abbreviations used: BLAST, basic local alignment search tool; CNS, central nervous system; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; [^{35}S]GTP[S], [^{35}S]guanosine 5′-[γ-thio]triphosphate; HTGS, high-throughput genome sequences; i3, third intracellular loop; RT-PCR, reverse transcriptase-PCR; TM, transmembrane domain; UTR, untranslated region.

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CCGCTGAA-CGCTTCG-3′; nt 299–337) and the 3′ reverse primer (5′-GGATCCTCACTTCCAGCAGTGCTCCAGGGA-GCT-3′; nt 1610–1636), which were designed according to the published sequence [4] (accession number AF140538). Thalamus mRNA (200 ng) was reverse-transcribed with oligo dT_{12-18} in accordance with the first-strand cDNA synthesis protocol of Amersham Pharmacia Biotech (Saclay, France). PCR reactions were performed in a volume of 100 μl containing native *Pfu* buffer, 0.25 mM dNTP, 2 μl of the single-stranded cDNA preparation, 3 × enhancer solution (Life Technologies, Cergy-Pontoise, France), 0.4 μM forward primer, 0.4 μM reverse primer and 2 units of the *Pfu* native polymerase (Stratagene, La Jolla, CA, U.S.A.) with a 30 cycle programme of 94 °C for 1 min, 65 °C for 2 min, 72 °C for 2 min and a final extension of 72 °C for 8 min. The amplified cDNA was then subcloned into *Eco*RI and *Bam*HI sites of the pcDNA3.1(+) vector (Invitrogen, San Diego, CA, U.S.A.). Sequence determinations were performed by dideoxy chain termination using an automated fluorescent Dye ABI 377 DNA Sequencer (Applied Biosystems, Courtaboeuf, France).

Tissue expressions of the H_3 isoforms were analysed by RT-PCR from 200 ng of mRNA (ClonTech Laboratories) or 2 μg of total RNA (Analytical Biological Services Inc., Wilmington, DE, U.S.A.), reverse-transcribed and amplified as described above. The 5′ forward primers F517 (nt 517–542) or F754 (nt 754–778) and the 3′ reverse primers B832 (nt 811–832) or B1406 (nt 1383–1406) were used for $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$, $H_{3(\Delta i3,415aa)}$, $H_{3(\Delta i3,365aa)}$, $H_{3(\Delta i3,\ 329aa)}$ and $H_{3(\Delta TM5+\Delta i3,326aa)}$ receptor amplifications. The PCR products were subcloned into a pGEMT-easy vector (Promega, Charbonnières, France) and sequenced. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was PCR amplified as described by the manufacturer (ClonTech Laboratories) and was used as a quantitative control for each cDNA amplification.

Southern- and Northern-blot analyses

Human genomic DNA was purchased from ClonTech Laboratories. The genomic DNA was digested with B*am*HI, P*st*I or S*ac*I. The fragments were subsequently separated by electrophoresis on a 0.8 % (w/v) agarose gel, transferred on to a nylon membrane (Hybond N^+) and hybridized with a [α-^{32}P]dCTP random labelled probe (Amersham Pharmacia Biotech) as described by the manufacturer (Express Hyb; ClonTech Laboratories). The probe corresponded to the open reading frame of the human $H_{3(445aa)}$ receptor [4], and 2000000 c.p.m./ml were used in 10 ml of hybridization buffer and incubated at 68 °C for 16 h. The blot was then washed twice at 25 °C in 2 × SSC (where 1 × SSC corresponds to 0.15 M NaCl/0.015 M sodium citrate) containing 0.05 % (w/v) SDS for 5 min each. The blot was then washed twice at 65 °C in 0.1 × SSC containing 0.1 % (w/v) SDS for 30 min each and exposed overnight. The human brain Northern blots obtained from ClonTech Laboratories were hybridized as described above. A β-actin probe (ClonTech Laboratories) was used as an internal control.

Sequence of the human H_3-receptor gene

The sequence of the entire human H_3-receptor gene was obtained by high-throughput genome sequences (HTGS) database screening using the basic local alignment search tool (BLAST) program [10] set up with default parameters (http://www.ncbi.nlm.nih.gov). The screening was performed with the complete human H_3-receptor cDNA sequence [4].

Stable CHO-K1 cell lines

CHO-K1 cells (American type culture collection, CCL61) were maintained in Ham-F12 medium supplemented with 10 % (v/v) foetal calf serum, 2 mM glutamine, 500 units/ml penicillin and 500 μg/ml streptomycin. The coding regions of the human $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$ receptor isoforms were subcloned into the pcDNA-neo expression vector (Invitrogen) and transfected into CHO-K1 cells using LIPOFECTAMINE™, as described by the manufacturer (Life Technologies). Stably transfected cells were selected with neomycin (500 μg/ml) and tested for their ability to either bind [^{125}I]iodoproxyfan or to inhibit adenylate cyclase. Only the best responding clones were selected for further studies.

Immunofluorescence microscopy

CHO-K1 cells were grown overnight on poly(D-lysine)-coated glass coverslips in Ham-F12 medium supplemented with 10 % (v/v) foetal calf serum. A sequence encoding the M5 flag epitope (DYKDDDDK; single-letter amino acid code) was introduced into the expression vector constructs at the C-terminus of the $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$ receptors. The pcDNA/H_3M5 vectors were then transfected into CHO-K1 cells using LIPOFECTAMINE™ (Life Technologies) according to the manufacturer's protocol. Following transfection for 24 h, cell nuclei were stained with 33258-Hoechst dye (Sigma) and the cells were fixed by immersion in cold acetone (−20 °C) for 10 min. Epitope-tagged H_3 isoforms were detected using a mouse monoclonal anti-Flag M2 antibody (Sigma) diluted to 17 μg/ml in Ham-F12 medium. Following incubation for 1 h at 37 °C, coverslips were extensively washed in PBS buffer and incubated with goat Cy3™ conjugated anti-mouse F(ab')$_2$ fragment IgG (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) diluted to 6 μg/ml in PBS for 30 min at 25 °C. Immunofluorescent staining was examined using a conventional inverted epifluorescence Leica microscope (40 × /10 × objective).

Cell membrane preparation

Cells grown to confluence were harvested in 2 mM EDTA/PBS and centrifuged at 1000 ***g*** for 5 min (4 °C). The resulting pellet was suspended in 20 mM Tris/HCl (pH 7.7) containing 5 mM EDTA, and was homogenized using a Kinematica polytron (Fisher Bioblock Scientific, Illkirch, France). The homogenate was then centrifuged at 95000 ***g*** for 30 min (4 °C) and the pellet was suspended in binding buffer [50 mM Na_2HPO_4/KH_2PO_4 (pH 7.5)]. Determination of protein content was performed according to the method of Lowry et al. [11]. Aliquots of membrane preparations were stored at −80 °C and were used for [^{125}I]iodoproxyfan or [^{35}S]guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTP[S]) binding experiments.

[^{125}I]Iodoproxyfan binding assay

Membranes (5 μg/ml) obtained from cells stably expressing $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$ or $H_{3(\Delta i3,365aa)}$ receptors were incubated for 1 h at room temperature in binding buffer in a final volume of 250 μl. For saturation binding experiments, [^{125}I]iodoproxyfan (2000 Ci/mmol; Amersham Pharmacia Biotech) concentrations ranged between 2 and 200 pM, whereas for competition studies 25 pM [^{125}I]iodoproxyfan was used. Non-specific binding was determined in the presence of 1 μM *R*(−)-α-methylhistamine. The reaction was stopped by rapid filtration through GF/B

unifilters pretreated with 0.1 % polyethyleneimine, followed by three ice-cold buffer washes [50 mM Tris/HCl (pH 7.4)]. The binding data were analysed by a non-linear regression curve-fitting (single site) procedure using the computer program PRISM (Graphpad Software Inc., San Diego, CA, U.S.A.) to yield IC_{50} values. The inhibition constants (K_i) were calculated according to the Cheng–Prusoff equation. The histaminergic ligands were purchased from Fisher Bioblock Scientific with the exception of $R(-)$-α-methylhistamine and ranitidine, which were purchased from Research Biochemical International (Saint-Quentin, France) and histamine, which was obtained from Sigma (Saint-Quentin, France). Ciproxifan was synthesized by Syntheval (Caen, France).

[^{35}S]GTP[S] binding assay

Membranes (15 μg/ml) were incubated in the presence of 0.1 nM [^{35}S]GTP[S] (1000 Ci/mmol; Amersham Pharmacia Biotech) for 30 min at room temperature in binding buffer [20 mM Hepes (pH 7.4) containing 3 μM GDP, 3 mM $MgCl_2$, 100 mM NaCl and 30 μg/ml saponin]. Non-specific binding was determined in the presence of 10 μM unlabelled GTP[S]. The reaction was stopped by rapid filtration through GF/B unifilters, followed by three washes with ice-cold binding buffer. For antagonist studies, membranes were preincubated with agonists and antagonists for 30 min before the addition of [^{35}S]GTP[S]. Agonist efficacy (E_{max}) was expressed relative to that of $R(-)$-α-methylhistamine (100 %), which was tested at the maximum effective concentration (1 μM) in each experiment. Agonist potency was expressed as pEC_{50}. Antagonist potency was expressed as pK_B:

$$K_B = IC_{50}/[1 + ([ago]/EC_{50}ago)]$$

where IC_{50} is the concentration of antagonist that gives 50 % inhibition of [^{35}S]GTP[S] binding in the presence of a fixed concentration of agonist ([ago]) and EC_{50}ago is the EC_{50} of the agonist when tested alone.

Ca^{2+} mobilization assay

Stable CHO-K1 cells expressing $H_{3(445aa)}$ or $H_{3(\Delta i3,369aa)}$ receptors were seeded (30000 cells/well, with a plating volume of 100 μl) into D-lysine-coated 96-well plates 24 h prior to the Ca^{2+} mobilization assay. Cells were then loaded with a calcium kit assay buffer (100 μl/well; Molecular Devices, Sunnyvale, CA, U.S.A.) containing 2.5 mM probenecid, and were incubated at 37 °C for 1 h in a 6 % CO_2 atmosphere. The intracellular Ca^{2+} mobilization in response to histaminergic agonists was analysed using a fluorescent imaging plate reader [FLIPR; Molecular Devices; argon laser; excitation at 488 nm]. Drugs were added to the assay at the 10 s time point.

RESULTS

Cloning of human H_3-receptor cDNA variants from human thalamus

Using two primers flanking the first methionine residue and the stop codon of the H_3 receptor [4], 11 cDNAs were amplified from human thalamus mRNA. Five of the cDNAs corresponded to the full-length open reading frame encoding the $H_{3(445aa)}$ receptor published by Lovenberg et al. [4] (Figure 1). Two cDNAs had a deletion at nt 549–590 [4] corresponding to the loss of 14 amino acids at the end of TM2 [$H_{3(\Delta TM2,431aa)}$; Figure 1]. Three cDNAs had deletions at nt 976–1323, 998–1087 or 1120–1359 [4] within i3, resulting in the loss of 116 [$H_{3(\Delta i3,329aa)}$], 30 [$H_{3(\Delta i3,415aa)}$] or 80 [$H_{3(\Delta i3,365aa)}$] amino acids respectively (Figure 1). One cDNA had a deletion at nt 886–1242 [4] with the loss of the putative TM5, as well as of 89 amino acids within i3, leading to a protein of 326 amino acids [$H_{3(\Delta i3+\Delta TM5,326aa)}$; Figure 1]. None of the observed deletions generated a frameshift. The cDNA sequences of the H_3-receptor variants isolated in the present study were otherwise 100 % identical in their coding regions to the cDNA previously described {$H_{3(445aa)}$ isoform [4]} The deduced amino acid sequences of all isolated human H_3 variants are summarized in Figure 1. In the case of the $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$ receptors, the deleted region displayed a donor (GTATG) and an acceptor (CAG) consensus sequence at the 5′ and 3′ ends respectively. With regard to the $H_{3(\Delta i3,329aa)}$ variant, only a consensus acceptor splice site (CAG) was present at the 3′ end.

Sequence and genomic organization of the H_3-receptor gene

The BLAST sequence alignment program [10] was used to screen the HTGS database with the complete $H_{3(445aa)}$ cDNA sequence [4]. The whole coding sequence was found to be included (100 % identity) in one human genomic clone (accession number AL078633) mapped on chromosome 20 and containing a total of 100 kbp. The schematic organization of the human H_3 gene deduced from sequence comparison between the AL078633 clone and the H_3 variants is shown in Figure 2(B). The gene spans more than 5.5 kbp (nt 15420–20693) and contains a minimum of four exons and three introns. A polyadenylation consensus signal site (AATAAA) was found at position 20688–20693 of the AL078633 clone.

The 5′ untranslated region (UTR) is encoded by exon 1. The coding region is encoded by the 3′ end of exon 1, exon 2, exon 3 and exon 4. The latter also encodes the 3′ UTR (Figure 2C). The coding region is interrupted by three introns whose sizes correspond to 1062, 1565 and 240 bp respectively. Analysis of the exon–intron boundaries revealed that each contained a donor and an acceptor consensus sequence [12], as well as a putative branch point site (CTRACT) located upstream from the acceptor splice sites (Table 1).

The mRNA encoding the reported $H_{3(445aa)}$ receptor conserves the third intron, whereas it is spliced from $H_{3(\Delta i3,365aa)}$ mRNA. In the case of $H_{3(\Delta TM2,431aa)}$ the splice acceptor site is located within exon 2, 42 nt downstream of the acceptor splice site of intron 1, thus producing a receptor with a deletion of the TM2 C-terminus.

We analysed the human genomic DNA by Southern blotting to confirm that the H_3 variants were coded by a single gene. Southern-blot analysis using the complete $H_{3(445aa)}$-receptor cDNA [4] as a probe followed by high stringency washes (Figure 3) indicated the presence of a unique 2.1 kbp *Pst*I restriction fragment, as well as two *Sac*I and *Bam*HI restriction fragments of 1.9–4.3 kbp and 2.6–5.6 kbp respectively. The size of the restriction fragments revealed by Southern-blot analysis was in agreement with the restriction map of the genomic clone AL078633 (Figures 2A and 3). The human genome thus contains a single H_3-receptor gene that can produce several receptor variants by a complex alternative splicing process.

Tissue expression

We investigated the distribution of the H_3-receptor mRNA variants by Northern-blot and RT-PCR analyses. Northern-blot experiments using the $H_{3(445aa)}$ cDNA coding region as a probe

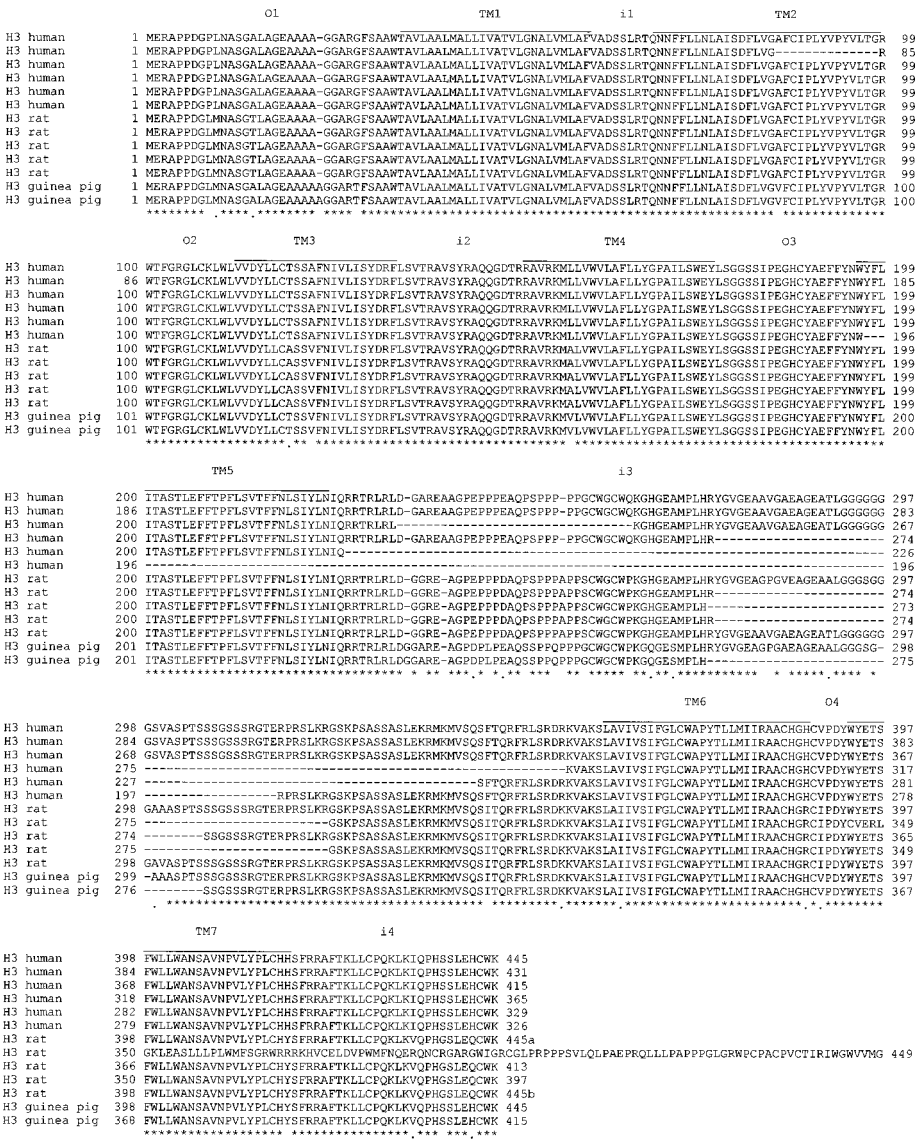

Figure 1 Alignment of deduced amino acid sequences of human, rat and guinea pig H_3-receptor variants

The six human H_3-receptor isoforms (445, 432, 415, 365, 329 and 326 amino acids) were isolated by RT-PCR from human thalamus mRNA as described in the Experimental section. The five rat H_3 receptor sequences (449, 445a, 445b, 413 and 397 amino acids) were extracted from GenBank® (accession number AB015646). The two guinea pig H_3-receptor isoforms (445 and 415 amino acids) were published by Tardivel-Lacombe et al. [9]. The deduced amino acid sequences are shown in single-letter amino acid code. The seven putative TMs (TM1–7), the four extracellular (o1–4) and the four intracellular (i1–4) domains are indicated. The clustalW algorithm was used to align human $H_{3(445aa)}$ and the other H_3 variants. Amino acid residues identical between the human, rat and guinea pig $H_{3(445aa)}$ receptors are indicated by asterisks. Similar amino acids are indicated by dots.

and a 7 day period of exposure revealed a single 2.7 kb mRNA species in human brain tissues (Figure 4A). No signal was observed in peripheral tissues after a 15 day period of exposure (results not shown). A strong hybridization signal was detected in thalamus, caudate nucleus, putamen and cerebellum (Figure 4A). The 2.7 kb mRNA species was also present at a significant level in the amygdala. Faint bands were detected in substantia nigra, hippocampus and cerebral cortex (Figure 4A). No signal could be seen in either corpus callosum or spinal cord. Northern-blot analysis was not sensitive enough to discriminate between the H_3-receptor splice variants differing by short deletions (42–357 bp). Therefore to investigate tissue distribution and the relative abundance of the H_3 splice variants, PCR analysis was performed on various human brain structures with two sets of primers (F517/B832 and F754/B1406; Figure 2C). The lengths of amplicons were consistent with those expected from the structures of the H_3 mRNA species. The specificity of PCR products was assessed by cloning and sequencing each amplicon. No signal was observed when either the mRNA or reverse transcriptase were omitted from the first-strand cDNA conversion, which suggests that the signals observed were not due to any contaminating genomic DNA (results not shown). A quantitative control (GAPDH) confirmed that each sample contained similar amounts of total cDNA (Figure 4B). The upper panel of Figure 4(B) shows that the F517/B832 primers amplified two cDNAs (315 and 273 bp), the size of which corresponded to $H_{3(445aa)}$ and $H_{3(\Delta TM2,431aa)}$ mRNAs respectively. PCR reaction with the F754/B1406 primers led to cDNA fragments whose

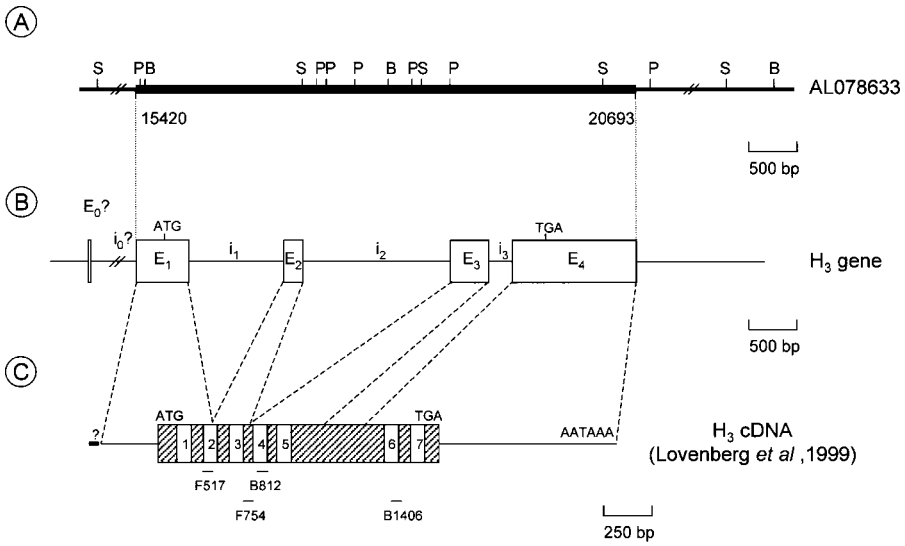

Figure 2 Schematic organization of the human H_3 histamine-receptor gene and cDNA

(**A**) Restriction map of the genomic DNA fragment from human chromosome 20 (accession number AL078633). B, *Bam*HI; P, *Pst*I; S, *Sa*cI. (**B**) Schematic structure of the H_3 gene with the location of exons ($E_{1–4}$) shown by boxes and the location of introns ($I_{1–3}$) shown by horizontal lines. The exons are numbered from the 5′ end of the gene with exon E_1 containing the first ATG codon. The initiation (ATG) and termination (TGA) codons are indicated. Exon E_0 and intron i_0 were deduced from the H_3 receptor cDNA described by Lovenberg et al. [4], but are not confirmed (see the Discussion section for further details). (**C**) Structure of the human H_3-receptor cDNA. The coding region is shown by boxes and the 5′- and 3′-UTRs are shown by horizontal lines. The putative TMs (1–7) are mentioned in the coding region. The translation initiation site (ATG), the termination site (TGA) and the polyadenylation signal sequence (AATAAA) are indicated. The forward (F) and reverse (B) primers used for RT-PCR analysis are shown under the cDNA structure.

Table 1 Exon–intron boundaries in the human histamine H_3-receptor gene

Nucleotides and amino acids are numbered according to the EMBL sequence number AF140538. Consensus splice and branch point sites are shown in bold.

	Exons			Introns			
Number	Size (bp)	Limits	Borders	Donor site	Branch point site	Acceptor site	Size (bp)
1	536	12–548 1–83	ATG CTC---CTC GTC G M E L V	**gtaaa**tc---------|	**ctggct**gcatggtcccactgtggccctactcccca	**cag**	1062
2	166	549–715 84–139	GC GCC----CGA GCG G A R A	**gtgag**tcc----------|	**ctgacc**agcctgcccttctg	**cag**	1565
3	404	716–1120 140–274	GTC TCA---CTG CAC AG V S L H R	**gtatg**gg----------|	**ctgtct**cgggga	**cag**	240
4	1312	1360–2672 354–445	G AAA----TGG AAG TGA K W K *					

* Stop codon.

sizes (652, 562, 412, 304 and 295 bp) corresponded to $H_{3(445aa)}$, $H_{3(\Delta i3,415aa)}$, $H_{3(\Delta i3,365aa)}$, $H_{3(\Delta i3,329aa)}$ and $H_{3(\Delta i3+\Delta TM5,326aa)}$ mRNAs respectively (Figure 4B, middle panel). However, the 2 % (w/v) agarose gel did not allow us to discriminate between the 304 and 295 bp PCR products. Thus the presence of $H_{3(\Delta i3,329aa)}$ and $H_{3(\Delta i3+\Delta TM5,326aa)}$ mRNAs was detected by further cloning and sequencing. The pattern of $H_{3(445aa)}$ mRNA expression, as assessed by RT-PCR, was comparable with that of the 2.7 kb mRNA detected by Northern-blot analysis. Strong $H_{3(415aa)}$ and $H_{3(\Delta i3,365aa)}$ mRNA expression was observed in brain regions that highly expressed the 2.7 kb mRNA, such as thalamus, caudate nucleus and cerebellum, whereas $H_{3(\Delta i3,329aa)}$ and $H_{3(\Delta i3+\Delta TM5,\ 326aa)}$ mRNAs were poorly expressed. In contrast, $H_{3(\Delta i3,329aa)}$ and $H_{3(\Delta i3+\Delta TM5,326aa)}$ mRNA expression levels were high in amygdala, substantia nigra, cerebral cortex and hypothalamus. The mRNA coding for the $H_{3(\Delta i3,415aa)}$ variant was not found in hypothalamus or substantia nigra.

Pharmacological properties of $H_{3(445aa)}$ and $H_{3(\Delta i3,365aa)}$ isoforms

Among the H_3-receptor variants, the $H_{3(445aa)}$ isoform, which matched that reported by Lovenberg et al. [4], was compared with the two isoforms $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$, which have deletions in TM2 or i3 respectively. The three isoforms were further investigated for their pharmacological properties. PCR fragments corresponding to the coding region of each H_3-receptor isoform were inserted into the pcDNA3.1 expression vector and used to stably transfect CHO-K1 cells. First, the subcellular localization of $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$ isoforms was studied with C-terminal M5 epitope-tagged recombinant H_3

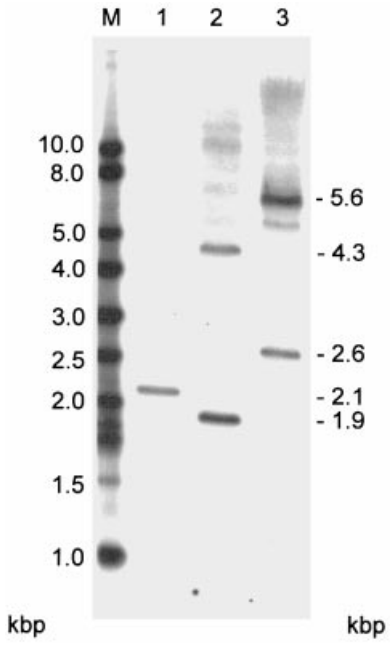

Figure 3 Southern-blot analysis of human genomic DNA

Human genomic DNA (8 μg/lane) was digested with *Pst*I (lane 1), *Sac*I (lane 2) or B*am*HI (lane 3). Hybridization analyses were carried out as described in the Experimental section with a probe corresponding to the coding region of the $H_{3(445aa)}$-receptor cDNA [4]. An 18 h autoradiography exposure is shown. M, molecular mass markers (Eurogentec, Seraing, Belgium).

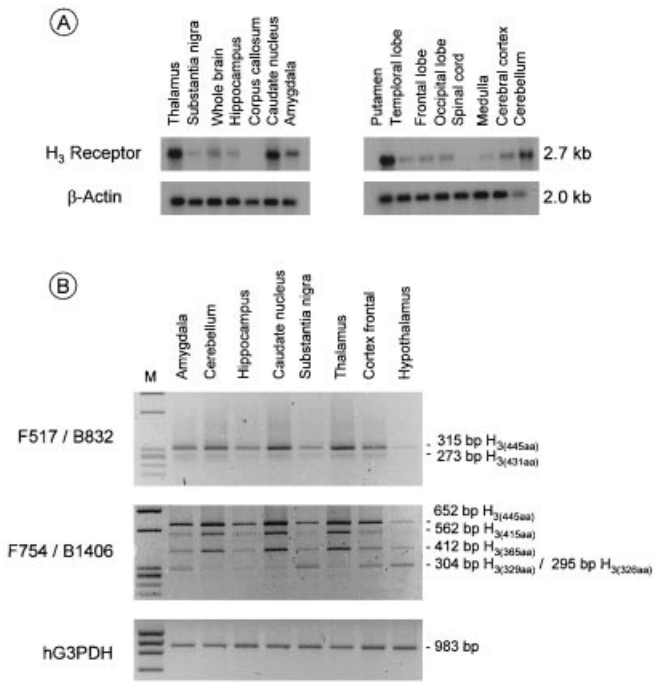

Figure 4 Distribution of mRNA encoding H_3-receptor isoforms in human brain tissues

(**A**) Northern-blot analysis of mRNA encoding H_3-receptor variants from human central tissues (2 μg of polyadenylated RNA/lane). The radiolabelled probe corresponds to the $H_{3(445aa)}$ full-length coding sequence [4]. Autoradiography exposure with an intensifying screen was carried out for 7 days at -80 °C. A β-actin probe was used as an internal standard. (**B**) mRNA (200 ng) of human brain tissues or total RNA (2 μg) from human frontal cortex and hypothalamus was amplified by RT-PCR using a common 5′ forward primer and a 3′ reverse primer flanking the intronic region to be analysed (see Figure 2C). After 30 PCR cycles for H_3-receptor variants and 20 PCR cycles for human GAPDH (hG3PDH), PCR products were analysed using a 2% (w/v) agarose gel stained with ethidium bromide. The lengths of amplicons were estimated by molecular mass markers (ΦX174/HaeIII; M) and are indicated in bp on the right. Each PCR product was purified and identified by sequencing on both strands. GAPDH amplification was used as an internal standard. Each picture is representative of three independent experiments.

isoforms transiently expressed in CHO-K1 cells. In fixed and permeabilized transfected cells, the fluorescent signal was detected at the surface and perinuclear compartments of cells expressing the M5-tagged H_3 isoforms (Figures 5A–5C), whereas no signal was observed in cells transfected with the vector alone (Figure 5D). These results demonstrate that the subcellular localization of the $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$ receptors is similar to that of the 'original' $H_{3(445aa)}$ isoform.

Membranes from CHO cells stably transfected with the $H_{3(445aa)}$ or $H_{3(\Delta i3,365aa)}$ cDNAs were able to bind [^{125}I]iodoproxyfan in a saturable manner with high affinity [$K_d = 28 \pm 5$ pM ($n = 4$) and 8 ± 1 pM ($n = 5$) respectively]. The expression levels of $H_{3(445aa)}$ and $H_{3(\Delta i3,365aa)}$ were 988 ± 23 and 439 ± 97 fmol/mg of protein respectively. CHO-K1 cells transfected with either pcDNA3.1 alone or with the $H_{3(\Delta TM2,431aa)}$ construct exhibited no specific binding (results not shown). The binding of [^{125}I]iodoproxyfan to cell membranes expressing either the $H_{3(445aa)}$ or $H_{3(\Delta i3,365aa)}$ isoform was inhibited with a similar rank order of potency by H_3 agonists or antagonists (Table 2).

[^{35}S]GTP[S] binding of $H_{3(445aa)}$ or $H_{3(\Delta i3,365aa)}$ receptors

In CHO-K1 cells expressing $H_{3(445aa)}$, $R(-)$-α-methylhistamine elicited a potent inhibition (99%) of forskolin-induced cAMP formation (results not shown). In contrast, $R(-)$-α-methylhistamine was inactive on CHO cells transfected with $H_{3(\Delta i3,365aa)}$ (results not shown). Despite the fact that H_3 agonists were unable to inhibit the forskolin-induced cAMP production in CHO cells expressing $H_{3(\Delta i3,365aa)}$, we investigated whether this receptor could stimulate GDP/GTP exchange by performing [^{35}S]GTP[S] binding on membranes from $H_{3(445aa)}$- or $H_{3(\Delta i3,365aa)}$-expressing cell lines. In the presence of $R(-)$-α-methylhistamine, a protein

Table 2 Ligand binding affinities of $H_{3(445aa)}$ and $H_{3(\Delta i3,365aa)}$ isoforms

Receptors were stably expressed in CHO-K1 cells. Dose–response isotherms were analysed by non-linear regression (one-site analysis). Results are presented as means ± S.E.M. of at least three independent experiments.

	pK_i	
Ligand	$H_{3(445aa)}$ receptor	$H_{3(\Delta i3,365aa)}$ receptor
H_3 agonists		
Histamine	8.24 ± 0.10	8.26 ± 0.09
$R(-)$-α-methylhistamine	8.73 ± 0.09	8.85 ± 0.05
N$^{\alpha}$-methylhistamine	8.96 ± 0.10	9.10 ± 0.01
Imetit	9.37 ± 0.22	9.66 ± 0.03
Immepip	9.60 ± 0.15	9.70 ± 0.08
H_3 antagonists		
Ciproxifan	7.26 ± 0.02	7.28 ± 0.13
Thioperamide	7.70 ± 0.07	7.66 ± 0.09
Clobenpropit	9.27 ± 0.05	9.23 ± 0.06
H_1/H_2 ligands		
Cimetidine	< 5	< 5
Pyrilamine	< 5	< 5
Ranitidine	< 5	< 5

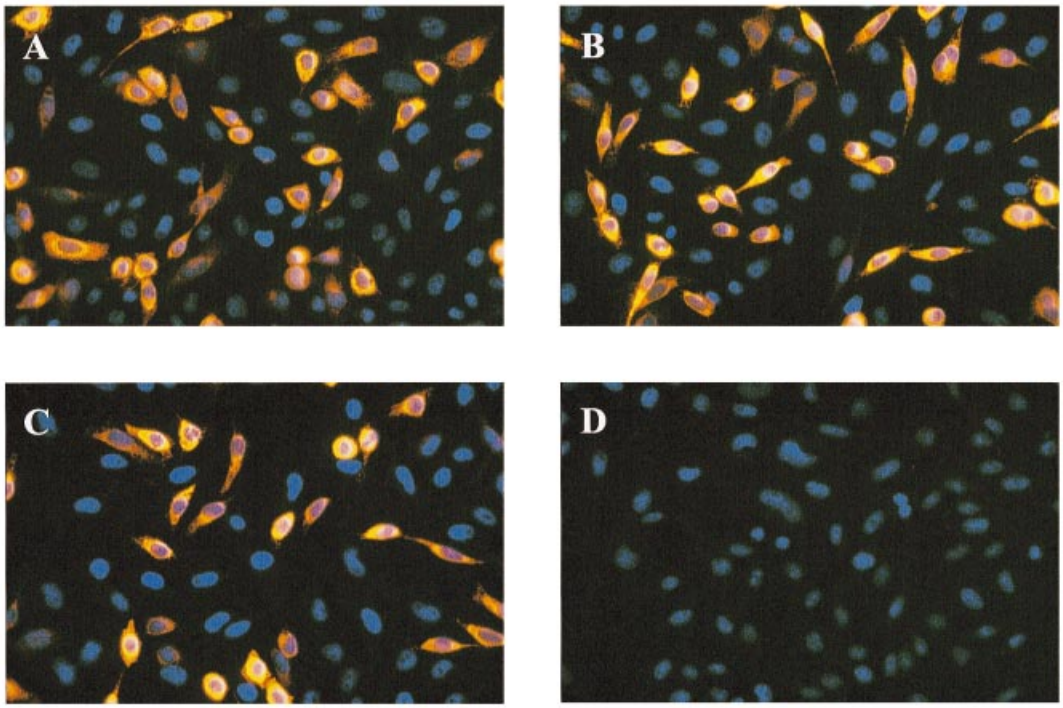

Figure 5 Subcellular localization of epitope-tagged $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$ isoforms

Immunofluorescence studies were performed with transfected CHO-K1 cells grown on coverslips as described in the Experimental section. Cells expressing $H_{3(445aa)}$ (**A**), $H_{3(\Delta TM2,431aa)}$ (**B**) and $H_{3(\Delta i3,365aa)}$ (**C**) isoforms were probed with the mouse monoclonal antibody M2 directed against the C-terminal epitope tag present in the recombinant receptors. Experiments were carried out with fixed and permeabilized cells. The fluorescent images were obtained using Cy3™ conjugated goat anti-mouse IgG secondary antibodies. CHO-K1 cell nuclei were stained with 33258-Hoechst dye. All photographs were taken at 400 × magnification. Each picture is representative of three independent experiments. CHO-K1 cells transfected with the vector alone were used as a negative control (**D**).

Table 3 Effects of ligands on [^{35}S]GTP[S] binding to CHO cell membranes stably expressing the $H_{3(445aa)}$ isoform

Dose–response isotherms of ligands were analysed by non-linear regression. Agonist potency is expressed as pEC_{50}; all the agonists were full agonists compared with stimulation by 1 μM *R*(−)-α-methylhistamine (100%). Antagonist potency to inhibit [^{35}S]GTP[S] binding induced by 30 nM *R*(−)-α-methylhistamine is expressed as pK_B. Results are presented as means ± S.E.M. of at least three independent experiments.

Ligand	[^{35}S]GTP[S] binding
Agonists	
Histamine	7.46 ± 0.04
R(−)-α-methylhistamine	8.57 ± 0.01
N^{α}-methylhistamine	8.70 ± 0.10
Imetit	8.82 ± 0.07
Immepip	9.29 ± 0.07
Antagonists	
Ciproxifan	7.62 ± 0.11
Thioperamide	8.12 ± 0.10
Clobenpropit	9.28 ± 0.06

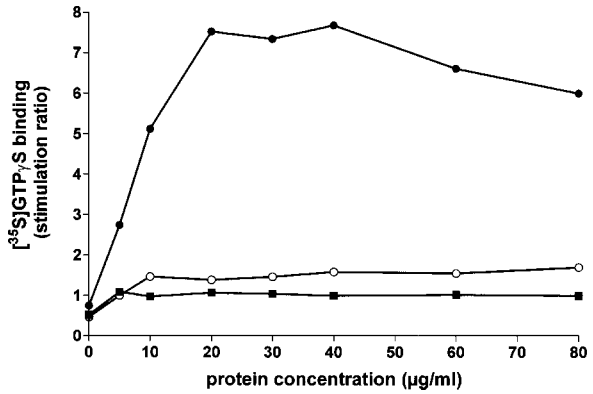

Figure 6 Dose–response effect of membranes from CHO cells stably expressing the $H_{3(445aa)}$ or the $H_{3(\Delta i3,365aa)}$ isoforms on *R*(−)-α-methylhistamine-induced specific [^{35}S]GTP[S] binding

Membranes from CHO-K1 cells (■) or from CHO cells stably expressing either the $H_{3(445aa)}$ receptor (●) or the $H_{3(\Delta i3,365aa)}$ receptor (○) were incubated in the presence of 10 μM *R*(−)-α-methylhistamine and 0.1 nM [^{35}S]GTP[S] as described in the Experimental section. Data are expressed as the ratio of [^{35}S]GTP[S] binding in the presence of the agonist compared with baseline. Representative curves are shown in which each point is the mean of triplicate determinations.

dose-dependent stimulation of [^{35}S]GTP[S] binding was observed with membranes expressing the $H_{3(445aa)}$ receptor (6-fold stimulation), but not with those expressing the $H_{3(\Delta i3,365aa)}$ receptor (Figure 6). Both the H_3 agonist and antagonist effects were further evaluated on membranes from CHO cells expressing $H_{3(445aa)}$ (Table 3). The agonists behaved as full agonists, compared with *R*(−)-α-methylhistamine (100% efficacy), with a rank order of potency similar to their binding affinity (Table 3). No effect on basal [^{35}S]GTP[S] binding was observed with antagonists (results not shown). However, clobenpropit, ciproxi-

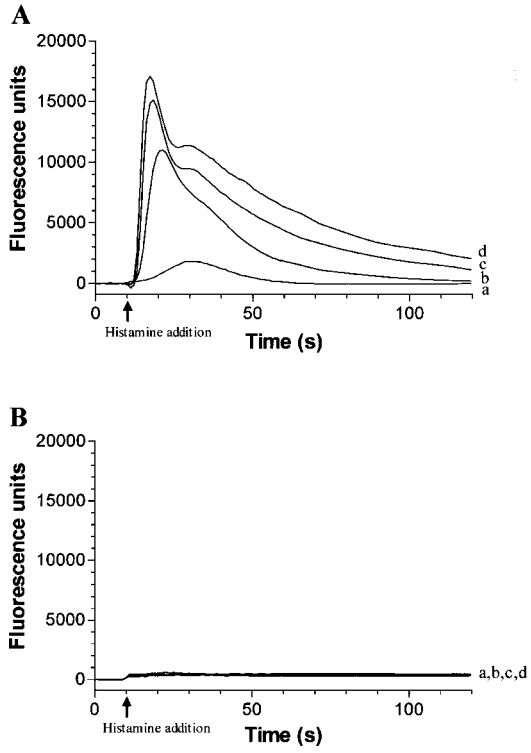

Figure 7 Intracellular Ca^{2+} mobilization induced by histamine in cells expressing the $H_{3(445aa)}$ or the $H_{3(\Delta i3,365aa)}$ receptor

Intracellular Ca^{2+} mobilization was measured in response to a range of histamine concentrations (a, 1 nM; b, 10 nM; c, 1 μM; d, 10 μM) in CHO cells expressing either the $H_{3(445aa)}$ (**A**) or the $H_{3(\Delta i3,365aa)}$ (**B**) receptor. Results shown are from a single experiment representative of three independent experiments performed in triplicate.

fan and thioperamide inhibited the $R(-)$-α-methylhistamine-induced [^{35}S]GTP[S] binding with pK_B values similar to their pK_i values (Tables 2 and 3).

Intracellular Ca^{2+} mobilization in CHO cells stably expressing $H_{3(445aa)}$ or $H_{3(\Delta i3,365aa)}$

Finally, we investigated the ability of both the $H_{3(445aa)}$ and $H_{3(\Delta i3,365aa)}$ isoforms to increase intracellular Ca^{2+} when stimulated by agonists. Using a fluorescent imaging plate reader to quantify intracellular Ca^{2+} responses, cells stably expressing either the $H_{3(445aa)}$ or $H_{3(\Delta i3,365aa)}$ receptors were incubated with fluo 3 acetoxymethyl ester and challenged with histamine. Figure 7 shows that increasing concentrations of histamine (1 nM to 10 μM) gave a consistent intracellular Ca^{2+} mobilization with cells expressing $H_{3(445aa)}$ (1700-fold stimulation), but not with those expressing $H_{3(\Delta i3,365aa)}$. Other agonists, such as $R(-)$-α-methylhistamine or Imetit did not induce $H_{3(\Delta i3,365aa)}$ receptor-mediated Ca^{2+} mobilization (results not shown). The histamine response was specific for $H_{3(445aa)}$ expression, since untransfected CHO cells did not elicit any response.

DISCUSSION

In the present study, the organization of the human histamine H_3-receptor gene is described. It consists of at least four exons and three introns. We also report the cloning and expression of various splice variants of the human H_3 receptor. Interestingly, most of these splice variants were characterized by a truncation within i3.

Human H_3 receptor gene

Sequence comparisons between splice variant cDNAs and the genomic DNA fragment (accession number AL078633) encoding the human H_3 gene revealed its complex organization. It was found to be at least 5.5 kb long and to contain three introns within its coding region. The comparison between the rat $H_{3(449aa)}$ variant, which presents a frameshift in the C-terminal region, and the human H_3 gene sequence suggests that a fourth intron at nt 19511–20208 may also exist (see Figures 1 and 2A). This putative fourth intron, which splits exon 4 in two, is flanked by consensus donor (GTACG) and acceptor (C/T_nAAAAG) sites. However, in human tissues, we did not find any variant corresponding to the rat $H_{3(449aa)}$ (Genbank® accession number AB015646). This complex genomic organization differs from that of the human H_1 and H_2 receptors, which are intronless within their coding region [13,14]. Furthermore, we observed that the first 11 bases from the 5′ UTR of the H_3 cDNA described by Lovenberg et al. [4] (E_0, see Figure 2B) did not match the upstream genomic sequence of the H_3 gene (nt 1–15420 in the AL078633 clone). Again, this observation suggests the existence of an additional large intron (> 15 kbp) in the 5′ UTR of the H_3 receptor (i_0, see Figure 2B), which remains to be fully characterized. Recently, it has been reported that the human histamine H_1 and H_2 receptor genes also contain a short first exon partially encoding the 5′ UTR (156 and 58 bp respectively) followed by a large intron (5.8 kbp and 19 kbp respectively) immediately downstream of the transcription start site [13,14]. Indeed, identification of the H_3 gene promoter awaits the full characterization of this first 5′ UTR-containing exon.

In the present study we have cloned six splice variants of the H_3 receptor [$H_{3(445aa)}$, $H_{3(\Delta TM2,\ 431aa)}$, $H_{3(\Delta i3,415aa)}$, $H_{3(\Delta i3,365aa)}$, $H_{3(\Delta i3,329aa)}$ and $H_{3(\Delta TM5+\Delta i3,326aa)}$] from human thalamus mRNA using RT-PCR. Among the cloned H_3 isoforms, the most frequently detected was the cDNA encoding the $H_{3(445aa)}$ receptor previously described by Lovenberg et al. [4]. The other cDNAs presented either a deletion in the putative TM2 [$H_{3(\Delta TM2,431aa)}$] or a large deletion in i3 [$H_{3(\Delta i3,415aa)}$, $H_{3(\Delta i3,365aa)}$, $H_{3(\Delta i3,329aa)}$ and $H_{3(\Delta i3,326aa)}$]. The comparison between the H_3 genomic sequence and the $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$ cDNAs showed that these variants resulted from splicing according to the GU-AG rule. The other variants used cryptic sites located within exon 3 [$H_{3(\Delta i3,415aa)}$] or exon 3–intron 3 [$H_{3(\Delta i3,329aa)}$ and $H_{3(\Delta i3,326aa)}$]. Moreover, all of these splice variants originated from the same gene, since the combination of Southern-blot analysis and the enzymic restriction map showed that the human genome contained a single H_3-receptor gene (see Figures 2A and 3).

The rat $H_{3(413aa)}$ and guinea pig $H_{3(415aa)}$ receptor variants utilize the same splice donor site as human $H_{3(\Delta i3,365aa)}$, but different acceptor sites (see Figure 1). Interestingly, sequence alignment between guinea pig $H_{3(415aa)}$ and human $H_{3(445aa)}$ receptor mRNAs showed that the flanking regions of the deletion observed in guinea pig $H_{3(415aa)}$ mRNA contained consensus donor and acceptor splice sites that are conserved (95% identity) in the human $H_{3(445aa)}$ cDNA. However, the human equivalent of the guinea pig $H_{3(415aa)}$ and rat $H_{3(413aa)}$ was not detected in the present study. These observations suggest that alternative splicing of the H_3 gene differs between the analysed species and/or tissues (human thalamus and guinea pig cerebral cortex [9]) or that other H_3 mRNA species might exist in the human.

Brain distribution of H_3-variant transcripts

The distribution of H_3 mRNAs using RT-PCR analysis revealed that the H_3 variants were mainly coexpressed in human brain, but that their relative expression level varied in a region-specific manner (see Figure 4B). It has also been reported that the relative expression of the two guinea pig $H_{3(445aa)}$ and $H_{3(415aa)}$ variants differed between cortex and striatum [9]. The various patterns of mRNA expression suggest that cellular mechanisms may regulate the rate of splicing and the final ratio of receptor isoforms. Comparison between Northern blot and RT-PCR results showed that the $H_{3(445aa)}$ mRNA was the most abundant transcript and displayed an expression profile similar to that of the 2.7 kb mRNA species (see Figure 4). It is highly expressed in thalamus, caudate nucleus and cerebellum and significantly expressed in amygdala, substantia nigra and cerebral cortex (Figure 4B). The important point in the present study is that, except for cerebellum, the $H_{3(445aa)}$ transcript distribution and expression level correlated with the H_3 binding site densities in rat brain, as also reported by Arrang et al. [8].

Pharmacological properties of H_3 variants

To determine the biological role of the H_3-receptor variants compared with the 'original' $H_{3(445aa)}$, three H_3 isoforms, namely $H_{3(445aa)}$, $H_{3(\Delta TM2,431aa)}$ and $H_{3(\Delta i3,365aa)}$, were expressed in CHO-K1 cells and their pharmacological properties were investigated. Immunofluorescence control experiments showed that the three tagged H_3-receptor isoforms exhibited similar subcellular localization and receptor expression levels (see Figure 5). Binding studies showed that $H_{3(\Delta TM2,431aa)}$, transiently expressed in CHO-K1 cells, was unable to bind [^{125}I]iodoproxyfan, whereas $H_{3(445aa)}$ displayed a high affinity ($K_d = 28 \pm 5$ pM) for this compound. These results suggest that the 14 amino acids belonging to TM2 are involved in the iodoproxyfan binding site. Alternatively, these amino acids may have an essential role in the integrity of receptor conformation. In contrast with $H_{3(\Delta TM2,431aa)}$, the $H_{3(\Delta i3,365aa)}$ isoform lacking 80 amino acids in i3, was found to bind [^{125}I]iodoproxyfan with an affinity ($K_d = 8 \pm 1$ pM) in the same range as the 'original' $H_{3(445aa)}$ receptor. Moreover, the pharmacological profile of this variant for both agonists and antagonists was similar to that of the 'original' H_3 receptor (see Table 2). These results are in agreement with the fact that the ligand binding site is likely to reside in the TMs [5] and that it was not affected by a change in the composition of i3.

Signal transduction of H_3-receptor variants

We investigated whether the truncation within i3 of the $H_{3(\Delta i3,365aa)}$ receptor could change the coupling efficiency to the G_i-protein or display differential G-protein interaction compared with the $H_{3(445aa)}$ isoform. Indeed, for the human dopamine D_2 receptor, the loss of 29 amino acids in i3 of the short D_{2S} isoform decreases the coupling efficiency to the $G_{\alpha i2}$-protein [15,16,17].

Our first results confirmed that $H_{3(445aa)}$ was coupled to the G_i-protein as described by Lovenberg et al. [4,5]. Indeed, in CHO cells expressing the $H_{3(445aa)}$ receptor, H_3 agonists either inhibited forskolin-induced cAMP production or increased [^{35}S]GTP[S] binding (see Figure 6). Moreover, as described for several G_i-coupled-receptors [18], the $H_{3(445aa)}$ receptor was also able to mediate intracellular Ca^{2+} mobilization through the release of the $\beta\gamma$ subunits from the G_i-protein (see Figure 7).

In contrast with the 'original' $H_{3(445aa)}$ receptor, agonist stimulation of the $H_{3(\Delta i3,365aa)}$ variant neither modified adenylate cyclase activity nor induced intracellular Ca^{2+} mobilization (see Figure 7). The $H_{3(\Delta i3,365aa)}$ isoform thus seems not to be coupled to $G_{i/o}$-, G_s- or $G_{q/11}$-proteins. These results, together with the fact that $R(-)$-α-methylhistamine did not stimulate [^{35}S]GTP[S] binding to the membrane of CHO cells expressing the $H_{3(\Delta i3,365aa)}$ receptor (see Figure 6), suggest that this variant could be biologically inactive. However, we cannot exclude that the lack of $H_{3(\Delta i3,\ 365aa)}$ receptor coupling may be due to inappropriate G-protein expression in CHO cells, and that the histamine-mediated signal does exist in native tissues expressing the $H_{3(\Delta i3,365aa)}$ receptor. Finally, these results demonstrate that the 80 amino acids near the C-terminal portion of i3 of the H_3 receptor participate in the agonist-mediated signal transduction through the G_i-protein.

Conclusion

In summary, several human H_3-receptor variants with a deletion in i3 were found to be generated from a complex H_3 receptor gene located on chromosome 20. Such alternative splicing produces receptor variants with impaired capabilities to mediate agonist responses. It can therefore be speculated that the histamine response may be abolished or modified through activation of alternative splicing mechanisms. In addition, heterodimerization between the $H_{3(\Delta i3,365aa)}$ receptor and the fully coupled $H_{3(445aa)}$ receptor could inhibit or change the histamine responses, as already seen for several other G-protein-coupled receptors, such as the α_{1A}-adrenoceptor and the γ-aminobutyric acid $(GABA)_B$ receptor [19,20]. Undoubtedly, the availability of these H_3 variants will facilitate further investigations of their potential roles in physiological and pathophysiological processes that involve the H_3 receptor.

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