Biochemical and Biophysical Research Communications **280,** 75–80 (2001)
doi:10.1006/bbrc.2000.4073, available online at http://www.idealibrary.com on IDEAL®

The Rat H_3 Receptor: Gene Organization and Multiple Isoforms

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Received November 23, 2000

Genomic DNA analysis revealed that the coding region of the rat histamine H_3 receptor comprises three exons interrupted by two introns of ~1 kb each. Several H_3 receptor mRNA variants were identified by PCR and cDNA cloning and sequencing. Four variants generated by pseudo-intron retention/deletion at the level of the third intracellular loop were designated $H_{3(445)}$, $H_{3(413)}$, $H_{3(410)}$, and $H_{3(397)}$, according to the length of their deduced amino acid sequence and display differential tissue expression. When expressed in CHO-K1 or Cos-1 cells, the $H_{3(445)}$, $H_{3(413)}$, and $H_{3(397)}$ were found to generate specific [^{125}I]iodoproxyfan binding of similar pharmacological profile. In addition, we identified two short variants, termed $H_{3(nf1)}$ and $H_{3(nf2)}$, which correspond to frame shift and stop codon interposition, respectively, and are presumably nonfunctional, among which $H_{3(nf2)}$ displays brain expression similar to that of the longer isoforms. © 2001 Academic Press

Key Words: **H_3 receptor; isoforms; regional distribution; gene structure; *in situ* hybridization; [^{125}I]iodoproxyfan binding.**

The histamine H_3 receptor (H_3R) was initially characterized as an autoreceptor regulating histamine synthesis and release in brain (1, 2) and cDNAs encoding the human (3), guinea pig (4), and rat (5–7) H_3R have only recently been cloned. Cloning of the guinea pig H_3R revealed, unexpectedly, the existence of two isoforms, termed H_{3L} and H_{3S}, differing by a stretch of 30 amino acids within the third intracytoplasmic loop of the receptor (4).

Most of the previous studies that established the localisations, signalling mechanisms and detailed pharmacology of the H_3R were performed in the rat, and a possible molecular heterogeneity of the receptor has been suggested (8, 9). Here, we have established the organization of the rat H_3R gene which indicates that two introns are located in the second transmembrane domain and second intracytosolic loop and report the existence of multiple isoforms of the rat H_3R.

[1] These authors contributed equally to this study.

MATERIALS AND METHODS

Molecular cloning and PCR analysis of rat H_3-receptor cDNA isoforms. RNAs from total rat brain (10 μg) were used for first strand cDNA synthesis using AMV reverse transcriptase (50 units, Finzymes) and 0.19 μM random primer $p(DN)_6$ (Roche). The obtained templates were amplified for 35 cycles (94, 56, and 72°C for 15, 15, and 30 s, respectively) using AmpliTaq Gold DNA polymerase (Perkin-Elmer) and primers 1 and 2, based on the sequence of the fourth transmembrane domain and the third intracellular loop of the rat H_3 receptor (5–7), respectively (primer 1: 5′-TGCTGTATGGGCCTGCCATCCTGAGTTGG-3′ and primer 2: 5′-CACCATCTTCATGCGCTTCTCCAGGGATGC-3′). The full-length cDNAs of the isoforms were obtained by PCR amplification of the first strand cDNA for 35 cycles (94, 56, and 72°C for 30 s, 30 s, and 1 min, respectively) using primers 3 and 4 corresponding to nt 1–33 of the rat H_3 receptor (5–7) and nt 1601–1637 of the human H_3 receptor (3), respectively (primer 3: 5′-ATGGAGCGCGCGCCGCCCGACGGGCTGATGAAC-3′ and primer 4: 5′-CTCACTTCCAGCAGTGCTCCAGGGAGCTGTGGGGCTG-3′). After migration of the PCR products on a 2% agarose gel, bands corresponding to the isoforms were extracted from the gel using NucleoSpin Extract 2 in 1 (Macherey-Nagel), subcloned into pCR-II vector (Invitrogen) and sequenced.

For cloning, screening of a rat striatal cDNA library was also performed at high stringency with a ^{32}P-labelled DNA fragment obtained by amplification of RNAs from rat cerebral cortex using primers based on the sequence of TM3 and the third intracellular loop (primer 5: 5′-CATCAGCTATGACCGATTCCTGTC-3′ and primer 6: 5′-AGTCAAGCTTGGAGCCCCTCTTGAGTGAGC-3′). Bluescript plasmids were recovered from 60 positive clones and their cDNA inserts sequenced.

When the expression of the isoforms was analyzed, PCR products amplified from RNAs of various rat brain regions and peripheral tissues with primers 1 and 2 were electrophoresed on a 2% agarose gel, blotted overnight onto nylon Hybond-N+-membranes (Amersham), and hybridized at 68°C for 1 h in ExpressHyb Solution (Clontech) with a ^{32}P-labelled cDNA probe (607 bp) (7).

Analysis of H_3-receptor genomic DNA. The genomic organization was analyzed by PCR analysis. Rat genomic DNA was amplified with

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Platinum Pfx DNA polymerase (Life Technologies) for 35 cycles (95, 60, and 68°C for 30 s, 30 s, and 5 min, respectively) using three sets of primers (0.8 μM each) designed along the H_3 receptor cDNA sequence. Primers 7 and 8 were used to amplify a fragment which encompassed TM1 and TM2 (primer 7: 5′-CTTCTCGGCAGCCTGGACCGCGG-TGCTG-3′ and primer 8: 5′-GTCAGCACGTAGGGTACATAC-AGTGGATG-3′). Primers 5 and 9 are based on the sequence of the 3′ end of TM3 and TM5 (primer 9: 5′-TGTTCAGGTAGATGCT-GAGGTTGAAGAAGG-3′). Primers 10 and 11 are based on the sequence of TM5 and TM6 and were used to amplify a fragment encompassing the third intracellular loop (primer 10: 5′-AGCGTTACCTTCTTCAACCTCAGC-3′ and primer 11: 5′-AGCAGAGCCCAAAGATGCTCAC-3′). PCR products were electrophoresed on a 1% agarose gel, subcloned and sequenced as described above.

Transient expression in Cos-1 cells. The cDNA inserts corresponding to the full length coding sequence of the $H_{3(445)}$, $H_{3(413)}$, and $H_{3(397)}$ were ligated into the mammalian expression vector pClneo (Promega) with Rapid DNA Ligation Kit (Roche) (isoforms designated according to the length of their deduced amino acid coding sequence). Cos-1 cells were grown to 60% confluence in Dulbecco's modified Eagle's Medium (Life Technologies) with 10% foetal calf serum (Pan Biotech GMBH) in 96 mm Petri dishes and transfected with 10 μg of pClneo-$H_{3(445)}$, pClneo-$H_{3(413)}$, or pClneo-$H_{3(397)}$ plasmids using 40 μl of SuperFect Reagent (Qiagen). After two days, the cells (100% confluence) were harvested and membranes prepared for binding assays.

Stable transfection of CHO-K1 cells. CHO-K1 cells were transfected with the pClneo-$H_{3(445)}$ or pClneo-$H_{3(413)}$ plasmids using SuperFect Reagent (Qiagen). Stable transfectants were selected with 2 mg/ml of G418. Two clones, named CHO($H_{3(445)}$) and CHO($H_{3(413)}$), expressing ~300 fmol of receptor per mg of protein were selected for further characterization and maintained in the presence of 1 mg/ml of G418 (7).

[^{125}I]Iodoproxyfan binding assays. Assays were performed as described previously (10). Briefly, aliquots of membrane suspensions from transfected Cos-1 or CHO-K1 cells (5–15 μg of protein) were incubated for 60 min at 25°C with 22–25 pM [^{125}I]iodoproxyfan alone or together with competing drugs (200 μl final volume). Nonspecific binding was determined using imetit (1 μM).

In situ hybridization histochemistry. Rats were killed by decapitation and their brain was removed rapidly, immediately frozen (−40°C) by immersion in monochlorodifluoromethane, and stored at −70°C. Sections were prepared on a cryostat (10 μM), treated with proteinase K (1 μg/ml) and hybridized for 12 h at 55°C in the presence of 50% formamide and 4×10^6 dpm of ^{33}P-labelled antisense or sense probes. Subsequently, sections were treated with RNase A (200 μg/ml) for 40 min at 37°C, rinsed in standard saline citrate and dehydrated. They were exposed for 1 or 2 weeks to β_{max} Hyperfilms (Amersham). For the hybridization probes, a partial coding sequence of the rat H_3 receptor, amplified from RNAs of rat cerebral cortex and primers 5 and 6, was used for labelling of H_3-receptor mRNAs. Primers 14 and 15 were used to amplify a probe (354 bp) designed for the specific labelling of the $H_{3(nf2)}$ isoform (primer 12: 5′-CTGGTGCAGAGTCGGGACAAAGCCCTTCC-3′ and primer 13: 5′-CAACAGGTCAGCAGCACTGGGCTGGAGGC-3′). The amplified fragments were subcloned into pGEM-T (Promega). ^{33}P-labelled antisense and sense strand RNA probes were prepared by *in vitro* transcription using a Riboprobe kit (Promega).

RESULTS

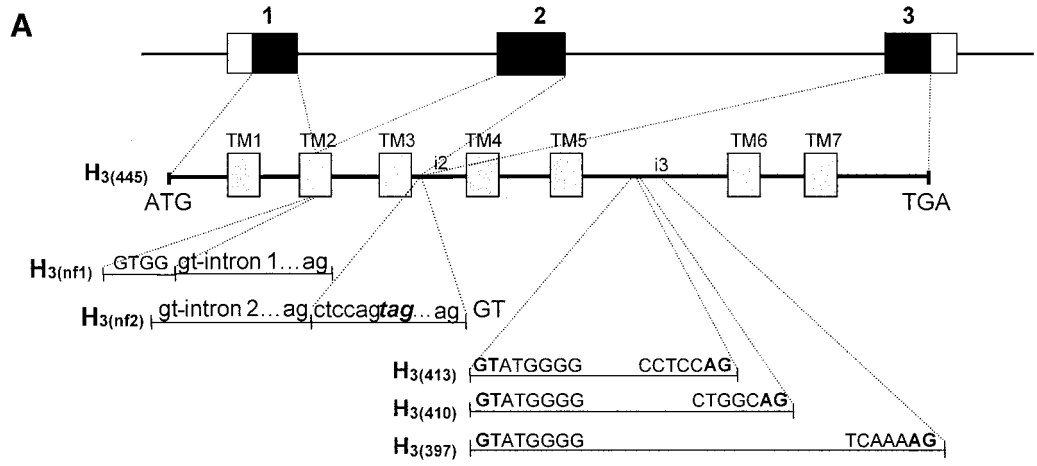
Identification of H_3 Receptor Isoforms

The existence of H_3-receptor isoforms was investigated by PCR analysis of cDNAs from total rat brain using specific primers 1 and 2. The gel-resolved DNA products comprised three fragments of ~530, 430, and 400 bp in length. Subsequent cloning and sequencing of these products revealed the existence of four isoforms of the rat H_3 receptor. The largest fragment ($H_{3(445)}$) corresponded to the previously published form of the H_3 receptor in human (3) that we named H_{3L} in guinea-pig (4) and rat (7). The 430-bp and 400-bp DNA fragments corresponded to three types of shorter fragments that differed by deletions of 96, 105, and 144-bp fragments (corresponding to nt 821–917, 821–926, and 821–995, respectively). Cloning of the corresponding full length cDNAs was performed using primers 3 and 4 and confirmed that the deletions did not change the open reading frame, leading to $H_{3(413)}$, $H_{3(410)}$, and $H_{3(397)}$ isoforms generated by deletions of 32, 35, and 48 amino acids, respectively, all located in the third intracellular loop of the H_3 receptor (Fig. 1).

Screening of a rat striatum cDNA library led to the isolation of several clones containing the full-length sequence of $H_{3(445)}$- and $H_{3(413)}$-receptor isoforms, that we described recently (7) and to the identification of two additional and presumably nonfunctional isoforms corresponding to a 94- and 141-amino acid protein, respectively. The first isoform, termed $H_{3(nf1)}$, was generated by a deletion of 4 nt in TM2 which results in a shift of the open reading frame, introducing a downstream stop codon. The second isoform, termed $H_{3(nf2)}$, was generated by an insertion of 396 nt located after nt 417 in the second intracellular loop and containing a stop codon (Fig. 1).

Analysis of H_3 Receptor Genomic DNA

The organization of the rat H_3R gene was studied by PCR analysis of rat genomic DNA. In agreement with the structure of the human H_3R gene that we recently disclosed (11), PCR amplifications performed with different primers designed in the coding region of the rat H_3 receptor, revealed that the rat H_3R gene also contains three exons interrupted by two introns (Fig. 1). A fragment of 1.2 kb was obtained with primers 7 and 8 from rat genomic DNA, i.e., was higher than that predicted from the cDNA sequence (0.2 kb) (data not shown). Nucleotide sequence analysis of the amplified fragment revealed an insertion of an intron at nt 250 in TM2. The size of the fragment amplified from rat genomic DNA with primers 5 and 9 (1.6 kb) was also higher than the size predicted for the corresponding cDNA sequence (0.3 kb). Its nucleotide sequence analysis showed that the second intron was inserted at nt 417 of the coding sequence, i.e., in the second intracellular loop (Fig. 1). Amplifications of genomic rat DNA performed downstream of intron 2 did not reveal the existence of additional introns. The size of the fragment amplified with primers 10 and 11, encompassing the third intracellular loop was in good agreement with the size predicted by the cDNA sequence (data not shown).



B

Exon	Splice donor	Intron	Splice acceptor	Exon
1	CGTGG**gt**aaagc	1	cccat**ag**GTGCC	2
2	GAGCT**gt**gagtc	2	ttctc**ag**GTCTC	3

FIG. 1. Exon/intron structure of the rH_3-receptor gene and rH_3R isoforms generated by retention/deletion of pseudo-introns. (A) Top, diagram of the rH_3R genomic DNA. Exons indicated by the boxes are numbered. Open boxes indicate nontranslated sequence. Bottom, structure of the $rH_{3(445)}$ receptor cDNA. Regions encoding transmembrane domains are represented by hatched boxes (TM1-TM7). i2 and i3 indicate the second and third intracytosolic loops, respectively. Deletions/insertions leading to two presumably non-functional H_3R isoforms, $H_{3(nf1)}$ and $H_{3(nf2)}$, and three functional shorter isoforms, $H_{3(413)}$, $H_{3(410)}$, and $H_{3(397)}$ are indicated. (B) Exon/intron junctions within the rH_3R gene.

Tissue Distribution of Rat H_3R Isoforms

The tissue distribution of the rat H_3 receptor isoforms was analyzed by Southern-blot of RT-PCR amplifications. Similar patterns were obtained after 25 or 35 cycles of amplification and revealed a differential expression of the isoforms in various brain regions. In the peripheral tissues, only the longer isoform was observed (Fig. 2).

The distribution of the H_3 receptor gene transcripts was also established by *in situ* hybridization histochemistry performed on sagittal sections of rat brain. Autoradiograms obtained with a selective antisense probe revealed a wide and heterogeneous distribution of H_3 receptor gene transcripts in the central nervous system, whereas no background signals were observed with the corresponding sense probe (Fig. 3 and data not shown). A high level of expression was observed in the olfactory tubercles, caudate putamen, nucleus accumbens, thalamus, anterior olfactory nucleus, and cerebral cortex in which a laminar distribution pattern was found. A high expression level was also detected in the tuberomammillary nucleus.

In situ hybridyzation performed with a riboprobe selective for the $H_{3(nf2)}$ isoform, revealed a general distribution pattern very similar to that obtained with the H_3 receptor riboprobe (Fig. 3B), with limited differences observed in the Ammon's horn where the expression of the $H_{3(nf2)}$ mRNA was stronger in CA_2 and CA_3 pyramidal layers.

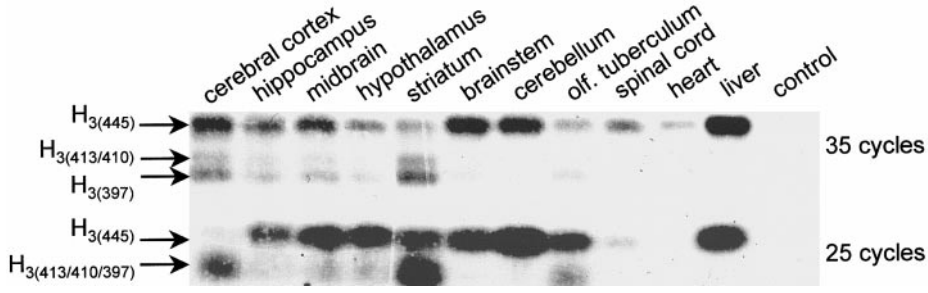


FIG. 2. Southern blots of RT-PCR analysis of H_3-receptor gene transcripts in various rat brain regions and peripheral tissues showing regional distribution of $H_{3(445)}$-, $H_{3(413)}$-, and $H_{3(397)}$-receptor RNAs (25 and 35 amplification cycles).

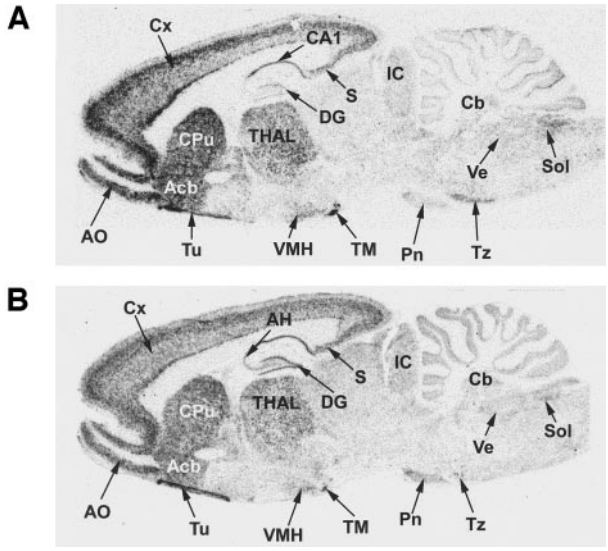


FIG. 3. *In situ* hybridization analysis of H_3 receptor mRNAs in the adult rat brain. Distribution of H_3-receptor gene transcripts (A) and $H_{3(nf2)}$-receptor gene transcripts (B) analyzed with a ^{33}P-labelled riboprobe in a sagittal section of the adult rat brain. Acb, accumbens nucleus; AH, Ammon's horn; AO, anterior olfactory nucleus; Cb, cerebellum; CPu, caudate putamen; Cx, cortex; DG, dentate gyrus; IC, inferior colliculus; Pn, pontine nucleus; S, subiculum; Sol, nucleus of the solitary tract; THAL, thalamus; TM, tuberomammillary nucleus; Tu, olfactory tubercle; Tz, nucleus of the trapezoid body; Ve, vestibular nucleus; VMH, ventromedial hypothalamic nucleus.

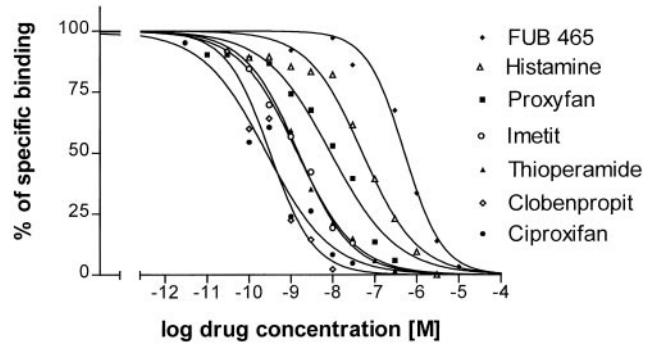


FIG. 4. Inhibition of [^{125}I]iodoproxyfan binding to the $rH_{3(397)}$ receptor by histamine H_3-receptor ligands. Membranes of transiently transfected Cos-1 cells were incubated with 22 pM [^{125}I]iodoproxyfan and drugs in increasing concentrations. Each point represents the mean value of three determinations.

Pharmacological Analysis of H_3R Isoforms

We recently described the pharmacological profiles of the rat $H_{3(445)}$ and $H_{3(413)}$ isoforms (6, 7). The pharmacological profile of the $H_{3(397)}$ isoform, described here for the first time, was established on membranes of Cos-1 cells following transient expression. Specific [^{125}I]iodoproxyfan binding was monophasic and saturable (B_{max} 300 fmol/mg protein). Nonlinear regression analysis using a one-site cooperative model led to a K_D value of 94 ± 16 pM. Various H_3 receptor agonists and antagonists inhibited [^{125}I]iodoproxyfan binding to the $H_{3(397)}$ receptor (Fig. 4). The deduced K_i values are reported in Table 1 and compared to values obtained from [^{125}I]iodoproxyfan binding studies performed using membranes of CHO cells stably expressing $H_{3(445)}$ (6) and $H_{3(413)}$. The pseudo Hill coefficients of the agonists, imetit and histamine, were close to 0.7 but did not significantly differ from unity for the antagonists (Table 1). [^{125}I]iodoproxyfan binding to membranes of Cos-1 cells expressing $H_{3(445)}$ was also performed for selected compounds, proving that the K_i values obtained from different cell lines (Cos-1 and CHO-K1) did not differ significantly. For example, K_i values of ciproxifan and thioperamide obtained with membranes of $H_{3(445)}$ transfected Cos-1 cells (1.5 and 6.2 nM, respectively) were in close agreement with values presented in Table 1. Moreover, binding studies performed with histamine, ciproxifan, and clobenpropit on membranes of Cos-1 cells expressing $H_{3(413)}$ receptors led to K_i values (100, 0.96, and 1.1 nM respectively) which were in excellent agreement with those obtained in CHO cells (Table 1 and (6)).

DISCUSSION

Nucleotide sequence analysis of genomic DNA revealed that the coding region of the rat H_3 receptor comprises three exons interrupted by two introns of ~1 kb each. The two introns are located at the same level as the two introns that we recently identified in the coding region of the human H_3-receptor gene (11), i.e., in the second transmembrane domain and second intracellular loop, respectively.

We identify here several isoforms of the rat H_3 receptor by cDNA cloning. Two of them, termed $H_{3(nf1)}$ and $H_{3(nf2)}$, do not contain a full length open reading frame and would correspond to truncated, i.e., presum-

TABLE 1

Compared Potencies of H_3-Receptor Ligands on the Inhibition of [^{125}I]Iodoproxyfan Binding to H_3-Receptor Isoforms Transfected in Cos-1 ($H_{3(397)}$) or CHO-K1 ($H_{3(413)}$ and $H_{3(445)}$) Cells[a]

	K_i (nM)		
Ligands	$H_{3(397)}$	$H_{3(413)}$	$H_{3(445)}$[b]
Histamine	28 ± 4	61 ± 8	20 ± 2
Imetit	1.1 ± 0.2	1.0 ± 0.2	0.33 ± 0.04
Thioperamide	1.7 ± 0.3	4.3 ± 1.2	6.5 ± 0.2
Ciproxifan	0.8 ± 0.1	0.96 ± 0.18	3.9 ± 0.2
Clobenpropit	0.5 ± 0.1	0.44 ± 0.08	1.4 ± 0.1
FUB 465	376 ± 24	399 ± 18	132 ± 12
Proxyfan	9.6 ± 2.4	5.6 ± 0.5	2.9 ± 0.2

[a] Each value represents the mean of 2–4 independent experiments with triplicate determinations each.

[b] Values taken from (6), except Imetit.

ably non functional isoforms. The $H_{3(nf1)}$ isoform is generated by the use of two potential splice donor dinucleotides (GT) at the splice junction site of intron 1. The $H_{3(nf2)}$ isoform is generated by the use of an alternative splice acceptor site within intron 2. The functional significance, if any, of their transcripts is unclear. $H_{3(nf1)}$ cDNAs were isolated from striatum and the general distribution pattern of $H_{3(nf2)}$ mRNAs analyzed by *in situ* hybridization is very similar to the pattern of H_3 receptor mRNAs, suggesting that they are expressed in the same neuronal populations and that they might be involved in the regulation of the expression and/or function of H_3 receptors.

We recently reported the deletion of a 30-amino acid fragment in the third intracellular loop of the receptor, which leads to a shorter isoform, termed H_{3S}, in the guinea pig (4). The retention/deletion of a corresponding 32-amino acid sequence at the same level of the human (11) and rat H_3 receptor also leads to the existence of H_{3L} and H_{3S} variants that we now propose to designate according to their reduced amino acid length, i.e., $H_{3(445)}$ and $H_{3(413)}$ isoforms. In addition to these previously known variants, we identify here $H_{3(410)}$- and $H_{3(397)}$-receptor isoforms in which the fragment deleted from the third intracytosolic loop was longer, including three and sixteen additional amino acids, respectively. We recently determined, by nucleotide sequence analysis of the human gene, that the deleted sequence is not flanked by introns. The absence of flanking introns in the rat gene is supported by PCR analysis of genomic DNA, since the size of the amplified fragment spanning the third intracellular loop, was similar to that expected from the corresponding rat cDNA sequence. Moreover, nucleotide sequence analysis revealed the presence of the same consensus splice donor (5′-GTATGGG-3′) together with various acceptor sites at the 5′ and 3′ ends of each deleted sequence, indicating that the $H_{3(413)}$, $H_{3(410)}$, and $H_{3(397)}$ isoforms are generated by deletion of a pseudo-intron, as we recently showed for the human receptor (11).

An important finding of this study is that the expression pattern of the various isoforms clearly differs, among both brain regions and peripheral tissues: in the latter, only the longer ($H_{3(445)}$) isoform was observed and the relative ratios of the isoforms dramatically varied among brain areas. Although the longer isoform was the more abundant in most brain regions, a significant expression level of the shorter variants was observed, except in the cerebellum where they were hardly detectable. The $H_{3(397)}$ variant was even predominant in striatum. These findings may indicate that the splicing mechanisms are regulated within the same neurons or that the various isoforms are differentially expressed among distinct neuronal populations in which they may subserve different functions.

We recently reported from functional studies that the pharmacological profiles of the rat $H_{3(445)}$ and $H_{3(413)}$ isoforms were rather similar (7). Binding studies confirm that the various isoforms, including the $H_{3(397)}$ variant, display limited pharmacological differences. However, these pharmacological differences of multiple variants displaying different expression patterns may partly account for the H_3-receptor heterogeneity previously reported in tissues from functional and binding studies (12–14).

ACKNOWLEDGMENT

This work was supported by the Deutsche Pharmazeutische Gesellschaft, DPhG-Stiftung zur Förderung des wissenschaftlichen Nachwuchses, Germany.

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