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Identification, Molecular Cloning, and Distribution of a Short Variant of the 5-Hydroxytryptamine$_{2C}$ Receptor Produced by Alternative Splicing

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SUMMARY

The actions of the neurotransmitter 5-hydroxytryptamine (5-HT) (serotonin) are mediated by multiple receptor subtypes. One of the prominent serotonin receptors in the brain is the 5-HT$_{2C}$ receptor (5-HT$_{2C}$-R). We report the occurrence of a second 5-HT$_{2C}$-R transcript, first identified using S1 nuclease protection of total RNA isolated from the choroid plexus. Analyses of the distribution of these two RNAs revealed that the short form is expressed in the same structures as the 5-HT$_{2C}$-R mRNA, including choroid plexus, striatum, hippocampus, hypothalamus, olfactory tubercles, and spinal cord. Cloning and sequence analyses revealed a second cDNA with a 95-nt deletion in the region coding for the putative second intracellular loop and the fourth transmembrane domain of the 5-HT$_{2C}$-R. This deletion leads to a frameshift in the coding sequence and the introduction of a premature stop codon. The predicted truncated protein (5-HT$_{2C\text{-}tr}$) contains 172 amino acids, with 153 residues at the amino terminus, identical to the 5-HT$_{2C}$-R, and 19 carboxyl-terminal amino acids that are unique. Although antibodies specific to the 5-HT$_{2C\text{-}tr}$ protein showed that the truncated form is expressed in a transfected fibroblast cell model system, there was no serotonergic ligand binding activity or phosphoinositide hydrolysis. Analyses of the 5-HT$_{2C}$-R gene revealed that the two transcripts arise from a single gene by differential splicing using alternative donor sites and a common 3′-splice acceptor. Polymerase chain reaction amplification of mouse and human brain cDNAs demonstrated the occurrence of the same splicing patterns in these species. Although this study demonstrates tissue-specific expression of two 5-HT$_{2C}$ mRNA splice variants in rat, mouse, and human, the significance of the truncated form in these three species remains to be established.

The diverse physiological effects of 5-HT (serotonin) in mammals are mediated by numerous receptors that have been classified into seven subfamilies on the basis of functional, pharmacological, and molecular differences (1). The 5-HT$_2$ receptor subfamily comprises three subtypes, 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ (formerly called 5-HT$_2$, 5-HT$_{2F}$, and 5-HT$_{1C}$, respectively), which are functionally linked to the phosphoinositide hydrolysis pathway (2). The structures of these three receptors have been identified by molecular cloning strategies, demonstrating that they belong to the large family of G protein-coupled receptors (3) and are encoded by distinct genes (4). In contrast to many G protein-coupled receptors, which are intronless, the 5-HT$_2$ receptor subfamily genes contain several introns and exons in their coding regions. In the current study, we focus on the 5-HT$_{2C}$-R, which is highly expressed in choroid plexus epithelial cells (5, 6), where it stimulates cGMP formation (7) in addition to phosphoinositide turnover (2). The 5-HT$_{2C}$-R is widely distributed throughout the brain and spinal cord (8–11) and has been implicated in the control of feeding behavior (12), the etiology of affective disorders (13), and the pharmacological actions of the atypical antipsychotic drug clozapine (14) and hallucinogens (15). The 5-HT$_{2C}$-R was recently shown to exhibit constitutive activity in a transfected cell line (16), suggesting that this receptor subtype may be involved in setting basal levels of phosphoinositide hydrolysis.

The molecular structure of the 5-HT$_{2C}$-R has been described in the rat (17), human (18), and mouse (4). The overall structure of the gene and the positions of intron/exon boundaries are conserved among these species (4, 19). Attempts to identify differentially spliced variants for the 5-HT$_{2C}$-R have been reported (20), but no alternatively spliced forms were identified. In the current study, we report the existence of a second transcript derived from the 5-HT$_{2C}$-R gene expressed in the same brain regions as the

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-HT$_{2C}$-R, 5-hydroxytryptamine$_{2C}$ receptor; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; nt, nucleotide.

5-HT_{2C}-R mRNA. Initially identified by S1 nuclease protection in rat brain, this transcript was subsequently cloned from a rat striatum cDNA library; this novel 5-HT_{2C} mRNA contains a deletion altering the amino acid reading frame and encodes a truncated protein (5-$HT_{2C\text{-}tr}$). Analyses of the rat 5-HT_{2C} gene show that the 5-$HT_{2C\text{-}tr}$ mRNA is generated by differential RNA processing in which the use of an alternative 5′-splice junction gives rise to an mRNA species containing a 95-nt deletion. PCR amplification of mouse and human brain cDNA demonstrates that this truncated form is conserved evolutionarily among all three species.

Materials and Methods

Recombinant DNA procedures. Recombinant DNA procedures were performed using standard techniques (21). Radioactive nucleotides and deoxynucleotides were obtained from New England Nuclear Research Products (Boston, MA). Except where indicated, restriction endonucleases and modifying enzymes were purchased from New England Biolabs (Beverly, MA), cell culture reagents from Life Technologies (Grand Island, NY), and chemicals from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

RNA extraction and S1 nuclease protection. A clonal fibroblast cell line (clone B 3–6) (16), stably transfected with 5-HT_{2C}-R cDNA (17), was used as a source of standard 5-HT_{2C}-R mRNA. To obtain total tissue RNA, 21-day-old or adult (250 g) male Sprague-Dawley rats (Sasco, Omaha, NE) were decapitated, and the brains were removed and placed on ice; choroid plexi were dissected according to Sanders-Bush *et al.* (22). Striatum, hippocampus, and other structures were dissected according to Glowinski and Iversen (23) and frozen in liquid nitrogen. Stomach fundus, liver, and kidney were removed; immediately frozen in liquid nitrogen; and minced before RNA extraction. Total RNA was isolated by centrifugation over a cesium chloride cushion as described previously (24). Briefly, structures were homogenized in buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.14 M β-mercaptoethanol) using a Brinkman Instruments Polytron (Westbury, NY), layered over a cushion of 5.7 M cesium chloride, and centrifuged at 260,000 × *g* for 18 hr at 20°. The resulting pellet was resuspended in diethyl pyrocarbonate-treated water, precipitated with ethanol, and quantified by measuring absorbance at 260 nm. Total RNA from B 3–6 cells was prepared according to Barker and Sanders-Bush (25).

Solution hybridization/S1 nuclease protection assays were performed according to Barker and Sanders-Bush (25) using a 570-nt ^{32}P-labeled riboprobe corresponding to a region extending from the beginning of the second intracellular loop through the third intracellular loop of the 5-HT_{2C}-R (nt 1143–1617); the predicted length of the protected fragment was 475 nt. A rat β-actin ^{32}P-riboprobe was used as an internal standard (265-nt riboprobe leading to a 143-nt protected fragment). Riboprobes were synthesized using the Promega Gemini II transcription kit (Madison, WI). Transcription was performed in presence of [α-^{32}P]CTP. After overnight hybridization and subsequent digestion with S1 nuclease, the protected fragments were visualized using 4% polyacrylamide/7 M urea gel electrophoresis. Intensities of the bands were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Quantification of the relative levels of 5-HT_{2C}-R and 5-$HT_{2C\text{-}tr}$ mRNAs was performed by correcting the PhosphorImager signal for the number of cytosine residues present in each protected fragment (89 for the 5-$HT_{2C\text{-}tr}$ protected fragment versus 111 for the 5-HT_{2C}-R).

cDNA library screening. A Sprague-Dawley rat striatum cDNA library (Lambda ZAPII, Stratagene, La Jolla, CA) was screened with a ^{32}P-radiolabeled cDNA probe encoding the first-through-second transmembrane-spanning domains of the 5-HT_{2C}-R (*AgeI*/*Msc*I fragment, 287 bp). Bacteriophage plaques (1.25 × 10^6 total) were screened at high stringency by filter hybridization, and positive plasmids were rescued in pBluescript SK(⁻) according to the manufacturer's instructions. Sequencing of the clones isolated from the cDNA library was accomplished by the dideoxynucleotide chain termination method using the fmol sequencing kit/*Taq* polymerase (Promega). The largest 5-$HT_{2C\text{-}tr}$ clone was selected, and both strands were completely sequenced using the *Taq* polymerase kit as well as an Applied Biosystems model 373A sequencer (Applied Biosystems, Norwalk, CT). Products resulting from PCR amplification of rat, mouse, and human cDNAs were sequenced directly, and four subclones of each reaction product [in pBluescript SK(⁻)] were again sequenced using T7 and T3 universal primers; no nucleotide differences were found between these two methods.

PCR amplification of rat, human, and mouse cDNA. Total RNA was prepared from brains of 21-day-old male Sprague-Dawley rats according the method described above. cDNA was synthesized using an oligo(dT) primer and avian myeloblastosis virus reverse transcriptase and then purified after hydrolysis of the remaining RNA and extraction of the cDNA by GENO-BIND beads (Clontech, Palo Alto, CA). Adult male Balb/c mouse brain and human caudate nucleus cDNA [prepared using an oligo(dT) primer and purified to remove interfering RNA and genomic DNA] were purchased from Clontech.

PCR reactions were performed using 1 ng of cDNA, 2.5 units of *Taq* DNA polymerase, 0.4 mM concentration of the dNTPs, 10 pmol of each primer, and buffer supplied by the manufacturer (Promega Corp) supplemented with 2.5 mM $MgCl_2$ (total volume, 50 μl). Amplification conditions were as follows: initial denaturation at 94° for 5 min followed by 35 cycles of denaturation (94° for 1 min), annealing (60° for 45 sec), and elongation (72° for 45 sec), followed by a final extension step at 72° for 5 min. The oligonucleotides used were rat: sense primer (nt 376–393 relative to the 5-HT_{2C}-R start codon) (17), 5′-TAT TTG TGC CCC GTC TGG-3′; antisense primer (727–752), 5′-CGA AGT AAC ATC AGA GTT TGA CGG CG3-′; mouse: sense primer (376–393), 5′-TAT TTG TGC CCC GTC TGG-3′ (4); antisense primer (607–634), 5′-CAT TGA GCA CGC AGG TAG TAT TAT TCA C-3′; and human: sense primer (373–390), 5′-TAT TTG TGC CCC GTC TGG-3′ (18); antisense primer (642–665), 5′-GCT ACG AAG GAC CCA ATA AGA ACG-3′. After amplification, cDNA fragments were resolved on a 2% agarose gel, isolated, and subcloned in a modified vector according to the method of Davis *et al.* (21). The isolated products were directly sequenced, as were several subclones of each of the fragments, to confirm the absence of *Taq* polymerase misincorporation.

Genomic Southern blot analyses. Ten micrograms of rat (21-day-old male Sprague-Dawley) genomic DNA was digested overnight with the following restriction enzymes: *Eco*RI, *Eco*RV, *Bam*HI, or a combination of *Eco*RI/*Eco*RV, *Eco*RI/*Bam*HI, or *Eco*RV/*Bam*HI. Buffers supplied by the manufacturer were used, and the incubation was performed in a final volume of 50 μl. Digested samples were electrophoresed on a 0.8% agarose gel at 50 V for 16 hr, transferred to a nylon membrane (BioRad, Richmond, CA) by capillary action, and fixed by baking in a vacuum oven for 30 min at 80°. Filters were probed with a ^{32}P-labeled random primed probe using a DNA template that contained a common exon to the 5-HT_{2C}-R and the 5-$HT_{2C\text{-}tr}$ (566–1508 relative to the 5-HT_{2C}-R start codon). No restriction sites corresponding to *Eco*RI, *Eco*RV, or *Bam*HI were present in the nucleotide sequence of the cDNA fragment used as probe. The choice of the probe was based on the published structure of the mouse and human 5-HT_{2C} gene (4, 19), assuming that the intron/exon positions were conserved in the rat 5-HT_{2C} gene.[1] To generate the DNA template, 1 ng of 5-HT_{2C} cDNA in pBluescript II(⁻) was amplified by PCR as described above using the sense primer [5′-CTA TCC CTG TGA TTG GAC-3′ (nt 566–583)] and the antisense primer [5′-CAG GGT TGC AAA GCT ATT-3′ (1508–1525)]. The 960-bp product was purified using an 0.9% agarose gel and labeled using the Random-Prime kit II from Stratagene. After hybridization and washing, blots were exposed to X-ray film for 4 days at −70°.

[1] J. Aimi, H.-T. Kao, and R. D. Ciaranello, unpublished observations.

Generation of affinity-purified 5-HT$_{2C\text{-}tr}$ antibodies. The 13-amino acid carboxyl-terminal peptide (NH_2-CDWTEGRKQSVRE-COOH) of the unique 19-amino acid 5-HT$_{2C\text{-}tr}$ sequence was used to generate and purify specific antibodies.[2] Briefly, the peptide was conjugated to carrier proteins and used to immunize a female New Zealand white rabbit (Myrtle's Rabbitry, Franklin, TN). Specific antibodies were purified from sera using the 2C-truncated peptide conjugated to a cysteine-reactive matrix (Sulfolink; Pierce Chemical, Rockford, IL). The affinity-purified antibodies are referred to as anti-2C-tr.

Expression of the 5-HT$_{2C\text{-}tr}$ cDNA in fibroblasts. The rat 5-HT$_{2C\text{-}tr}$-encoded cDNA was subcloned into the *Eco*RI site of the eukaryotic expression vector pCMV2 (26). Transient transfection of NIH-3T3 fibroblasts was performed by electroporation at 270 V and 960 μF using a BioRad Gene Pulser transfection apparatus (Richmond, CA). Cells were cultured and harvested 48 hr later, and crude membrane homogenates were prepared as described previously (25, 27). In parallel, NIH-3T3 cells were transfected with the 5-HT$_{2C}$-R cDNA subcloned in pCMV2 (27) and used as positive controls. Expression of the 5-HT$_{2C\text{-}tr}$ or 5-HT$_{2C}$-R mRNAs was verified by S1 nuclease protection analyses as described above. Binding experiments were performed using a filtration assay as described previously (25, 27, 28).

For immunoblots, extracts were prepared from NIH-3T3 cells transiently expressing either 5-HT$_{2C}$ or 5-HT$_{2C\text{-}tr}$ cDNA. Membrane fractions were prepared as described previously (29), and proteins were extracted with 1× electrophoresis sample buffer [62.5 mM Tris, pH 6.8, 2% SDS, 10% (v/v) glycerol]. Insoluble material was removed by centrifugation at 14,000 × *g* for 10 min at room temperature. Protein concentrations were determined with the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard. Soluble proteins were electrophoresed in SDS-polyacrylamide gels and transferred from the gels to nitrocellulose (Hoeffer Pharmacia Biotech, San Francisco, CA) in a modified Towbin buffer (25 mM Tris, 192 mM glycine, pH ~8.4). Blots were blocked with 3% bovine serum albumin in blot buffer.

Blots were probed with purified antibodies at a concentration of 5 μg/ml. The truncated protein and full-length 5-HT$_{2C}$-Rs were detected with anti-2C-tr and anti-2C (29) antisera, respectively. Immunoreactive protein was visualized with an alkaline phosphatase-conjugated secondary antibody (Dako, Carpintera, CA) in a phosphatase system as described previously (29).

Results

Identification of a second 5-HT$_{2C}$-R transcript by S1 nuclease protection. To quantify rat 5-HT$_{2C}$-R mRNA, a ^{32}P-labeled antisense RNA probe was designed, and S1 nuclease protection assays were performed with total RNA prepared from choroid plexus. RNA isolated from a fibroblast cell line transfected with the 5-HT$_{2C}$-R cDNA (B 3–6) yielded a 5-HT$_{2C}$-R single band corresponding to the expected 475-nt protected fragment (Fig. 1); however, RNA from rat choroid plexus yielded two bands: the predicted 475-nt fragment and an additional fragment of ~400 nt (Fig. 1). These results suggested the existence of a second 5-HT$_{2C}$-R-related RNA transcript in choroid plexus. Based on the structure of the 5-HT$_{2C}$ gene (4, 19), the antisense RNA probe used for these studies was thought to span two exons within the 5-HT$_{2C}$ coding region. To further confirm the existence of two transcripts, PCR amplification of rat brain cDNA was performed using primers situated on the two exons within the region

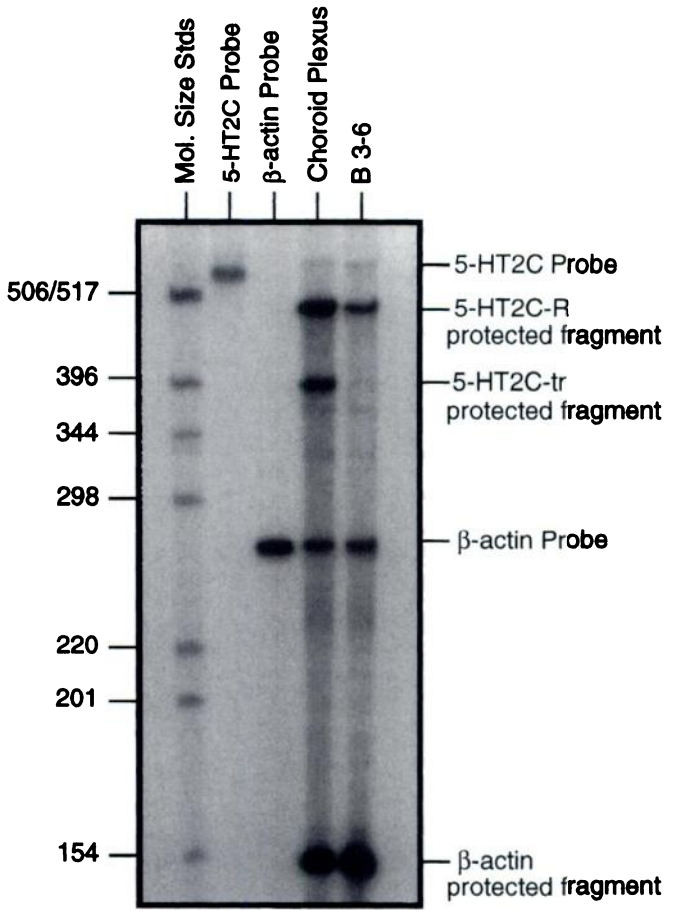

Fig. 1. S1 nuclease protection analysis of 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ mRNAs. Fifteen micrograms of total RNA isolated from rat choroid plexus or a clonal cell line of NIH-3T3 fibroblasts stably transfected with the 5-HT$_{2C}$-R cDNA (B 3–6) was hybridized with a ^{32}P-labeled antisense RNA probe and digested with S1 nuclease. Protected fragments were analyzed by polyacrylamide gel electrophoresis and then autoradiographed. β-Actin mRNA was used as an internal control; 1500 cpm of each undigested probe was also analyzed on the gel. Molecular size standards are expressed in bp and were ^{32}P-labeled using T4 polynucleotide kinase. This gel is representative of six different experiments with identical results.

encoding the antisense riboprobe. PCR analyses revealed the generation of two amplification reaction products: one fragment of 377 bp corresponding to the expected size for the 5-HT$_{2C}$-R and a shorter fragment of ~280 bp (data not shown). Sequence analyses of the shorter fragment revealed it to be a 5-HT$_{2C}$-related cDNA that contained a 95-bp deletion at nt 458 of the 5-HT$_{2C}$-R open reading frame. Additional analyses showed that the length of the protected fragment corresponding to the shorter 5-HT$_{2C}$ mRNA (5-HT$_{2C\text{-}tr}$) in the S1 nuclease protection experiment was 380 bp, which is in good agreement with the sequence of the smaller PCR reaction product.

Cloning of a truncated form of the rat 5-HT$_{2C}$-R. To isolate a full-length cDNA corresponding to the shorter 5-HT$_{2C}$-related transcript, a rat striatum library was screened using a radiolabeled probe recognizing both the 5-HT$_{2C}$-R cDNA and 5-HT$_{2C\text{-}tr}$ cDNAs. Two independent clones were isolated and shown to have the 95-bp deletion seen in the PCR reaction products, whereas seven clones were isolated that lacked the deletion and corresponded to the previously published sequence for the 5-HT$_{2C}$-R.

[2] J. R. Backstrom and E. Sanders-Bush. Generation of anti-peptide antibodies against serotonin 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors: Comparison between rabbit and chicken. Submitted for publication.

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                    TM III
                ...
                ...His Leu Cys Ala Ile Ser Leu Asp Arg Tyr Val Ala Ile Arg Asn Pro Ile Glu His Ser Arg Phe Asn Ser Arg      169
rat 5-HT2C-R    ...CAC CTC TGC GCC ATA TCG CTG GAC CGG TAT GTA GCA ATA CGT AAT CCT ATT GAG CAT AGC CGG TTC AAT TCG CGG     +507

rat 5-HT2C-tr   ...CAC CTC TGC GCC ATA TCG CTG GAC CG . . . . . .                                                      +458
                ...His Leu Cys Ala Ile Ser Leu Asp Arg. . . . . .                                                      153

                                                    TM IV
                Thr Lys Ala Ile Met Lys Ile Ala Ile Val Trp Ala Ile Ser Ile Gly Val Ser Val Pro Ile Pro Val Ile Gly      194
rat 5-HT2C-R    ACT AAG GCC ATC ATG AAG ATT GCC ATC GTT TGG GCA ATA TCA ALA GGA GTT TCA GTT CCT ATC CCT GTG ATT GGA     +582

rat 5-HT2C-tr   . . . . . .G AGT TTC AGT TCC TAT CCC TGT GAT TGG      +486
                . . . . . Ser Phe Ser Ser Tyr Pro Cys Asp Trp       162

                Leu Arg Asp Glu Ser Lys Val Phe Val Asn Asn Thr Thr Cys Val Leu Asn Asp Pro Asn Phe Val Leu Ile Gly...    219
rat 5-HT2C-R    CTG AGG GAC GAA AGC AAA GTG TTC GTG AAT AAC ACC ACG TGC GTG CTC AAT GAC CCC AAC TTC GTT CTC ATC GGG...    +657

rat 5-HT2C-tr   ACT GAG GGA CGA AAG CAA AGT GTT CGT GAA TAA CAC CAC GTG CGT GCT CAA TGA CCC CAA CTT CGT TCT CAT CGG G...  +562
                Thr Glu Gly Arg Lys Gln Ser Val Arg Glu STOP                                                           172
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Fig. 2. Partial nucleotide and deduced amino acid sequences of the rat 5-HT$_{2C\text{-}tr}$ cDNA compared with those of the rat 5-HT$_{2C}$-R cDNA (17). The position of the third and the fourth transmembrane domains are indicated (*TM III* and *TM IV*) for the 5-HT$_{2C}$-R. *Italics*, specific amino acid sequence of the 5-HT$_{2C\text{-}tr}$. *Numbers on the right*, amino acid and base number, with 1 and +1 being the first amino acid and base of the initiating codon, respectively. *Dotted line*, deleted portion of the 5-HT$_{2C\text{-}tr}$. Only the portion relevant to the deletion is shown, the sequence of the 5-HT$_{2C\text{-}tr}$ cDNA clone extends 1060 nt upstream and 1124 nt downstream and is identical to the published 5-HT$_{2C}$-R sequence (17). The complete sequence has been submitted to GenBank (accession no. U35315).

One of the 5-HT$_{2C\text{-}tr}$ clones was completely sequenced. The entire open-reading frame was identical to the sequence published by Julius *et al.* (17) except for a 95-nt deletion at nt 459–553 in the 5-HT$_{2C}$-R open reading frame. The deletion corresponds to the region encoding the putative second intracellular loop and the fourth transmembrane domain of the 5-HT$_{2C}$-R and leads to a frameshift in the downstream sequence and a premature stop codon. The predicted protein contains 172 amino acids, with 153 residues at the amino terminus identical to the 5-HT$_{2C}$-R and 19 amino acids on the carboxyl terminus, subsequent to the frameshift, that are unique (Fig. 2). A Kyte and Doolittle hydropathicity plot showed the 5-HT$_{2C\text{-}tr}$-specific region to be hydrophilic (data not shown) and unlikely to be membrane associated or forming additional transmembrane domains.

Distribution of the 5-HT$_{2C\text{-}tr}$ mRNA. The regional distribution of the 5-HT$_{2C\text{-}tr}$ mRNA in comparison to the 5-HT$_{2C}$-R mRNA was examined with an S1 nuclease protection assay of various brain regions and peripheral organs. Total RNA was hybridized with the antisense ^{32}P-labeled riboprobe used above and digested with S1 nuclease. The regional distribution of the 5-HT$_{2C\text{-}tr}$ in various rat brain structures is presented in Fig. 3. The highest level of

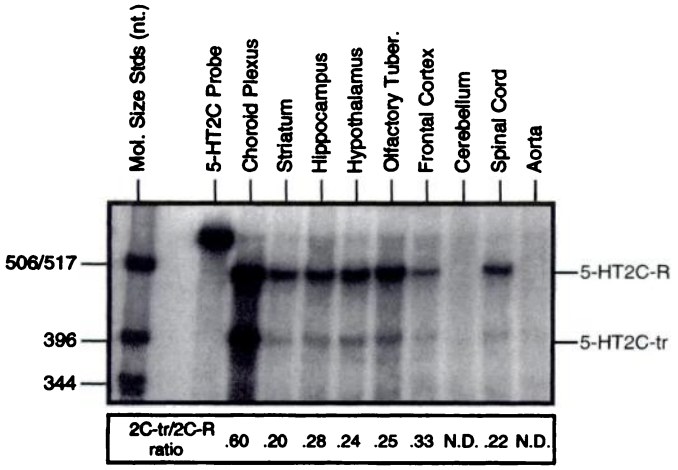

Fig. 3. S1 nuclease analysis of the 5-HT$_{2C\text{-}tr}$ and 5-HT$_{2C}$-R mRNA distribution. Fifteen micrograms (choroid plexus) or 30 μg of total RNA was hybridized overnight with a ^{32}P-labeled antisense RNA and digested with S1 nuclease. Protected fragments corresponding to the 5-HT$_{2C}$-R mRNA (475-bp fragment) and 5-HT$_{2C\text{-}tr}$ mRNA (380 bp) were visualized with 4% polyacrylamide/7 M urea gel electrophoresis. The undigested probe (1500 cpm) was also analyzed on the gel. Molecular size markers (1-kb DNA ladder; GIBCO BRL, Gaithersburg, MD) were ^{32}P-labeled with T4 polynucleotide kinase. Ratios of transcription of 5-HT$_{2C\text{-}tr}$ to 5-HT$_{2C}$-R mRNA were obtained by using a PhosphorImager and calculated by normalizing the amount of ^{32}P nucleotides corresponding to each fragment. Ratios correspond to the illustrated gel and are representative of three different experiments, with no variation of >15%.

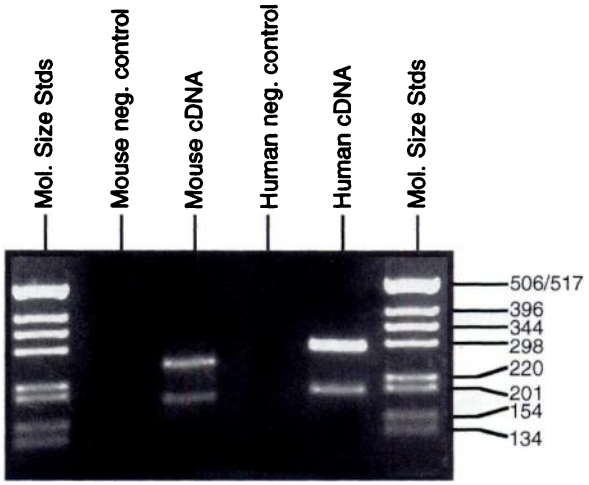

Fig. 4. PCR amplification of mouse and human 5-HT$_{2C}$ cDNA using specific primers flanking the 95-nt deletion observed in the rat. Mouse brain or human caudate nucleus cDNA (1 ng) was amplified by PCR using the primers described in Materials and Methods and analyzed on a 2% agarose gel stained with ethidium bromide. Negative controls containing all of the reagents except cDNA were amplified in parallel; no band was visible in these samples.

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                ...His Leu Cys Ala Ile Ser Leu Asp Arg Tyr Val Ala Val Arg Ser Pro Val Glu His Ser Arg Phe Asn Ser Arg      169
mou.5-HT2C-R    ...CAC CTC TGC GCC ATA TCG CTG GAC CGG TAT GTA GCA GTG CGT AGT CCT GTT GAG CAT AGC CGG TTC AAT TCG CGG     +507

mou.5-HT2C-tr   ...CAC CTC TGC GCC ATA TCG CTG GAC CG . . . . . .                                                      +458
                ...His Leu Cys Ala Ile Ser Leu Asp Arg. . . . . .                                                      153



                Thr Lys Ala Ile Met Lys Ile Ala Ile Val Trp Ala Ile Ser Ile Gly Val Ser Val Pro Ile Pro Val Ile Gly      194
mou.5-HT2C-R    ACT AAG GCC ATC ATG AAG ATT GCC ATC GTT TGG GCA ATA TCA ATA GGA GTT TCA GTT CCT ATC CCT GTG ATT GGA     +582

mou.5-HT2C-tr   . . . . . .G AGT TTC AGT TCC TAT CCC TGT GAT TGG      +486
                . . . . . Ser Phe Ser Ser Tyr Pro Cys Asp Trp       162



                Leu Arg Asp Glu Ser Lys Val Phe Val Asn Asn Thr Thr Cys Val Leu Asn Asp Pro Asn Phe Val Leu Ile Gly...    219
mou.5-HT2C-R    CTG AGG GAC GAA AGC AAA GTG TTC GTG AAT AAT ACT ACC TGC GTG CTC AAT GAC CCG AAC TTC GTT CTC ATC GGG...    +657

mou.5-HT2C-tr   ACT GAG GGA CGA AAG CAA AGT GTT CGT GAA TAA TAC TAC CTG CGT GCT CAA TGA CCC GAA CTT CGT TCT CAT CGG G...  +562
                Thr Glu Gly Arg Lys Gln Ser Val Arg Glu STOP                                                           172
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Fig. 5. Partial nucleotide and deduced amino acid sequences of mouse 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ cDNAs. *Underlined*, nucleotides and amino acids not identical to the rat sequence. *Dotted line*, deleted region in the 5-HT$_{2C\text{-}tr}$. *Italics*, specific sequence of the 5-HT$_{2C\text{-}tr}$. *Numbers on the right*, amino acid and nucleotide numbers, with 1 and +1 being the first amino acid and nucleotide of the initiation codon, respectively, according to Foguet *et al.* (4).

5-HT$_{2C\text{-}tr}$ mRNA was found in choroid plexus, with intermediate levels in striatum, hippocampus, hypothalamus, olfactory tubercles, and spinal cord. Expression of the 5-HT$_{2C\text{-}tr}$ was low in frontal cortex and not detected in cerebellum and aorta. In addition, no detectable level of 5-HT$_{2C}$-R or 5-HT$_{2C\text{-}tr}$ mRNAs was found in kidney or liver (data not shown). The distribution of the 5-HT$_{2C\text{-}tr}$ mRNA follows the distribution of the 5-HT$_{2C}$-R; no area was found to have only one of these RNA species. The frequency of isolating cDNA clones encoding the 5-HT$_{2C}$-R and the 5-HT$_{2C\text{-}tr}$ from the rat striatal library suggested that the relative level of expression for the truncated form was ~28% of that observed for the 5-HT$_{2C}$-R in the rat striatum. This result agrees with the relative levels of the two transcripts in striatum, as determined by S1 nuclease protection (Fig. 3). In all brain regions, the amount of 5-HT$_{2C}$-R mRNA was higher than that of 5-HT$_{2C\text{-}tr}$ mRNA. However, the ratio of 5-HT$_{2C\text{-}tr}$ mRNA to 5-HT$_{2C}$-R varied from 0.60 in choroid plexus, an epithelial tissue, to 0.20–0.30 in neuronal tissues.

Identification of 5-HT$_{2C\text{-}tr}$-R transcripts in mouse and human brain. To investigate whether the 5-HT$_{2C\text{-}tr}$ form is conserved among different species, PCR amplification of cDNA from mouse brain and human caudate nucleus was performed using sets of primers specific to the respective cDNA of each species and situated on positions analogous to the two sides of the 95-nt deletion present in the rat. Using mouse brain cDNA as template, two products were obtained (Fig. 4); the shorter amplified product (163-bp) had the 95-nt deletion characteristic of the 5-HT$_{2C\text{-}tr}$, whereas the remainder of its nucleotide sequence was identical to the published sequence of the mouse 5-HT$_{2C}$-R (4); the longer fragment (258-bp) was identical to the mouse 5-HT$_{2C}$-R. As in the rat, the 95-nt deletion alters the open reading frame, and the predicted amino acid sequence contains 19 new amino acids before a stop codon is reached (Fig. 5). The amino acid sequence of these 19 residues is similar to that observed in the rat.

To determine whether the 5-HT$_{2C\text{-}tr}$ mRNA variant is also present in the human brain, PCR amplification was performed using strategies similar to those described for the mouse. As with rat and mouse cDNAs, two products were amplified (Fig. 4); the larger PCR reaction product (293 bp) was identical to that published for the human 5-HT$_{2C}$-R (18), whereas the shorter fragment had a 95-nt deletion identical to that previously found in rat and mouse (Fig. 6). This deletion alters the reading frame of the downstream coding sequence, but unlike mouse and rat cDNAs, the human 5-HT$_{2C\text{-}tr}$ nucleotide sequence lacks the stop codon TAA in position 514–516. The human 5-HT$_{2C\text{-}tr}$ mRNA encodes a putative 248-amino acid protein with 96 unique residues on the carboxyl terminus. Hydropathic analysis of these specific amino acids was incompatible with a transmembrane-spanning topology.

5-HT$_{2C\text{-}tr}$ is produced by alternative splicing. The presence of two distinct 5-HT$_{2C}$ cDNAs could result from unique but related 5-HT$_{2C}$ genes or alternative splicing of a single primary RNA transcript. To distinguish between these possibilities, Southern blot analyses of rat genomic DNA were performed. Hybridization to a single DNA fragment for each restriction digest (Fig. 7) indicated that only a single 5-HT$_{2C}$ gene exists, suggesting that 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ mRNAs are produced by alternative RNA processing. The genomic organization of the mouse and human 5-HT$_{2C}$ genes has been determined (4, 19), and the locations of the intron/exon boundaries are highly conserved. The genomic structure of the rat 5-HT$_{2C}$ gene is similar to that of the human and mouse in its organization and positions of the three introns.[3] The sequence divergence between the 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ cDNAs corresponds precisely to an exon/intron boundary (Fig. 8). In addition, the sequence of the 5′ end of the deleted region of the 5-HT$_{2C}$ gene corresponds to the consensus sequence for a 5′ (donor) splice site (G/GTAGT, Fig. 8) (30). These results support the conclusion that the 5-HT$_{2C\text{-}tr}$ mRNA is an alternatively spliced product of 5-HT$_{2C}$ gene-encoded pre-mRNA in which the use of alternative 5′-splice sites and a common 3′-splice acceptor gives rise to two distinct 5-HT$_{2C}$ mRNA transcripts.

Expression of the 5-HT$_{2C\text{-}tr}$ and 5-HT$_{2C}$-R in fibroblasts. To evaluate functional differences between the 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ proteins, the corresponding full-

[3] J. Aimi, H.-T. Kao, and R. D. Ciaranello, unpublished observations.

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                ...His Leu Cys Ala Ile Ser Leu Asp Arg Tyr Val Ala Ile Arg Asn Pro Ile Glu His Ser Arg Phe Asn Ser Arg      168
hum.5-HT2C-R    ...CAC CTC TGC GCT ATA TCG CTG GAT CGG TAT GTA GCA ATA CGT AAT CCT ATT GAG CAT AGC CGT TTC AAT TCG CGG     +504

hum.5-HT2C-tr   ...CAC CTC TGC GCT ATA TCG CTG GAT CG . . . . . .                                                      +455
                ...His Leu Cys Ala Ile Ser Leu Asp Arg. . . . . .                                                      152



                  Thr Lys Ala Ile Met Lys Ile Ala Ile Val Trp Ala Ile Ser Ile Gly Val Ser Val Pro Ile Pro Val Ile Gly      193
hum.5-HT2C-R      ACT AAG GCC ATC ATG AAG ATT GCT ATT GTT TGG GCA ATT TCT ATA GGT GTA TCA GTT CCT ATC CCT GTG ATT GGA     +579

hum.5-HT2C-tr     . . . . . .G TGT ATC AGT TCC TAT CCC TGT GAT TGG      +483
                  . . . . . Cys Ile Ser Ser Tyr Pro Cys Asp Trp       161



                  Leu Arg Asp Glu Glu Lys Val Phe Val Asn Asn Thr Thr Cys Val Leu Asn Asp Pro Asn Phe Val Leu Ile Gly      218
hum.5-HT2C-R      CTG AGG GAC GAA GAA AAG GTG TTC GTG AAC AAC ACG ACG TGC GTG CTC AAC GAC CCA AAT TTC GTT CTT ATT GGG     +654

hum.5-HT2C-tr     ACT GAG GGA CGA AGA AAA GGT GTT CGT GAA CAA CAC GAC GTG CGT GCT CAA CGA CCC CAA TTT CGT TCT TAT TGG      +558
                  Thr Glu Gly Arg Arg Lys Gly Val Arg Glu Gln His Asp Val Arg Ala Gln Arg Pro Lys Phe Arg Ser Tyr Trp      186



                  Ser Phe Val Ala Phe Phe Ile Pro Leu Thr Ile Met Val Ile Thr Tyr Cys Leu Trh Ile Tyr Val Leu Arg Arg      243
hum.5-HT2C-R      TCC TTC GTA GCT TTC TTC ATA CCG CTG ACG ATT ATG GTG ATT ACG TAT TGC CTG ACC ATC TAC GTT CTG CGC CGA     +729

hum.5-HT2C-tr     GTC CTT CGT AGC TTT CTT CAT ACC GCT GAC GAT TAT GGT GAT TAC GTA TTG CCT GAC CAT CTA CGT TCT GCG CCG      +633
                  Val Leu Arg Ser Phe Leu His Thr Ala Asp Asp Tyr Gly Asp Tyr Val Leu Pro Asp His Leu Arg ser Ala Pro      211



                  Gln Ala Leu Met Leu Leu His Gly His Thr Glu Glu Pro Pro Gly Leu Ser Leu Asp Phe Leu Lys Cys Cys Lys      268
hum.5-HT2C-R      CAA GCT TTG ATG TTA CTG CAC GGC CAC ACC GAG GAA CCG CCT GGA CTA AGT CTG GAT TTC CTG AAG TGC TGC AAG     +804

hum.5-HT2C-tr     ACA AGC TTT GAT GTT ACT GCA CGG CCA CAC CGA GGA ACC GCC TGG ACT AAG TCT GGA TTT CCT GAA GTG CTG CAA      +708
                  Thr Ser Phe Asp Val Thr Ala Arg Pro His Arg Gly Thr Ala Trp Thr Lys Ser Gly Phe Pro Glu Val Leu Gln      236



                  Arg Asn Thr Ala Glu Glu Glu Asn Ser Ala Asn Pro Asn Gln Asp Gln Asn Ala Arg Arg Arg Lys Lys Lys Glu...    293
hum.5-HT2C-R      AGG AAT ACG GCC GAG GAA GAG AAC TCT GCA AAC CCT AAC CAA GAC CAG AAC GCA CGC CGA AGA AAG AAG AAG GAG...   +879

hum.5-HT2C-tr     GAG GAA TAC GGC CGA GGA AGA GAA CTC TGC AAA CCC TAA CCA AGA CCA GAA CGC ACG CCG AAG AAA GAA GAA GGA G...  +784
                  Glu Glu Tyr Gly Arg Gly Arg Glu Leu Cys Lys Pro STOP                                                   248
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Fig. 6. Partial nucleotide and deduced amino acid sequence comparisons of the human 5-HT_{2C}-R and 5-$HT_{2C\text{-}tr}$ cDNAs. *Underlined*, nucleotides and amino acids not identical to the rat sequences. *Dotted line*, deleted region of the 5-$HT_{2C\text{-}tr}$. *Italics*, amino acids unique to the 5-$HT_{2C\text{-}tr}$ were deduced according to the nucleotide sequence of the human 5-$HT_{2C\text{-}R}$ (18). *Numbers on the right*, amino acid and base numbers, with 1 and +1 being the first amino acid and nucleotide of the initiation codon, respectively.

length cDNAs were transiently transfected into NIH-3T3 fibroblasts. S1 nuclease protection assays verified that mRNAs corresponding to the respective cDNAs were present; fibroblasts transfected with the 5-$HT_{2C\text{-}tr}$ or 5-HT_{2C}-R cDNA yielded the expected 380-or 475-nt-protected fragments, respectively. In contrast to membranes from cells transfected with the 5-HT_{2C}-R, no specific radioligand binding activity could be detected in membranes prepared from cells transfected with the 5-$HT_{2C\text{-}tr}$ cDNA using three different serotonergic radioligands: [^{3}H]mesulergine, [^{3}H]ketanserin, and [^{3}H]5-HT(data not shown). In addition, 5-HT activates phosphoinositide hydrolysis in cells expressing 5-HT_{2C}-R, but it failed to stimulate the breakdown of phosphoinositides in cells transfected with the 5-$HT_{2C\text{-}tr}$ construct (data not shown).

Immunoblotting of extracts from transfected fibroblasts revealed several immunoreactive bands (17, 19, 30, and 39–40 kDa) in cells transfected with the 5-$HT_{2C\text{-}tr}$ mRNA as well as several bands after transfection with the 5-HT_{2C}-R mRNA (Fig. 9). The predicted size of the truncated protein was 19.1 kDa. The higher bands probably correspond to *N*-glycosylated products. A consensus *N*-linked glycosylation sites is found in the common amino terminus and, for the full-length protein, produces mass shafts of 11–28 kDa (29). Similar mass shafts were found for the 5-$HT_{2C\text{-}tr}$ protein.

Discussion

Alternative splicing of mRNA precursors represents a common mechanism for increasing the flexibility of eukaryotic gene expression by the generation of structurally distinct isoforms from a single genomic locus. Recent studies have indicated that alternative splicing is responsible for the generation of a number of G protein-coupled receptor isoforms [e.g., in the dopamine receptor family (31) and the prostaglandin EP3 receptors (32)]. In some cases, cassettes are inserted or removed from the original sequence. For example, two isoforms of dopamine D_2 receptor have been identified that differ by the inclusion of 29 amino acids in the third intracellular loop, producing subtle alterations in G protein coupling (33, 34). Alternative splicing can also alter the predicted reading frame of a protein, producing a form that is truncated due to the presence of a premature stop codon. Numerous alternatively spliced variants of the dopamine D_3 receptors are truncated proteins, having two or more transmembrane-spanning domains (35, 36).

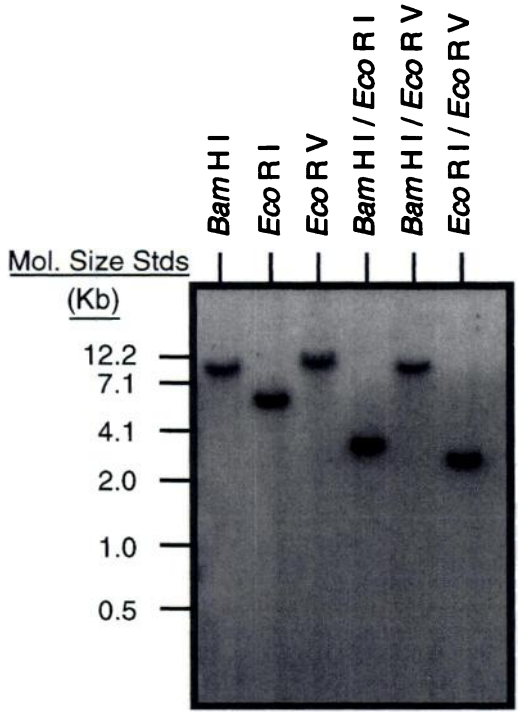

Fig. 7. Southern blot analysis of rat genomic DNA. Rat genomic DNA was digested with the indicated enzymes and separated on agarose gel. After transfer to a nylon membrane, the blot was probed with the ^{32}P-labeled DNA fragment described in Materials and Methods. This probe recognizes an exonic portion common to the 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ DNAs. This gel is representative of three experiments with identical results.

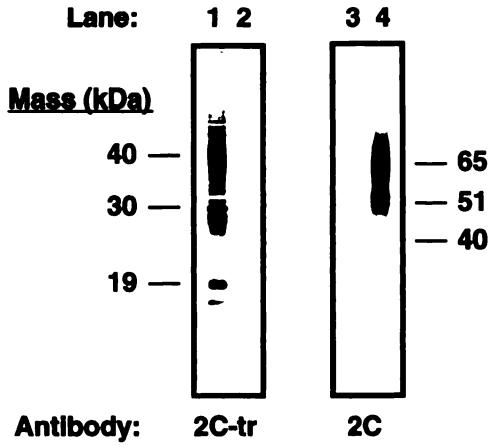

Fig. 9. Immunoreactive proteins from transfected cells. SDS-soluble proteins were prepared from NIH-3T3 fibroblasts transfected with 5-HT$_{2C\text{-}tr}$ mRNA (*lanes 1 and 3*) or 5-HT$_{2C}$-R (*lanes 2 and 4*); 75 μg of protein/lane was loaded for Western blots. *Left* (12.5% gel), probed with anti-2C-tr antibodies. *Right* (10% gel), probed with anti-2C antibodies. Relative masses of the immunoreactive proteins are indicated.

In the current study, we demonstrate that a novel mRNA isoform of the 5-HT$_{2C}$ gene occurs in the brains of rats, mice, and humans as a result of alternative splicing. This new RNA transcript has a 95-nt deletion that changes the reading frame in comparison to previously described 5-HT$_{2C}$-R mRNA (17) and leads to a truncated protein, referred to as 5-HT$_{2C}$ truncated (5-HT$_{2C\text{-}tr}$). The predicted protein in the rat contains the first two transmembrane domains of the 5-HT$_{2C}$-R as well as a unique 19-amino acid carboxyl terminus. The occurrence of two distinct 5-HT$_{2C}$ mRNAs was first identified by S1 nuclease protection analyses of total RNA from rat choroid plexus. A cDNA corresponding to this shorter RNA species was subsequently cloned from a rat striatum cDNA library and found to be identical to the 5-HT$_{2C}$-R, except for a 95-bp deletion. High expression of the two 5-HT$_{2C}$ mRNA transcripts was found in choroid plexus, with intermediate levels in hypothalamus, hippocampus, and olfactory tubercles. The distributions of the 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ mRNAs are in agreement with the distribution of the 5-HT$_{2C}$-R, as determined by *in situ* hybridization studies (8–11). The 5-HT$_{2C\text{-}tr}$ mRNA is evolutionarily conserved among rat, mouse, and human. The predicted amino acid sequence of the carboxyl terminus of the rat and mouse 5-HT$_{2C\text{-}tr}$ is identical; however, the predicted sequence of the novel human carboxyl terminus differs markedly in length and represents more than one third of the total protein.

The genomic organization of the mouse and human 5-HT$_{2C}$ genes is similar with regard to exon size and position of exon/intron boundaries (4, 19). Preliminary data from our laboratory as well as unpublished observations[4] have shown that this organization is also conserved in the rat. The 95-nt sequence occurs at an intron/exon boundary with the 5′ end of the deleted segment corresponding to a consensus sequence for a 5′ splice (donor) site. These data suggest that the two transcripts are produced as a result of differential RNA processing in which two alternative 5′-splice (donor) sites compete for a common 3′-splice (acceptor) site. This conclusion is consistent with the results of Southern blot analyses of rat genomic DNA, which ruled out two different but closely related genes. Alternative production of 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ mRNAs may represent a regulated cellular event

[4] J. Aimi, H.-T. Kao, and R. D. Ciaranello, unpublished observations.
[5] H. Canton and E. Sanders-Bush, unpublished observations.

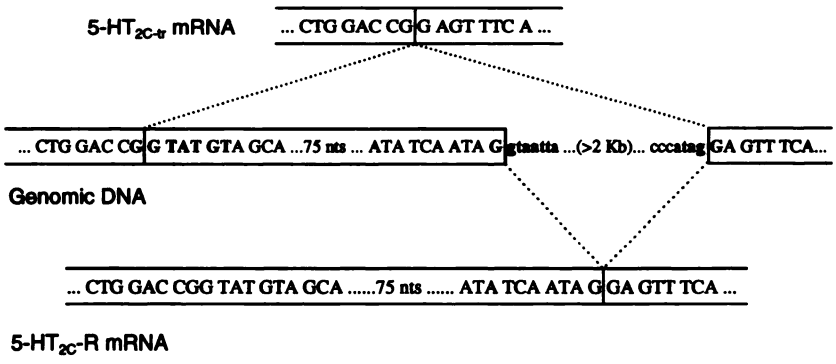

Fig. 8. Genomic organization of the mouse 5-HT$_{2C}$ gene and proposed splicing mechanism leading to the production of the 5-HT$_{2C}$-R and 5-HT$_{2C\text{-}tr}$ mRNAs. *Uppercase letters*, coding sequences. *Boxed*, exonic regions. *Lowercase letters*, intronic sequences. *Bold letters*, consensus sequences corresponding to the two 5′-splice (donor) and the common 3′-splice (acceptor) sites. The mouse genomic sequence is from Foguet *et al.* (4). The intron/exon boundaries as well as the presence of two alternative donor sites are similar in the rat[5] and human 5-HT$_{2C}$ gene (19).

as demonstrated by the differential expression levels of the transcripts in choroid plexus versus brain, perhaps reflecting a difference between neurons and epithelial cells.

The production of a novel mRNA isoform could have biological implications in a number of cellular processes such as modification of protein localization and/or function, novel protein activities, modification of RNA stability, and translational efficiency, as well as effects during development. With regard to the alternatively spliced 5-HT_{2C} mRNA, two obvious questions arise: Is the alternative mRNA actually translated into protein, and if so, does the novel protein share properties with the full-length protein? To address these questions, we used fibroblasts transfected with the 5-$HT_{2C\text{-}tr}$ mRNA. Polyclonal antibodies generated to the unique carboxyl-terminal tail of the putative 5-$HT_{2C\text{-}tr}$ protein were used to probe Western blots of cell extracts and revealed the presence of a protein with the predicted molecular weight in transfected fibroblasts but not in untransfected cells. Subsequent biochemical analyses did not reveal any serotonergic binding activity or phosphoinositide hydrolysis activity. We conclude that the 5-$HT_{2C\text{-}tr}$ mRNA can be translated into protein; however, the protein does not function as a 5-HT receptor. The latter result was not unexpected, considering that the truncated mRNA variant encodes for only the first two transmembrane-spanning domains. Several truncated muscarinic m3 receptors, including one with a topography similar to that of the 5-$HT_{2C\text{-}tr}$, have been shown to be expressed in transfected cells but have no ligand binding activity; however, when two halves of the receptor are coexpressed, the truncated proteins interact to form a functional receptor (36). It is intriguing to consider that such interactions may occur naturally or that truncated products of G protein-coupled receptors may form dimers with full-length proteins to regulate localization or function.

The occurrence of the 5-$HT_{2C\text{-}tr}$ mRNA transcript could result from cellular errors in splicing fidelity; however, the abundance and differential expression of this RNA transcript, as well as its conservation in multiple species, make this possibility seem unlikely. Even though no function has been demonstrated for the truncated 5-HT_{2C} protein, alterations in expression of the 5-$HT_{2C\text{-}tr}$ relative to the 5-$HT_{2C\text{-}R}$ could be biologically significant. A nonsense mutation in the first exon of the dopamine D_4 receptor, which encodes a truncated nonfunctional protein, has been associated with disturbances of the autonomic nervous system, somatic ailments, and obesity (37). The fact that both 5-HT_{2C} mRNA transcripts are present in the limbic system and hypothalamus, combined with the possible involvement of the 5-HT_{2C}-R in the control of feeding behavior and affective disorders (13), suggests that further investigations concerning the biological relevance of the novel product of the 5-HT_{2C} gene are necessary. In addition, the finding that the alternative spliced mRNA, which is abundant in brain, encodes a protein that does not bind serotonergic radioligands may explain the discrepancies between the levels and distribution of 5-HT_{2C}-R mRNA and 5-HT_{2C}-R binding sites (8). In these and other *in situ* hybridization studies (9–11) of the 5-HT_{2C}-R mRNA, the hybridization probe was nonselective, hybridizing with both full-length and truncated transcripts. Because only the full-length protein is capable of binding 5-HT ligands, previous conclusions concerning the localization of the mRNA encoding functional 5-HT_{2C}-Rs must be revised.

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