CALL FOR PAPERS | Comparative Genomics

Alternative splicing and exon duplication generates 10 unique porcine 5-HT₄ receptor splice variants including a functional homofusion variant

Joris H. De Maeyer,¹,² Jeroen Aerssens,² Peter Verhasselt,³ and Romain A. Lefebvre¹

¹Heymans Institute of Pharmacology, Ghent University, Ghent; and Departments of ²Internal Medicine and ³Functional Genomics, Johnson & Johnson Pharmaceutical Research and Development, Beere, Belgium

Submitted 14 February 2008; accepted in final form 15 April 2008

De Maeyer JH, Aerssens J, Verhasselt P, Lefebvre RA. Alternative splicing and exon duplication generates 10 unique porcine 5-HT₄ receptor splice variants including a functional homofusion variant. Physiol Genomics 34: 22–33, 2008. First published April 22, 2008; doi:10.1152/physiolgenomics.00038.2008.—5-HT₄ receptors are present in human and porcine atrial myocytes while they are absent from the hearts of small laboratory animals. The pig is therefore the only available nonprimate animal model in which to study cardiac 5-HT₄ receptor function under physiological conditions. While several human splice variants of the 5-HT₄ receptor have been described, the splicing behavior of this receptor in porcine tissue is currently unknown. Here we report on the identification of nine novel COOH-terminal splice variants of the porcine 5-HT₄ receptor, which were named 5-HT₄(a₂), 5-HT₄(b₂), 5-HT₄(c₂), 5-HT₄(d₂), 5-HT₄(e₂), 5-HT₄(f₂), 5-HT₄(g₂), 5-HT₄(h), and 5-HT₄(i). The internal h-variant was found in combination with several COOH-terminal exons. In addition, splice variants were found that comprised duplicated exons fused to the common region of the 5-HT₄ receptor, thereby providing evidence for a duplication of the porcine HTR4 gene. One of these variants putatively encoded a nine transmembrane-spanning domain homofusion receptor, 5-HT₄(9TM); also the other variants with a duplicated region might translate into functional, transcriptionally fused dimeric 5-HT₄ receptor variants. The elucidation of the genomic context confirmed that the variants were not genomic artefacts but originated from alternative splicing. This was further corroborated by a functional analysis of the variants 5-HT₄(d₂), 5-HT₄(e₂), and 5-HT₄(9TM). To our knowledge, our data are the first to report on a functional GPCR mRNA splicing, which causes an extra level of functional diversity (Fig. 1). Since the initial cloning of two splice variants from rat brain (13), several variants of 5-HT₄ receptors have been described by cDNA cloning in human and other species. The divergence in the sequence of these splice variants usually starts after amino acid Leu⁵⁸⁸, resulting in COOH-terminal splice variants. 5-HT₄(a₂), 5-HT₄(b₂), 5-HT₄(c₂), 5-HT₄(d₂), 5-HT₄(e₂), 5-HT₄(f₂), 5-HT₄(g₂), and 5-HT₄(h) have been cloned from humans (Fig. 1); 5-HT₄(a₂), 5-HT₄(b₂), 5-HT₄(c₂) in rat; and 5-HT₄(a₂), 5-HT₄(b₂), 5-HT₄(c₂), and 5-HT₄(h) in mouse (6, 7). Sequencing of the human 5-HT₄ receptor gene (HTR4) revealed that the splice variants were indeed splice variants from the same gene (Fig. 1). Exons corresponding to the different COOH-terminal variants were found in the genomic context. Furthermore, the existence of the so-called exons “gef” suggests the existence of the putative human variants 5-HT₄(e₂) and 5-HT₄(f₂), which is underscored by their mRNA expression (3). In addition, one splice variant, 5-HT₄(b₂), has an insertion of 14 amino acids in the second extracellular loop of the receptor. This variant was first described in porcine blood vessels (40). Meanwhile, the 5-HT₄(b₂) variant has been found in human tissue, but so far only in combination with COOH-terminal exon b (3). Although this differential splicing of the human HTR4 gene attracted a lot of attention in the field, which led to the
and 5-HT4(9TM)) were functionally characterized. Moreover, we found splice variants composed of duplicated exons, one of which resulted in a putatively 9TM terminal exons of variant a, r, and m (5-HT4(ha), 5-HT4(hr), internal h-variant was found in combination with the COOH-terminus and a variable intracellular COOH terminus, caused by alternative splicing. The known splice variants are indicated. The inclusion of exon h in the mRNA results in an insertion of 14 amino acids in the 2nd extracellular loop of the receptor.

characterization of many splice variants in vitro, the full-length nature of some transcript variants has not been determined. Additionally, specific antibodies against the different 5-HT4 receptor splice variants have not been generated yet, and no experimental evidence exists that all the different identified splice variants are translated in vivo.

The pig is currently the only available animal model in which to study human cardiac 5-HT4 receptor function (17, 18), mainly because in physiological conditions, rodents lack functional 5-HT4 receptors on the heart. Nevertheless, no studies addressed the occurrence of 5-HT4 receptor splice variants in the pig. Except for the published porcine cDNA sequence (GenBank: NM_001001267) that corresponds to the functional 5-HT4 receptors on the heart. Nevertheless, no studies addressed the occurrence of 5-HT4 receptor splice variants in the pig. Except for the published porcine cDNA sequence (GenBank: NM_001001267) that corresponds to the human 5-HT4 receptor-specific sequence located between the nested forward primer (transmembrane regions are shown as black oval boxes) with an extracellular NH2 terminus and a variable intracellular COOH terminus, caused by alternative splicing. The known splice variants are indicated. The inclusion of exon h in the mRNA results in an insertion of 14 amino acids in the 2nd extracellular loop of the receptor.

MATERIALS AND METHODS

Tissues

Female piglets (10–11 wk, 22–27 kg) and newborn female piglets (2–3 days, 1.6–2.8 kg), obtained from local farms, were deeply anesthetized with an intravenous (50 mg/kg) or intraperitoneal (100 mg/kg) pentobarbital sodium (Kela, Hoogstraten, Belgium) injection, respectively. After exsanguination, the heart, esophagus, stomach, duodenum, ileum, distal colon, and adrenal gland were dissected, thoroughly washed under continuous oxygenation, and placed in ice cold phosphate-buffered saline (PBS; Invitrogen, Merelbeke, Belgium). The gastrointestinal tissues were dissected into the mucosal-submucosal (mucosa) fraction and the muscular-muenteric plexus fraction (confirmed by histological examination). Tissues were cut into small pieces, rapidly frozen in liquid N2, and stored at −70 °C. The study was approved by the ethical committee from Johnson & Johnson Pharmaceutical Research & Development, a division of Janssen Pharmaceutica (Beerse, Belgium).

General Molecular Biological Methods

Unless otherwise stated, all PCR reactions were performed in a final reaction volume of 25 μl containing 22.5 μl AccuPrime pf mix (0.55 U of AccuPrime pf polymerase, buffer components, 330 μM dNTPs, 1.1 mM MgSO4; Invitrogen), 200 nM primers and 1 μl of template DNA. PCR amplifications were performed on a Tetrad-2 Thermal Cycler (Bio-Rad, Hercules, CA).

DNA sequencing was carried out by cycle sequencing using BigDye Terminator (Applied Biosystems) and M13F (5’-GT-A-TAA-A-CGA-CGG-CTT-G-A-3’) and M13R (5’-CAG-GAA-ACA-GCT-ATG-AC-3’) sequencing primers. Sequences were analyzed on an ABI PRISM 3730 sequencer (Applied Biosystems) and assembled using Sequencher version 4.6 (GeneCodes, Ann Arbor, MI).

3’-RACE Amplification and Subcloning of 3’-RACE Products

The SMART RACE (Clontech, Mountain View, CA) protocol was adapted to amplify the 3’-ends of porcine 5-HT4 receptor cDNAs. Rapid amplification of 3’-cDNA ends (RACE)-ready cDNA was reverse transcribed from 1 μg of total RNA isolated from the mucosal and muscular fraction of the proximal stomach and the adrenal gland of newborn piglets as well as from the left and right atrium of newborn and young piglets, using a modified Lock-Docking oligo(dT) primer (5’-AAG-CAG-TGG-TAT-TAT-AA-CAG-AGA-GTA-C(T)30VN-3’) and PowerScript Reverse Transcriptase (Clontech) or Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturers’ instructions. The first-strand reaction products were diluted with Tricine-EDTA buffer to a final volume of 110 μl. Two forward primers were designed based on the published porcine 5-HT4 receptor cDNA sequence (GenBank: AY566638): F865 and F843 (Supplemental Table S1).1 We used 1.25 μl of cDNA in a PCR reaction (5 min at 95°C followed by 20 cycles of 15 s at 95°C, 30 s at 52°C, and 1 min at 68°C, with a final extension step of 7 min at 68°C) using F865 in conjunction with the Universal Primer A Mix (UPA; long: 5’-CTA-ATA-CGA-CTC-CTA-ATA-GGG-CAA-GCA-GTG-GTA-ATA-GGG-CAG-GAT-3’; short: 5’-CTA-ATA-CGA-CTC-CTA-ATA-GGG-C-3’). We used 1 μl of this reaction as template in a nested amplification round (5 min at 95°C followed by 25 cycles of 15 s at 95°C, 30 s at 54°C, and 1 min at 68°C, followed by 7 min incubation at 68°C) with F843 and the Nested Universal Primer A (NUP; 5’-AAG-CAG-TGG-TAA-CAG-CAG-GAT-3’). The target sequences of UPA and NUP lie within the adapter sequence that is ligated to the 3’-end of the RACE-ready cDNA. PCR products were separated on a 2% agarose gel, and product bands were excised and purified using the QIAquick Gel Extraction Kit (Qiagen Benelux, Venlo, The Netherlands). Purified PCR products were ligated into pCR-Blunt II-TOPO plasmid vector and transformed into TOP10 One Shot Electrocompe nt Cells (Invitrogen). For each gel-extracted PCR product, 6–12 colonies were picked randomly, and plasmid DNA was prepared from overnight grown cultures using the QIAprep Turbo BioRobot Kit on a BioRobot 8000 Workstation (Qiagen). The plasmids were screened for the presence of the 5-HT4 receptor-specific sequence located between the nested forward primer F843 and the common 3’-splice site of exon 5 on position 1118 of the open reading frame (ORF).

1 The online version of this article contains supplemental material.
Tissue Localization Studies

Total RNA was prepared from porcine peripheral tissues using the Qiagen RNA isolation kit. cDNA was prepared from 1 μg total RNA using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). The cDNAs specific for the splice variants identified in this study were detected using a nested PCR amplification. To normalize the quantities of cDNA from different tissue sources used as input in the PCR reactions, 10 ng of cDNA was PCR amplified with primers specific for the porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-ACC-ACC-ATC-CTG-GCC-ATC-ACC-3' and 5'-TCC-ACC-ACC-CTG-TTG-CTG-CAA-3'). Next, a first reaction was performed using ~10 ng of cDNA (deduced from GAPDH results) together with the exon 5-specific primer F865 and a splice variant-specific reverse primer (Supplemental Table S1). Products of this first reaction were used as templates for a nested PCR amplification with primer F843, and variant-specific 3'-end primer (Supplemental Table S1). All PCR reactions in tissue distribution studies were performed using the following protocol: 5 min at 95°C followed by 30 cycles (15 s at 95°C, 30 s at 55°C, 1 min at 68°C) and a final elongation (7 min at 68°C). The absence of a PCR product does not exclude the presence of the variant in the tissue: since we used a nested PCR approach, false negative results may appear when the concentration of target sequence is below the threshold of detection, set by the conditions of the first amplification round. To analyze the tissue distribution of the 5-HT4(a) variant, a single competitive PCR reaction was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems): 10 min at 94°C followed by 41 cycles (30 s at 94°C, 30 s at 56°C, 1 min at 72°C) and a final elongation (8 min at 72°C) with primers flanking the h-exon: an exon 4-specific forward primer (Fh) and an exon 5-specific reverse primer (Rh). This results in coamplification of variants with or without the h-exon, and gives information on their relative amount because any variable affecting PCR amplification has the same effect on both molecular species.

Cloning of Full-Length cDNAs and Generation of Fusion Proteins With the EGFP

Full-length cDNAs of 5-HT4(a) and 5-HT4(r) were generated by RT-PCR, using cDNA generated with Superscript III and random hexamers (Invitrogen) (5-HT4(a): F1022/R968, 5-HT4(r): F1022/R1014; Supplemental Table S1). The resulting PCR products were gel fractionated, excised, purified, and used as a template in a second reaction with a nested reverse primer (R906 and R1024 respectively; Supplemental Table S1). Products were again size-separated on an agarose gel, excised, purified, cloned into pENTR-D-TOPO (Invitrogen) and sequence verified. The full-length cDNA of 5-HT4(9TM) was assembled by ligation. The HaelII restriction fragment of PCR product F843/R1039 (Supplemental Table S1) was ligated (T4 DNA ligase; Roche Diagnostics, Mannheim, Germany) with the HaelII digested PCR product F1022/R1028 (Supplemental Table S1). The resulting ligation product was cloned into pENTR-D-TOPO and transformed into TOP10 One Shot Electrocompetent Cells. Several colonies were picked; Miniprep DNA was prepared using the QIAprep Spin MiniPrep Kit (Qiagen), and the presence of an insert was verified by restriction enzyme digestion; the sequence was verified by sequencing. The PCR products of the porcine 5-HT4(a), 5-HT4(r), and 5-HT4(9TM) were then shuttled into the mammalian expression vector pDEST40 by LR clonase (Invitrogen). The plasmids containing the full length sequences were used to generate a full-length sequence with a reverse primer in which the TGA stop codon was mutated to remove the translation termination signal and to provide an in-frame sequence with enhanced green fluorescent protein (EGFP). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), cloned into pENTR-D-TOPO and sequence verified. These products were subcloned into an in house generated pEGFP-N1/pDEST expression vector to generate NH2-terminal fusions to the EGFP.

Cell Culture and Transfection

HEK-293 tsA cells were grown in DMEM (Invitrogen) containing 10% fetal calf serum (Hyclone, Logan, UT), 105 IU/l penicillin (Serva, Heidelberg, Germany), 0.1 g/l streptomycin (Serva), 0.1 g/l pyruvate (Sigma, St. Louis, MO), and 0.292 g/l 1-glutamine (Sigma), under 5% CO2 at 37°C. Cells were transfected overnight in suspension using Lipofectamine 2000 reagent (Invitrogen) and cultured posttransfection in DMEM containing 10% diazylated fetal calf serum (Invitrogen).

Membrane Preparations and Radioligand Binding

Cells were cultured on 145 mm Petri dishes and incubated in medium containing 5 mM sodium butyrate (Acros Organics, Geel, Belgium) for 20 h before experiments to boost receptor expression before membrane preparation for radioligand saturation binding (33). Cells were washed twice with ice-cold PBS. The cells were then scraped from the plates with a cell scraper, suspended in 50 mM Tris-HCl buffer (pH 7.4), and harvested by centrifugation for 10 min at 16,000 g. The pellet was resuspended in 5 mM Tris-HCl (pH 7.4) and homogenized with an ultra Turrax homogenizer. The resulting membranes were collected by centrifugation at 25,000g for 20 min and stored in 50 mM Tris HCl buffer (pH 7.4) or 50 mM HEPES-NaOH (pH 7.4), at a protein concentration of ~1 mg/mL. The Bradford protein assay (Bio-Rad) was used for protein determination with bovine serum albumin as a standard.

Assay mixtures (0.5 ml) contained 50 μl of the tritiated ligand [either the 5-HT4 receptor antagonist [3H]GR-113808 or the agonist [3H]5-HT (Amersham Biosciences, Little Chalfont, UK)] and 0.4 ml of the membrane preparation (at 0.003, 0.006, or 0.013 mg/ml protein for [3H]GR-113808 binding of 5-HT4(a), 5-HT4(r), or 5-HT4(9TM), respectively, and 0.025, 0.075, or 0.13 mg/ml protein for [3H]5-HT binding of 5-HT4(a), 5-HT4(r), or 5-HT4(9TM), respectively). Furthermore, 50 μl of solvent was added for total binding or 50 μl of 10 μM SB-207266 for [3H]GR-113808 binding or 50 μl of 10 μM GR-113808 for [3H]5-HT binding, to determine nonspecific binding. The [3H]GR-113808 assay buffer was 50 mM HEPES-NaOH (pH 7.4). The [3H]5-HT assay buffer was 50 mM Tris-HCl (pH 7.4) containing 10 mM MgCl2, 1 μM pargyline, and 1 μM paroxetine. The mixture was incubated for 1 h at 25°C. The incubation was terminated by rapid filtration over Whatman GF/B filters, presoaked in 0.1% polyethylenimine, followed by three washing steps with 50 mM HEPES-NaOH (pH 7.4) for [3H]GR-113808 binding or 50 mM Tris-HCl (pH 7.4) for [3H]5-HT binding. Ligand concentration-isotherms were obtained with twelve concentrations of [3H]GR-113808 ranging from 0.003 to 3 nM or 12 concentrations of [3H]5-HT ranging from 0.2 to 14 nM. Ligand concentration-binding isotherms were calculated by nonlinear regression analysis. Data were fit to one-and two-binding site models. The fit with the lowest squares of sum of residuals (F-test) was chosen as the best fit of the data. The null hypothesis was rejected at a P < 0.05 level. All data are reported as means ± SE.

Measurements of cAMP Formation

After an overnight incubation in medium containing diazylated fetal calf serum, cells that were transiently transfected with 5-HT4(a), 5-HT4(r), and 5-HT4(9TM), were used for cAMP measurements performed by homogeneous time-resolved fluorescence (Cisbio International, Bedford, MA) (12). We added 25 μl of cell suspension (10,000 cells per well) and 25 μl of either vehicle or compound (1 μM–10 μM), all in stimulation buffer composed of HBSS (Invitrogen) plus 1 mM isobutylmethylxanthine (Sigma), 5 mM HEPES (Sigma), 1 mM MgCl2 (Merck, Darmstadt, Germany), and 1 g/l BSA (Sigma) to the wells of a black Costar 96-well plate and incubated that for 30 min at room temperature. In experiments where the effect of 5-HT was assessed in the presence of 0.3 μg MR-113808, we preincubated cells with the antagonist (or its solvent) for 20 min, before adding 5-HT.
assess the effect of direct activation of adenyl cyclase, 3 \( \mu M \) forskolin (Sigma) was added to the transfected cells and incubated for 30 min at room temperature. Effects were quantified as the 665 nm/620 nm ratio and reported on a standard curve to deduce respective cAMP concentrations. cAMP concentrations [one (5-HT\(_{4a}(\alpha)\) experiment or two experiments (5-HT\(_{4a}^{(1)}\text{TM})\) with \( n = 6 \) independent wells assayed for each concentration point] were used to fit the data to a three-parameter logistic model using GraphPad Prism version 4.02. To compare the curve location parameters of 5-HT and prucalopride, the fit of a three-parameter logistic model with common curve location parameters was compared with the fit of a three-parameter logistic model with separate curve location parameters. The goodness of fit of both models was compared with an F-test (assessed by the sum of squares), adjusting for differences in the number of degrees of freedom. If the F-test resulted in a \( P \) value <0.05, the individual curve location parameters (the maximal effect \( \alpha \), pEC50) were compared by a \( t \)-test, and the corresponding \( P \) values are shown.

Confocal Microscopy

HEK-293 tA cells (8.10\(^6\) cells) were transfected with 3 \( \mu g \) of the fusion constructs of 5-HT\(_{4a}(\alpha)\), 5-HT\(_{4d}(\alpha)\), 5-HT\(_{4a}^{(1)}\text{TM}\) with EGFP, diluted, and grown on polylysine-treated Lab-Tek chambered cover glasses (Nunc, Naperville, IL) at a density between 40,000 and 80,000 cells/cm\(^2\). At 48 h posttransfection, the cells were labeled with Alexa Fluor 594 wheat germ agglutinin (Molecular Probes, Eugene, OR) to label the plasma membrane, fixed, and covered with a drop of Vectashield antifade solution containing DAPI (Vector Laboratories, Burlingame, CA) and a cover glass. Cells were analyzed using the Zeiss LSM 510Meta confocal imaging system.

Data Mining

The following bioinformatics tools were used: genome browsing (http://www.ensembl.org/index.html; http://trace.ensembl.org; http://www.sanger.ac.uk/cgi-bin/Projects/S_scrofa/BESSearch.cgi) and tmembrane topology prediction (http://www.ebs.db.dk/services/TMHMM-2.0/; http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html; http://bioinfo.st.hirosaki-u.ac.jp/~ConPred2/); alignments between the porcine splice variants found in our study and the human HTR4 genomic region were performed using several alternative alignment algorithms: Spidey, an mRNA-to-genomic alignment program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/), ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html), and EMBOSS-Align (Smith-Waterman algorithm; http://www.ebi.ac.uk/emboss/align). To allow for interspecies comparisons, Spidey was run with lowered mismatch, gap opening, and gap extension penalties. When using ClustalW and EMBOSS-Align, the gap opening penalty and gap extension penalty were varied during the optimization of the alignments.

RESULTS

Identification of Porcine 5-HT\(_4\) Receptor Splice Variants

A 3′-RACE strategy was applied in an attempt to identify porcine 5-HT\(_4\) receptor splice variants expressed in the adrenal gland, proximal stomach, and the aorta. The 3′-end of the majority of the positive clones in the 3′-RACE experiments corresponded to the 5-HT\(_{4a}\) splice variant (Fig. 2A), based on the homology of its translated amino acid sequence to the human, rat, and mouse 5-HT\(_{4a}\) protein (Fig. 3A). A large diversity in the 3′-untranslated region (UTR) of 5-HT\(_{4a}\) was found with multiple combinations of noncoding exons. Alignment of these noncoding exon sequences to the human HTR4 gene, suggested their genomic location downstream of the coding exon a (Supplemental Fig. S1).

![Fig. 2](http://physiolgenomics.physiology.org/)

Suinspiciously, none of our RACE clones corresponded to the published sequence of the porcine 5-HT\(_{4}\) being the only porcine 5-HT\(_4\) sequence currently found in public databases. However, we identified a variant (5-HT\(_{4}(b2)\)) that showed exactly the same nucleotide sequence as 5-HT\(_{4}(b)\) up to position 1147, but which diverged downstream and was followed by a ~900 bp long UTR. At the protein level, the terminal six amino acids of 5-HT\(_{4}(b)\) were substituted by two valine residues in 5-HT\(_{4}(b2)\) (Fig. 3B). Nevertheless, a PCR that was set up with specific primers designed to amplify the extreme 3′-end of the coding sequence of the published porcine 5-HT\(_{4}(b)\) variant only, produced a single band of expected size, suggesting the presence of 5-HT\(_{4}(b)\) (data not shown).

Besides 5-HT\(_{4}(a)\) and 5-HT\(_{4}(b)\), none of the COOH-terminal variants currently known in other species were identified in the RACE experiments (nor in RT-PCR experiments with a reverse primer based on the human sequences; see supplemental data). However, several sequences were found that comprised the common sequence up to exon 5 but did not correspond to any known COOH-terminal splice variant of the 5-HT\(_4\) receptor. In accordance with the current nomenclature of 5-HT\(_4\) receptor splice variants (http://www.iuphar-db.org/GPCR/ReceptorDisplayForward?receptorID=2329), we named these new porcine variants 5-HT\(_{4}(d)\), 5-HT\(_{4}(k)\), 5-HT\(_{4}(l)\), 5-HT\(_{4}(m)\), 5-HT\(_{4}(n)\), 5-HT\(_{4}(o)\), 5-HT\(_{4}(p)\), 5-HT\(_{4}(q)\), and 5-HT\(_{4}(r)\).
5-HT4(o), 5-HT4(p), 5-HT4(q), and 5-HT4(r) (Figs. 2A, 3B). All the new COOH-terminal variants but 5-HT4(m) could be aligned to the human HTR4 genomic region (Supplemental Fig. S1). Sequence identities were, however, rather low, in the range of 60–70%, indicating the low conservation of these sequences between human and porcine DNA. For comparison, the sequence identity of exon 2 (90%), 3 (90%), 4 (85%), h (84%), 5 (91%), a (83%), and b (99%) are much higher (Supplemental Fig. S2). The presence of the new variants in human tissue is therefore unlikely. A BLAST search of the known human variants against porcine genomic shotgun sequences resulted in the identification of the porcine homologs of the "gef" exons (SS_WGS-375j07.q1k). Similar as in human, the sequence is preceded by a splice acceptor site (AG), and 5-HT4(g) might thus be present in porcine tissue. Based on this shotgun sequence, 5-HT4(e) and 5-HT4(f) cannot be generated in the pig since the required alternative acceptor sites are not present in the porcine sequence (Supplemental Fig. S3).

Identification of Splice Variants Composed of Duplicated Exons of the Porcine 5-HT4 Receptor, Including 5-HT4(9tm) and 5-HT4(15tm) (Figs. 2A, 3B). All the new COOH-terminal variants but 5-HT4(o) could be aligned to the human HTR4 genomic region (Supplemental Fig. S1). Sequence identities were, however, rather low, in the range of 60–70%, indicating the low conservation of these sequences between human and porcine DNA. For comparison, the sequence identity of exon 2 (90%), 3 (90%), 4 (85%), h (84%), 5 (91%), a (83%), and b (99%) are much higher (Supplemental Fig. S2). The presence of the new variants in human tissue is therefore unlikely.

A BLAST search of the known human variants against porcine genomic shotgun sequences resulted in the identification of the porcine homologs of the “gef” exons (SS_WGS-375j07.q1k). Similar as in human, the sequence is preceded by a splice acceptor site (AG), and 5-HT4(o) might thus be present in porcine tissue. Based on this shotgun sequence, 5-HT4(o) and 5-HT4(r) cannot be generated in the pig since the required alternative acceptor sites are not present in the porcine sequence (Supplemental Fig. S3).

In addition to the splice variants described above, the RACE experiments with cDNA from left atrium revealed splice variants composed of (partial) duplicates of one or more exons of the porcine HTR4 gene. For example, variants in which the common splice donor site of exon 5 was followed by exons 4–5 or exons 4-h-5 (Fig. 2B: variant 1). Exon 5 was only partially duplicated and followed by a short sequence that contained an in-frame stop codon, a polyadenylation signal, and a poly-A tail. The duplicated exons were in frame with the common sequence. Consequently, translation results in duplication of part of the transmembrane region of the receptor: the sequence between the second and the third intracellular loop, including two transmembrane helices, is repeated after the common intracellular COOH terminus (Supplemental Fig. S2 and Fig. 4A). This homofusion variant is predicted to contain nine transmembrane helices by all prediction servers tested (Fig. 4, B and C) and we therefore named this variant 5-HT4(9TM). Additional evidence for the existence of this variant was found by the amplification of products with an exon succession 5-4-5 (variant 1, 5-HT4(9TM)) and 5-5 (variant 2) using primers located within exon 5 (F1045-R1046) (Fig. 2B).

The existence of variants that contain duplicated exons was further supported by the identification of a putative variant 5-HT4(15TM). A nested PCR reaction (F865-R1016 and F843-R1015) designed to amplify 5-HT4(9TM) generated a 400 bp PCR side product with the exon succession 5-2-3-4-5 (Fig. 2B, variant 3). When extrapolating to a putative ORF, this receptor is predicted to possess 15 transmembrane helices and would be a dimer of two 5-HT4 receptors (Supplemental Fig. S4). The middle helix “restores” the correct orientation of the COOH-terminal receptor to the cell membrane. The existence and expression of such dimer 5-HT4 receptor needs, however, to be confirmed.

Finally, the existence of splice variants composed of duplicated exons fused to the complete common 5-HT4 region was given a solid base by the amplification of a variant with a rank order of exons (1)-2-3-4-5-1-4, which suggests a duplicated region located downstream of the HTR4 gene. This variant was
found in a heminested PCR reaction on the muscular fraction of the ileum using F1022 (exon 1 from ATG onwards) and R1015 in a first amplification round, and forward primers located in exon 2 (F932) and exon 3 (F934 or F858) in a second round (Fig. 2B, variant 4).

**Genomic Context of the Newly Identified HTR4 Exons**

To confirm that the newly identified variants originated from alternative splicing of genomic exons, we elucidated the genomic context of the new cDNA nucleotide sequences. Several of the 5-HT$_4$ UTR exons could be traced down to their genomic context on chromosome 2q29 of the porcine genome using the Ensembl Trace Server (e.g., end sequences of BACs CH242-19O19.T7 and PigE-146O22.T7; detailed results not shown). The common exons 1, 4, and 5 were also mapped in this genomic region (BACs CH242-91N19.SP6, CH242-369H11.T7, and CH242-400M6.T7 respectively; Supplemental Table S2).

The end sequences of BACs CH242-338C6.T7 and CH242-478H10.T7 on the one hand and the genomic shotgun sequence SS_WGS-258F21.q1k on the other hand provide additional evidence for the presence of two different b-variants that are derived from separate exons: an alternative splice-donor site in the b-exon may lead to the alternative 5-HT$_4$ variant h, while the unspliced exon b generates the known 5-HT$_4$b sequence (Fig. 5A, Supplemental Fig. S1C).

5-HT$_4$(j), (k), (l), (o) variants were all found within the same genomic region (Fig. 5B). Porcine exons j, k, and o are organized similarly as exons g, e, and f in the human genome, i.e., the porcine COOH-terminal variants o and k are generated on the basis of three alternative splice acceptor sites within exon j (in the coding sequence for variant o and in the UTR sequence for k). 5-HT$_4$(j) differs from 5-HT$_4$(o) by the presence of 5 extra nucleotides, resulting in a frame shift. The stretch of adenosines preceding the splice acceptor site of 5-HT$_4$(o) presumably served as the binding place for the oligo(dt) primer during the construction of the RACE-ready cDNA. Therefore, 5-HT$_4$(j) is likely part of exon j, and the 3'-UTR sequence of j, k, and o may all include the sequence of l. The absence of a polyadenylation signal at the 3'-end of variants j, k, and o supports this view. Based on sequence similarity searches, homologous sequences to variants j, k, l, and o can be found in the human HTR4 gene ~1 kb downstream of exon d (Supplemental Fig. S1B).

Porcine splice variant 5-HT$_4$(hm) was found with two alternative UTRs originating from internal splicing of exon m, both containing the canonical polyadenylation signal (Fig. 5C). None of these sequences showed homology to the human genome. 5-HT$_4$(hp) was generated from an alternative splice acceptor site within one of the 5-HT$_4$(a) UTR variants, which causes a UTR exon to become a coding exon (Fig. 5D). The end sequence of BAC clone CH242-19O19.T7 contains part of the p sequence. The COOH-terminal sequence of 5-HT$_4$(q) (Fig. 5E) was also found within the publicly available genomic sequences. Unexpectedly, however, an insertion of 26 nucleotides was present in the cDNA of 5-HT$_4$(q) compared with this genomic sequence. The porcine homologs of the human "gef" sequences were also identified in porcine genomic sequences (Fig. 5F). A homologous sequence to this porcine exon r was found between exon 5 and exon i on the human HTR4 gene (Supplemental Fig. S1B).

Finally, in support of our assumption that the identified 5-HT$_4$ variants did not represent genomic sequences, we determined the intron sequence located at the 3'-end of exon 5 from porcine BAC clone CH242-369H11 and confirmed that it did not correspond to any of the sequences found in this study, nor to an exon duplication (data not shown).

**Tissue Distribution of COOH-Terminal Splice Variants of the Porcine 5-HT$_4$ Receptor**

We then examined the expression profile in different tissues of the variant COOH-terminal sequences by RT-PCR. Except for the 5-HT$_4$(j) variant, all other variants identified in the RACE experiments were confirmed in at least one tissue (Fig. 6A). The applied method does unfortunately not allow for a quantitative analysis of the results, and false negative results cannot be excluded when the concentration of target sequence was below the threshold of detection. Splice variants 5-HT$_4$(a) and 5-HT$_4$(b) are the predominant variants in terms of expression level and distribution across various tissues, followed by 5-HT$_4$(m), 5-HT$_4$(r), and 5-HT$_4$(h) (Fig. 5A). The h-exon was found in combination with the COOH terminus of the a, m, and r variant, resulting in 5-HT$_4$(hr), 5-HT$_4$(hml), and 5-HT$_4$(hr) (Fig. 2A; not analyzed for other variants).

**Cloning and Functional Characterization of the Porcine 5-HT$_4$(a), 5-HT$_4$(r), and 5-HT$_4$(h) Splice Variants**

**Cloning and confocal imaging.** Full-length cDNAs from 5-HT$_4$(a), 5-HT$_4$(r), and 5-HT$_4$(h) were generated using RT-PCR. To compare the pharmacological profiles of the 5-HT$_4$(a), 5-HT$_4$(r), and 5-HT$_4$(h) variants, the corresponding pDEST40 constructs were transiently transfected into HEK-293 tsA cells. In addition, to visualize cellular distribution, these variants were fused to EGFP and transfected into HEK-293 tsA cells (Fig. 7A). EGFP-fused 5-HT$_4$(a) and 5-HT$_4$(r) were clearly expressed at the cell membrane. In addition, also intracellular structures (presumably ER and Golgi) were stained, which was most pronounced in 5-HT$_4$(r), 5-HT$_4$(h), was mainly located intracellularly, suggestive for a different topology and less effective membrane trafficking or instability of this variant compared with the other two variants; weak membrane staining was, however, also observed.

**Binding.** The receptors were analyzed by radioligand binding assays on crude membrane preparations using two radioligands: the reference antagonist [${}^{3}$H]GR-113808 and the natural agonist of the 5-HT$_4$ receptor, [${}^{3}$H]5-HT. The concentration binding isotherms of the antagonist [${}^{3}$H]GR-113808 to 5-HT$_4$(a), 5-HT$_4$(r), and 5-HT$_4$(h) receptors showed rectangular hyperbolae, and the resulting linear Scatchard plots revealed a single high-affinity binding site. The calculated $K_D$ and $B_{max}$ values are presented in...
Table 1. In contrast, the binding data of [³H]5-HT to 5-HT₄(a) and 5-HT₄(r) were significantly better fitted by a two-site than a one-site binding model [$P < 0.001$ for 5-HT₄(a), $P < 0.05$ for 5-HT₄(r)]. This was also clear from the nonlinear Scatchard plots, which indicated the existence of a high- as well as a low-affinity binding site for 5-HT₄(a) and 5-HT₄(r). At the 5-HT₄(9TM) variant, [³H]5-HT binding occurred at a single low-affinity site (Table 1).
Signal transduction. To investigate the functional properties of the 5-HT4(a), 5-HT4(r), and 5-HT4(9TM) receptor variants, we examined their ability to activate adenylyl cyclase by measuring the formation of cAMP in transiently transfected HEK-293 tsA cells. All three variants constitutively stimulated cAMP formation (6-, 2-, and 1.5-fold vs. mock transfected for 5-HT4(a), 5-HT4(r), and 5-HT4(9TM) respectively). Of note, the response to forskolin in the 5-HT4 receptor-transfected cells was lower compared with mock-transfected cells. This was most prominent for 5-HT4(9TM), with a 50% reduction of the forskolin response (Table 2). The cAMP formation was analyzed upon challenging the different transfected cells with 5-HT, prucalopride, or GR-113808 (Fig. 7B). 5-HT and the 5-HT4 receptor agonist prucalopride were more potent on 5-HT4(a) than on 5-HT4(r) and 5-HT4(9TM) (Table 2). While both agonists were equivalently efficacious on 5-HT4(r), prucalopride was more efficacious than 5-HT on 5-HT4(a) while a small but significantly higher efficacy of 5-HT compared with prucalopride was seen at 5-HT4(9TM). The effects of 5-HT on all variants could be antagonized by GR-113808 (0.3 μM), indicating that indeed, the observed effects are mediated by 5-HT4 receptors. GR-113808 itself induced a concentration-dependent increases in cAMP in all 5-HT4 receptor-transfected cells (results not shown). However, only at 5-HT4(a) the maximal effect exceeded a 1 nM increase above basal (4.7 nM).

DISCUSSION

In the present study we identified nine novel COOH-terminal splice variants of the porcine 5-HT4 receptor. In addition, several splice variants were identified in which duplicated exons were fused to the common region of the 5-HT4 receptor, one of which translated into a putative nine transmembrane (TM) domain homofusion receptor, 5-HT4(9TM). To our knowledge, this study is the first to report on a GPCR with more than 7 TM domains. The elucidation of the genomic context confirmed that the variants originated from alternative splicing, and were no genomic artefacts. Finally, the existence of 5-HT4(a), 5-HT4(r), and 5-HT4(9TM) was further corroborated by a functional analysis of these variants.

Although 5-HT4 receptors are expressed in embryonic murine hearts (16) and in failing rat hearts (36), the porcine heart is currently the only available tool for studying cardiac 5-HT4 receptors under physiological conditions in a nonprimate animal model. However, the findings of this study suggest that additional validation at the molecular level might be needed to give a solid basis for the use of swine as a model for human 5-HT4 receptor function. In human, several 5-HT4 receptor variants are produced by alternative splicing of a complex gene of >185 kb that includes at least 14 exons (6). While the in vivo relevance and functional role of the multiplicity of 5-HT4 receptor splice variant remains to be elucidated, dif-

---

**Fig. 5.** Genomic context of porcine 5-HT4 receptor splice variants. Open boxes represent cDNA sequences, while black lines represent corresponding genomic sequences, derived from the Ensemble Trace Server. Flanking genomic sequences are shown, as well as consensus polyadenylation signals. A: the 5-HT4(a) variant originates from the use of an alternative splice donor site within the b-terminal exon, resulting in the splicing of the end of exon b. This results in an alternative end of 5-HT4(a), while an unspliced exon generates the known 5-HT4(b) sequence (underlined). B: organization of the COOH-terminal sequences of 5-HT4(9TM), which are located in the same genomic region. The presence of a poly(A)-stretch (underlined) between k and l presumably served as a binding site for oligo-dT during cDNA synthesis. C: 5-HT4(9TM), was found with different untranscribed region (UTR) originating from internal splicing of part of the UTR sequence. D: 5-HT4(9TM) was generated from an alternative splice acceptor site within one of the 5-HT4(a) UTR variants. E: for 5-HT4(9TM) unexplained insertion of 26 nucleotides was found in the cDNA sequence compared with the genomic sequence, indicated by a grey box. F: alignment of an exon in the 3’-UTR of 5-HT4(a) to the porcine genome.
differences between 5-HT₄ receptor splice variants are becoming increasingly recognized in heterologous expression systems (5, 6). These differences occur at the level of receptor palmitoylation (29, 32), and constitutive receptor activity (8). Additionally, human 5-HT₄ receptors interact with specific intracellular proteins in a splice variant-specific manner (15), which further supports the view that such isoforms are in charge of fine regulation of signal transduction and are not the result from transcriptional leakage (5). This study is the first to address the splicing behavior of the porcine 5-HT₄ receptor.

Using BAC end sequences positive for HTR4 sequences from the porcine genome project, we annotated the HTR4 gene since we identified intronic sequences in the public databases that surrounded the exons, excluding the possibility that the variants originated from intron retention. On the other hand, we detected only few of the splice variants that have been reported in human. Based on the porcine genomic homolog of the “gef” exons, it can be anticipated that of these three variants, only 5-HT₄(9TM) might be present in porcine tissue that have not been described in human tissue or any other species. Similar as in human, rat and mouse, these new variants (5-HT₄(b2, j, k, l, m, o, p, q, r)) differ in their sequence from Leu358 onwards, resulting in COOH-terminal splice variants that have not been reported in the human HTR4 gene. Our data provide evidence that these variants were splice variants from a single gene since we identified intronic sequences in the public databases that surrounded the exons, excluding the possibility that the variants originated from intron retention. On the other hand, we detected only few of the splice variants that have been reported in human. Based on the porcine genomic homolog of the “gef” exons, it can be anticipated that of these three variants, only 5-HT₄(9TM) might be present in porcine tissue.

Table 1. Summary of saturation binding analysis of membrane preparations from HEK-293 tsA cells transiently transfected with porcine 5-HT₄ receptor splice variants using [³H]GR-113808 or [³H]5-HT

<table>
<thead>
<tr>
<th></th>
<th>5-HT₄(a)</th>
<th>5-HT₄(r)</th>
<th>5-HT₄(9TM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]GR-113808</td>
<td>4126 ± 105</td>
<td>639 ± 20</td>
<td>195 ± 5</td>
</tr>
<tr>
<td>[³H]5-HT</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. Saturation binding was performed in triplicate using [³H]GR-113808 at concentrations from 0.02 to 3 nM and [³H]5-HT from 0.2 to 1.5 nM. Bₘₐₓ (maximum binding capacity, fmol/mg) and Kᵦ (nM) values were obtained from curve fitting of ligand concentration binding isotherms using nonlinear regression analysis. n.a., not applicable.

Table 2. Potency and efficacy of 5-HT and prucalopride on the cAMP formation in HEK-293 tsA cells transiently transfected with 5-HT₄(a), 5-HT₄(r) and 5-HT₄(9TM)

<table>
<thead>
<tr>
<th></th>
<th>5-HT₄(a)</th>
<th>5-HT₄(r)</th>
<th>5-HT₄(9TM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC₅₀ (max)</td>
<td>9.01 ± 0.08</td>
<td>7.63 ± 0.07</td>
<td>7.66 ± 0.06</td>
</tr>
<tr>
<td>pEC₅₀ (nM)</td>
<td>10.7 ± 0.2</td>
<td>9.3 ± 0.2</td>
<td>9.6 ± 0.2</td>
</tr>
<tr>
<td>Prucalopride</td>
<td>8.60 ± 0.12*</td>
<td>7.57 ± 0.10</td>
<td>7.65 ± 0.07</td>
</tr>
<tr>
<td>max (nM)</td>
<td>15.2 ± 0.5*</td>
<td>9.2 ± 0.34</td>
<td>8.3 ± 0.2*</td>
</tr>
</tbody>
</table>

Results are derived from curves fitted by nonlinear regression analysis. Values are presented as means ± SE of one (5-HT₄(a), 10) experiment or two experiments (5-HT₄(9TM)) with n = 6 independent wells assayed for each concentration point. Basal cAMP levels were 0.5 ± 0.05 nM (mock), 3 ± 0.2 nM (5-HT₄(a)), 1.0 ± 0.1 nM (5-HT₄(r)), and 0.7 ± 0.03 nM (5-HT₄(9TM)). Forskolin (3 μM) increased cAMP levels to 50 ± 3 nM (mock), 41 ± 3 nM (5-HT₄(a)), 31 ± 3 nM (5-HT₄(r)), and 27 ± 1 nM (5-HT₄(9TM)). Assays were performed with a fixed number of (living) cells per well (10,000 cells). Comparison of the potency and efficacy of an agonist between splice variants should be done with care because experiments were performed using transiently transfected cells and transfection efficiencies might vary between the different splice variants. *P < 0.001 vs. corresponding values for 5-HT.
tissues. Although no porcine genomic sequences were retrieved that are homologous to the other human variants, their existence cannot be ruled out due to the unfinished status of the porcine genome project.

Splice variants in extracellular loops of G protein-coupled receptors are rare (20). One of these is the 5-HT aggregates receptor, which has an extra insertion of 14 amino acids in its second extracellular loop. The first description of a partial 5-HT splice sequence originates from porcine cDNA (40), and this variant has also been found in human, albeit only in combination with the b-terminal exon (5-HT) (3). However, the authors suggested that the h-exon is probably also expressed in combination with various other COOH-terminal exons. Here, we provide experimental evidence that porcine 5-HT splice variants exist in combination with other COOH-terminal tails, 5-HT, 5-HT, and 5-HT. Interestingly, a tissue distribution analysis of the exon h-containing variants revealed that the mucosal fractions along the entire gastrointestinal tract, in contrast to the muscle layers containing the myenteric plexus, predominantly expressed variants with the h-exon. In mice, a similar distribution pattern of some 5-HT receptor splice variants has been described. Expression of 5-HT and 5-HT mRNA, normalized to the expression of synaptophysin to correct for the proportion of transcripts derived from neurons, was more pronounced in the submucosa than in the longitudinal muscle with adherent myenteric plexus (25). In contrast, the expression of mRNA encoding 5-HT and 5-HT was myenteric plexus specific (25). The preferential mucosal localization of exon h-containing splice variants might correlate with their presence in enterochromaffin cells (EC) and/or intrinsic primary afferent neurons. These are two well-known locations of 5-HT receptors, where they are involved in autoregulatory feedback of 5-HT release from EC cells and in the peristaltic reflex, respectively (14, 38).

In addition, our data demonstrate that at least part of the porcine HTR4 gene is duplicated in the genome, which can give rise to splice variants comprising duplicated exons. All of these variants contained duplicated exons from the common part of the receptor (exon 1 to 5). One of these duplication variants is predicted to be a homofusion protein containing nine putative TM domains (5-HT), which we also demonstrated to be functionally active. Several other findings argue in favor of a duplicated genomic region: 1) A diversity of splice variants that each contained different duplicated exons were obtained; 2) Multiple combinations of duplicated exons were found, but the duplicated exons always retained the same order; 3) The variants were found using different primer combinations, located in different exons; 4) Expression of the duplicated variants was found in multiple tissues from different animals; 5) The junction site is the common splicing site Leu supporting the fact that the duplication variants resulted from alternative splicing mechanisms; 6) The presence of porcine COOH-terminal splice variants that have not been found in human suggests that this genomic region is not well conserved between pig and human. This is further supported by the low sequence similarity with their human homolog sequences.

In addition, some circumstantial evidence can be found that would be in line with a duplication of the porcine HTR4 gene. The human chromosome 5 corresponds to porcine chromosomes 2 and 16, with the human 5q13–q32 region (74.0–148.2 Mb) corresponding to the porcine region 2q22–q28 (22, 39). In the human genome, the HTR4 gene is located at 5q32 (147.8–148.0 Mb), thus close to the breakpoint between the two synteny blocks in human chromosome 5. This is clearly illustrated by the location of the CSF1R and GPX3 genes. While both genes map closely to the HTR4 gene on human chromosome 5 (at 149.4 Mb and 150.4 Mb, respectively), the CSF1R maps at the porcine chromosome 2q telomere, while GPX3 maps at porcine chromosome 16 (22). In line with these results, the porcine HTR4 gene is located at the telomeric end of chromosome 2 (at 2q28 or q29). Of note, the mapping of the porcine HTR4 gene to SSC 2q28 by Shimogiri et al. (39) contrasts with the porcine physical map in the Ensembl database, where the HTR4 gene is mapped to SSC 2q29. Enrichment of segmental duplications have been shown earlier in regions of breaks of synteny between human and mouse genomes (2). In addition, the duplication frequency in subtelomeric regions is greater than the genome average (42).

The functionally active 5-HT receptor variant forms a partial 5-HT receptor dimer that is directly transcribed from the genome. This variant is proposed to possess a 9TM topology by a number of different prediction algorithms, including ConPred II. In this method sequences are first run through ConPred elite, which has an accuracy of almost 100% (1). Based on these modeling approaches only, however, we cannot exclude that 5-HT has a 7TM topology with a large COOH-terminal tail containing the duplicated sequences. The 9TM domain structure needs experimental confirmation using for instance a series of truncated fusion constructs of 5-HT (4). It has been postulated that new protein topologies may originate during evolution through multistep gene rearrangements, involving duplication, fusion, and truncation, which may lead to fully functional intermediate proteins (41). Besides, evidence is accumulating that in human and in other mammalian genomes, copy number polymorphisms explain a substantial amount of genetic variation and may account for a significant proportion of normal phenotypic variation (11). It has been suggested that subtelomeres could be a place where new genes are created by duplication and subsequent modification processes and could be the source for phenotypic variation among individuals (24). This is illustrated by a study on the transcriptional activity of multiple copies of the subtelomerically located olfactory receptor gene OR-A (24). Our data might fit into these theories.

The porcine splice variants showed some degree of tissue specificity. Some of the predominant variants in terms of expression level and distribution across various tissues were selected for a functional analysis: the homofusion receptor 5-HT and 5-HT, one of the new COOH-terminal variants 5-HT and 5-HT, which is present throughout species examined. These experiments confirmed, at least for these variants, that the variants identified in this study were expressed at the cell membrane and that they shared some characteristics with human 5-HT receptors: 1) 5-HT-EGFP fusion constructs were expressed at the cell membrane; 2) Two affinity sites for [1H]5-HT were revealed on porcine 5-HT and 5-HT receptors (GPCR fraction and noncoupled receptor fraction), similar to what has been reported for the human 5-HT and 5-HT variants (33); in contrast, only a single affinity site was found for 5-HT at 5-HT (39). Possibly the low receptor density of 5-HT might mask the few high affinity sites;
3) [3H]GR-113808 bound to these receptors as a typical antagonist (capable of binding the GPCR fraction and the non-coupled receptor fractions) with Kᵢ values consistent with previous findings on 5-HT₄ receptor-transfected cells and different brain tissues (33). Nevertheless, GR-113808 behaved as a weak agonist at these splice variants. Such a behavior has been previously reported with 5-HT₄ receptor antagonists, including GR-113808, in cell systems transfected with 5-HT₄ receptors (3, 33); 4) The variants all constitutively stimulated the formation of cAMP. The role of the COOH terminus in the constitutive activity of 5-HT₄ receptors has been previously reported (8); 5) Prucalopride behaved as a superagonist (more efficacious than 5-HT) at the porcine 5-HT₄ receptor (33), while it was equi-efficacious with 5-HT at the 5-HT₄ variant and a partial agonist at 5-HT₄(9TM). Such a reversal of efficacy order cannot be attributed to differences in receptor expression levels. At the human 5-HT₄ receptor prucalopride behaved as a full agonist compared with 5-HT in inducing cAMP formation but as a superagonist when Ca²⁺ influx was measured (33). The 5-HT₄ receptor agonist cisapride has also been shown to behave as a superagonist in mouse colliculi neurons (10).

Together, our results add to the complexity of the 5-HT₄ receptor. As discussed above for the human variants, at present one can only speculate on the functional significance of such a diversity of splice variants. Some of the variants identified in this study are only weakly expressed at the mRNA level and might thus not lead to functionally significant protein levels. However, given the multitude of variants, a tight regulation of their differential expression can be expected, and the expression of some of these variants might only be triggered by certain external stimuli or time factors.

In conclusion, our data provide evidence for a duplicated porcine HTR₄ gene. Alternative splicing generates a homofusion GPCR with a predicted 9TM topology. These results urge for functional studies on 5-HT₄ receptor-related pharmacology obtained in the pig. Validation at the molecular level might be warranted before extrapolating results to humans, as drug binding properties at the expressed set of splice variants can be altered. Nevertheless, in vitro and/or in vivo functional data obtained in the porcine model could still be highly similar to their human counterpart, especially because all 5-HT₄ receptor splice variants characterized so far are positively coupled to cAMP, and the COOH-terminal variants appear to affect mainly the fine regulation of the signal transduction cascade.

ACKNOWLEDGMENTS

We thank Ronald De Hoogt for technical assistance in genomic technologies, Erwin Fraipont for assistance with binding and cAMP experiments, Paul Van Gompel for help with cell culture, and Pieter Peeters for help and advice on confocal microscopy.

Present addresses: J. De Maeyer, Movetis N.V., Veedijk 58, 2300 Turnhout, Belgium; J. Aerssens, Tibotec bvba, Generaal De Wittelaan L11 B3, 2800 Mechelen, Belgium.

GRANTS

The study was financially supported by Interuniversity Attraction Poles Programme P5/20, Belgian Science Policy and the Fund for Scientific Research Flanders (6.0061.08).

REFERENCES


