0026-895X/99/010054-14$3.00/0

MOLECULAR PHARMACOLOGY, **56:**54–67 (1999).

Humanization of Mouse 5-Hydroxytryptamine$_{1B}$ Receptor Gene by Homologous Recombination: In Vitro and In Vivo Characterization

P. BONAVENTURE,[1,2] L. UMANS,[1] M. H. M. BAKKER, P. CRAS, X. LANGLOIS, W. H. M. L. LUYTEN, A. A. H. P. MEGENS, L. SERNEELS, F. VAN LEUVEN, and J. E. LEYSEN

Department of Biochemical Pharmacology, Janssen Research Foundation, Beerse, Belgium (P.B., M.H.M.B., X.L., J.E.L.); Research Institute Neurosciences, Vrije Universiteit Amsterdam, the Netherlands (P.B., J.E.L.); Experimental Genetics Group, Centrum voor Menselijke Erfelijkheid, Vlaams Interuniversitair Instituut voor Biotechnologie, Katholieke Universiteit Leuven, Campus Gasthuisberg, Leuven, Belgium (L.U., L.S., F. Van L.); Department of Pathology, University of Antwerp, Edegem, Belgium (P.C.); Department of Functional Genomics, Janssen Research Foundation, Beerse, Belgium (W.H.M.L.L.); and Department of General In Vivo Pharmacology, Janssen Research Foundation, Beerse, Belgium (A.A.H.P.M.)

Received January 26, 1999; accepted March 21, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

We replaced the coding region of the murine 5-hydroxytryptamine (5-HT)$_{1B}$ receptor by the human 5-HT$_{1B}$ receptor using homologous recombination in embryonic stem cells and generated and characterized homozygous transgenic mice that express only the human (h) 5-HT$_{1B}$ receptor. The distribution patterns of h5-HT$_{1B}$ and murine (m) 5-HT$_{1B}$ receptor mRNA and binding sites in brain sections of transgenic and wild-type mice were identical as measured by in situ hybridization histochemistry and radioligand receptor autoradiography. When measured in parallel under identical conditions, the h5-HT$_{1B}$ receptor expressed in mouse brain had the same pharmacological characteristics as that in human brain. Stimulation by 5-HT$_{1B}$ agonists of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate binding in brain sections demonstrated the functional coupling of the h5-HT$_{1B}$ receptor to G proteins in mouse brain. In tissue slices from various brain regions, electrically stimulated [^{3}H]5-HT release was not modified by 5-HT$_{1B}$ agonists in tissue from either transgenic and wild-type mice; a 5-HT$_{1B}$ antagonist enhanced electrically stimulated [^{3}H]5-HT release in wild-type mouse brain, but was ineffective in the transgenics. The centrally active 5-HT$_{1A}$/5-HT$_{1B}$ agonist RU24969 induced hypothermia but did not increase locomotor activity in the transgenic mice. The ineffectiveness of RU24969 in the transgenic mice could be due to the lower affinity of the compound for the h5-HT$_{1B}$ receptor compared with the m5-HT$_{1B}$ receptor. The present study demonstrates a complete replacement of the mouse receptor by its human receptor homolog and a functional coupling to G proteins. However, modulation of [^{3}H]5-HT release could not be shown. Furthermore, behavioral effects were not clearly observed, which may be due to a lack of appropriate tools.

The neurotransmitter 5-hydroxytryptamine (5-HT) is involved in many physiological functions (Hoyer et al., 1994). In mammals, the multiple actions of 5-HT are mediated by the interaction with at least 13 molecularly distinct receptors (Saudou and Hen, 1994). Among them, the 5-HT$_{1B}$ receptor has gained particular interest. It belongs to the superfamily of G protein-coupled receptors, and its principal signal transduction pathway occurs via negative coupling to adenylyl cyclase (Adham et al., 1992; Hamblin et al., 1992; Maroteaux et al., 1992; Weinshank et al., 1992). The 5-HT$_{1B}$ receptors modulate serotonergic transmission by a presynaptic autoreceptor function (Middlemiss et al., 1988; Hamblin et al., 1992). They also appear to function as presynaptic heteroreceptors and have been shown to regulate the release of norepinephrine, glutamate, acetylcholine, and γ-aminobutyric acid (Raiteri et al., 1986; Hoyer and Middlemiss, 1989; Molderings et al., 1990; Hen, 1992). Furthermore, they may also play a role in the presynaptic inhibition of neuropeptide release (substance P and calcitonin gene-related peptide) (Buzzi et al., 1991; Moskowitz, 1992; Bonaventure et al., 1998a). The 5-HT$_{1B}$ receptors are widely distributed in the brain but are particularly abundant on axon terminals in the

The experimental Genetics Group, Centrum voor Menselijke Erfelijkheid, Vlaams Interuniversitair Instituut voor Biotechnologie, Katholieke Universiteit Leuven was supported by grants from FWO-Vlaanderen, the Interuniversity Attraction Pole program (IUAP) of the Belgian government, and the Biotechnology Program of the Flemish government (IWT/VLAB/COT-008). L.U. was a postdoctoral research fellow of the Katholieke Universiteit Leuven Research Fund.

[1] These two authors contributed equally to this work.

[2] Current address: R. W. Johnson Pharmaceutical Research Institute, 3535 General Atomic Court, Suite 100, San Diego, CA 92122.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); 5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetraline; ES, embryonic stem; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HYG, hygromycin; h5-HT, human 5-hydroxytryptamine; ISHH, in situ hybridization histochemistry; m5-HT, murine 5-hydroxytryptamine, PCR, polymerase chain reaction; PGK, phosphoglycerate kinase.

output structures of the basal ganglia (globus pallidus and substantia nigra) (Waeber et al., 1989; Bruinvels et al., 1993; Boschert et al., 1994; Langlois et al., 1995; Bonaventure et al., 1997, 1998b). Because of their role in the regulation of multiple neurotransmitters and neuropeptides, 5-HT$_{1B}$ receptors could be a target for the development of new therapeutic agents in the field of depression and migraine, among others (Halazy et al., 1997).

Despite an amino acid sequence identity of more than 90% with the human (h)5-HT$_{1B}$ receptor, the rodent 5-HT$_{1B}$ receptors display different ligand binding properties than their human homolog. For example, the antimigraine agent alniditan binds with a 50-fold higher affinity to the human compared with the rodent 5-HT$_{1B}$ receptor, whereas CP93129 and certain adrenergic antagonists bind more potently to the rodent receptor (Hamblin et al., 1992; Bach et al., 1993; Leysen et al., 1996; Zgombick et al., 1997). These differences in pharmacological properties result from an amino acid substitution at position 355, being asparagine in rat and mouse and threonine in the human receptor (Metcalf et al., 1992; Oksenberg et al., 1992; Parker et al., 1993).

As a consequence of this species difference, rats and mice are not suitable or representative for testing the pharmacological properties of h5-HT$_{1B}$ receptor agonists and antagonists. Transgenic animal technology has provided tools to delete or replace genes that encode receptors; hence, genes can be "humanized," and the function of the human protein can be studied in the transgenic animal. Mutant mice lacking a functional gene encoding the 5-HT$_{1B}$ receptor have been generated by homologous recombination, resulting in mice with increased aggressive behavior (Saudou et al., 1994).

In the present study, an animal model to investigate the h5-HT$_{1B}$ receptor was generated using a "knock-in" technology (i.e., by "humanizing" the mouse locus), producing mice that express the h5-HT$_{1B}$ receptor instead of its murine counterpart but from the same genetic locus. We report the characterization and functional authentication of the h5-HT$_{1B}$ receptor as expressed in the brain of homozygous transgenic animals. The distribution patterns of h5-HT$_{1B}$ and murine (m)5-HT$_{1B}$ receptor mRNA and binding sites in transgenic and wild-type mice were compared by quantitative in situ hybridization histochemistry (ISHH) and receptor autoradiography throughout the brain with two different radioligands (i.e., the antagonist [^{3}H]GR125743, which recognizes both m5-HT$_{1B}$ and h5-HT$_{1B}$ receptors [Mengod et al., 1996; Audinot et al., 1997; Domenech et al., 1997] and the agonist [^{3}H]alniditan, which is selective for h5-HT$_{1B}$ receptors [Leysen et al., 1996]. Additionally, [^{3}H]GR125743 concentration binding curves were obtained from brain sections containing the substantia nigra and globus pallidus of wild-type and transgenic animals. Differential identification was performed by measuring the potency of several compounds [alniditan, rauwolscine, 8-hydroxy-2-dipropylaminotetraline (8-OH-DPAT), CP93129, RU24969, pindolol, propranolol, sumatriptan, and 5-HT) to inhibit binding of [^{3}H]GR125743 in transgenic and wild-type mice and in human brain sections by quantitative receptor autoradiography. In transgenic mouse brain, the functional coupling of the h5-HT$_{1B}$ receptor to G protein activation was established by quantitative autoradiography of [^{35}S]guanosine-5′-*O*-(3-thio)-triphosphate (GTPγS) binding after stimulation by nonselective agonists [5-carboxamidotryptamine (5-CT) and 5-HT] or agonists selective for the mouse (CP93129) or human (alniditan) 5-HT$_{1B}$ receptors. Modulation of electrically evoked [^{3}H]5-HT release by a selective 5-HT$_{1B}$ antagonist (SB224389) was studied in slices of transgenic mouse cortex, hippocampus, and striatum. In vivo effects (i.e., RU24969-induced locomotor activity and effects on body temperature) that are reported to be modulated by 5-HT$_{1B}$ receptor activation (Ramboz et al., 1996; Hagan et al., 1997) were also investigated in these "humanized" mice.

Experimental Procedures

Materials. The mouse strain genomic library was obtained from Genome Systems (St. Louis, MO). The pSG5 expression vector, pBS (KS−) vector, and *Pful* DNA polymerase were purchased from Stratagene (La Jolla, CA). Trizol reagent was from GIBCO BRL (Merelbeke, Belgium).

[^{3}H]Alniditan (41.4 Ci/mmol) was synthesized at Janssen Pharmaceutica (Beerse, Belgium). [^{3}H]GR125743 (73 Ci/mmol), [^{35}S]UTP (1250 Ci/mmol), and [^{35}S]GTPγS (1053 Ci/mmol) were purchased from Dupont-NEN (Zaventem, Belgium). GDP and GTPγS were purchased from Boehringer Mannheim (Mannheim, Germany). [^{3}H]5-HT (106 Ci/mmol), Hyperfilm-^{3}H, Hyperfilm MP, Hyperfilm-βmax, ^{14}C and ^{3}H standards strips were obtained from Amersham (Paisley, UK). All other reagents were from Merck (Overijse, Belgium) or Sigma (Bornem, Belgium). The compounds listed in Table 3 were obtained from various commercial sources or kindly donated by the companies of origin.

Generation of h5-HT$_{1B}$ Receptor Transgenic Mice. The replacement or knock-in construct was based on the h5-HT$_{1B}$ receptor gene (Jin et al., 1992), cloned in a pUC18 vector and on a genomic BAC clone containing the homologous mouse gene isolated from a mouse 129 strain genomic library (clone address BAC-231-B2). Both genes are intronless and thus easily manipulated. A 1173-bp *Eco*RI/*Bam*HI fragment containing the h5-HT$_{1B}$ receptor gene was subcloned in the pSG5 expression vector and amplified with adaptor primers containing suitable restriction sites (*Nco*I, underlined): forward primer 5′-ATAGCTAGCAGGCCTGCCACC**ATG**GAGGAACCGGGTGCTCAG-3′ including the ATG translation start codon of the human receptor gene (reverse complement, CAT, in bold) and reverse primer 5′-CCAGCCATGGTAAGATACATTGATGAGTTTGGACA-3′, located 3′ of the polyadenylation signal in pSG5. A 1.4-kb *Nco*I fragment encoding the human receptor gene with the SV40 polyadenylation signal of the vector was isolated.

A 6.2-kb *Eco*RI fragment of the 129 mouse genomic BAC clone, containing the m5-HT$_{1B}$ receptor gene, was subcloned in the pBS (KS−) vector creating vector pmHTR. The 5′ promoter region was amplified with, as forward primer, the T3 primer of the pBS vector and reverse primer 5′-TGCACCTNAGGC**CAT**GGCTCTCCTCGTCCTGGCTG-3′, including the ATG translation startcodon (in bold) of the mouse receptor gene, creating *Sau*I (in italics) and *Nco*I (underlined) restriction sites. The resulting 2-kb amplicon was digested with *Not*I and *Sau*I and ligated into the pmHTR vector from which most of the gene was deleted by double digestion with *Not*I (site located in the polylinker of the pBS vector) and *Sau*I (site located in the 3′ untranslated region of the m5-HT$_{1B}$ gene). Thus, about 2 kb of the mouse promoter region was conserved in this vector, and the mouse coding sequences with about 1.2 kb of the 3′ untranslated sequence (Maroteaux et al., 1992) were deleted. The created *Nco*I site then received the 1.4-kb *Nco*I fragment encoding the human receptor decoding sequences. The construct was completed by insertion in the *Sau*I site of the 2-kb cassette containing the phosphoglycerate kinase (PGK)-hygromycin (HYG) minigene used as a positive selection marker in embryonic stem (ES) cells. In summary, the construct contained (from 5′ to 3′ direction) a 2-kb fragment of the m5-HT$_{1B}$ gene promoter, a 1.2-kb fragment encompassing the h5-HT$_{1B}$ gene

coding sequence, about 200 bp representing the SV40 3′ untranslated region including its polyadenylation signal, the 2-kb cassette of the PGK gene promoter and HYG selection marker gene, and finally about 1.5 kb of the 3′ untranslated region of the m5-HT_{1B} receptor gene (Fig. 1). All PCR amplifications were performed with *Pfu*I DNA polymerase, and the entire construct was sequenced before linearization by *Sal*I restriction and introduction into ES cells by electroporation.

The ES cells (line E14, Hooper et al., 1987), grown on mitomycin STO (mouse fibroblast cell line, thioguanine and ouabain resistant) feederlayers, were positively selected in medium containing HYG (100 μg/ml). Colonies were picked, expanded, and genotyped by Southern blotting as previously described (Umans et al., 1995).

The following DNA fragments were isolated from the original mouse genomic BAC clone and used as probes for genotyping the ES cell lines: a 1.2-kb *Kpn*I/*Eco*RI genomic DNA fragment located 5′ of the gene (L probe, Fig. 1) and a 0.6-kb *Eco*RI/*Eco*RV genomic DNA fragment located 3′ of the gene (R probe, Fig. 1). In addition, a 1.6-kb *Bg*III fragment of the PGK HYG cassette was used as an internal probe (Umans et al., 1995).

Homozygous deficient mice were obtained, and the presence of the mutated allele and its Mendelian inheritance were demonstrated by Southern blotting on isolated tail-DNA as described previously (Umans et al., 1995).

Routine genotyping was done by polymerase chain reaction (PCR) on genomic DNA, isolated from mouse tail biopsy samples as described previously (Umans et al., 1995). The PCR mixture contained (total volume of 50 μl) 0.2 μM concentration of each primer, 200 μM concentration of each dNTP, 50 mM KCl, 10 mM Tris·HCl, 1.5 mM $MgCl_2$, and 1.25 U of *Taq* DNA polymerase. Both the wild-type m5-HT_{1B} receptor allele and the targeted allele containing the h5-HT_{1B} receptor gene were amplified with forward primer

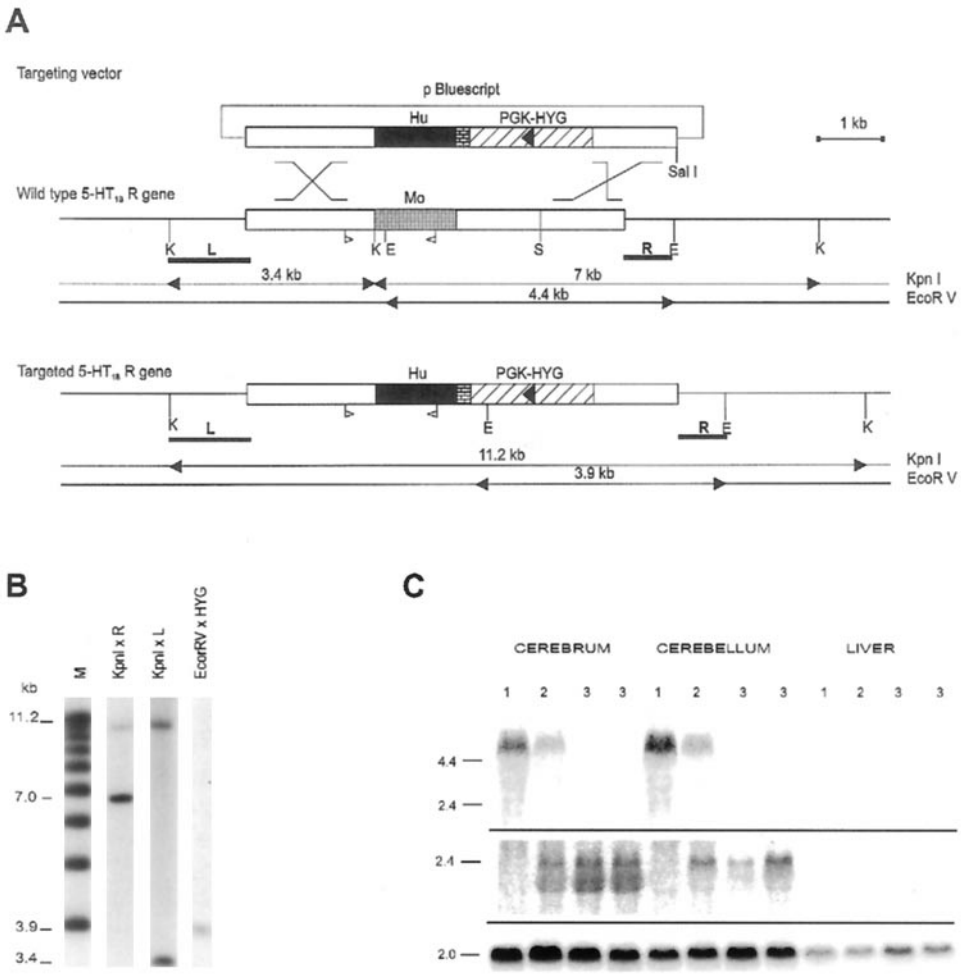

Fig. 1. Transgenic targeting construct, genotyping of ES cells, and analysis of mRNA expression. A, recombinant DNA construct used to target the m5-HT_{1B} receptor gene and replace it with the h5-HT_{1B} receptor gene. The black box represents the coding sequence of the intronless h5-HT_{1B} receptor gene; the hatched box represents the HYG marker gene, which was embedded in the *Sau*I restriction site (S); and the small box with bricks represents the SV40 polyadenylation signal. The unique *Sal*I restriction site used to linearize the construct is also indicated. The wild-type m5-HT_{1B} receptor gene (intronless) containing the coding sequence is represented by the gray box. Relevant restriction sites are shown: *Kpn*I (K), *Eco*RV (E), and *Sau*I (S). R (right) and L (left) represent the position of the genomic DNA probes, located externally to the genomic region that was targeted and used in the construct (white box). The predicted structure of the targeted 5-HT receptor gene contains the elements from the targeting vector (described above) and predicts the loss of the wild-type 3.4- and 7-kb *Kpn*I fragments and of the 4.4-kb *Eco*RV fragment, while generating a 11.2-kb *Kpn*I fragment and a 3.9-kb *Eco*RV fragment (▲, all boundaries). △, position of the primers used for PCR amplification (sequences given in *Experimental Procedures*). B, example of Southern blot of DNA isolated from one of the four selected ES cell lines, digested with *Kpn*I or *Eco*RV as indicated, and hybridized with the different probes (R, L, and HYG). See text and A for details. The leftmost lane (M) represents radiolabeled DNA markers with the approximate size indicated. C, Northern blot of total RNA isolated from the cerebrum, cerebellum, and liver of wild-type mice (lanes 1), heterozygous targeted mice (lanes 2), and homozygous targeted, humanized 5-HT_{1B} mice (lanes 3). The blot was hybridized with the m5-HT_{1B} receptor probe (top), the h5-HT_{1B} receptor probe (middle), and a β-actin cDNA probe (bottom).

5′-CAGCCAGCAAACTCCTTC-3′ (position 79) and reverse primer 5′-GAGACTCGCACTTTGACTTGG-3′ (position 1421) (Maroteaux et al., 1992). This generated a 1342-bp amplicon for the wild-type allele and a 1351-bp amplicon from the targeted allele, which were digested with *Eco*RV, resulting in diagnostic bands of 867 and 475 bp for the wild-type allele and the intact 1351-bp amplicon for the targeted allele (position of primers is indicated in Fig. 1).

RNA Extraction and Northern Blotting. Total RNA was extracted and purified from cerebrum, cerebellum, spleen, and liver of wild-type, heterozygous, and homozygous mice. The isolated organs were immediately frozen in liquid nitrogen and stored at −70°C until RNA extractions were performed with Trizol reagent. Tissue (100 mg) was mechanically homogenized (Virtis) in 1 ml of reagent and centrifuged at 16,000*g* for 15 min at 4°C. Chloroform (0.2 ml) was added to the supernatant, shaken vigorously, and left at ambient temperature for 3 min. After centrifugation at 16,000*g* for 10 min at 4°C, the aqueous phase containing RNA was mixed with 0.5 ml of isopropyl alcohol. After 15 min at ambient temperature, the RNA was pelleted at 16,000*g* for 10 min at 4°C. The RNA pellet was washed in 1 ml of 75% ethanol, resuspended in deionized formamide, stored at −70°C. The concentration of RNA in the samples was measured spectrophotometrically at 260 nm. Then, 10 μg of total RNA was separated and blotted as described previously (Lorent et al., 1994). The h5-HT$_{1B}$ receptor mRNA transcript was detected with a probe that represented the entire 1.3-kb human cDNA. The m5-HT$_{1B}$ receptor probe was a 0.7-kb fragment generated by PCR with forward primer 5′-CATTTACCAGGACTCCATCGC-3′ (position 651 in the mouse cDNA) and reverse primer 5′-GAGACTCGCACTTT-GACTTGG-3′ (position 1421 in the mouse cDNA) (Maroteaux et al., 1992). Equal loading of mRNA was authenticated by hybridization with a 2-kb β-actin cDNA probe (Umans et al., 1995).

Tissue Preparation for ISHH and Radioligand Autoradiography. Transgenic or wild-type mice (20–35 g) were decapitated. Brains were immediately removed from the skull and rapidly frozen in dry-ice-cooled 2-methylbutane (−40°C). Then, 20-μm-thick frontal sections were cut using a Reichert Jung 2800E cryostat-microtome (Cambridge Instruments, Cambridge, UK) and thaw-mounted on adhesive microscope slides (Super Frost). The sections were kept at −70°C until use. The sectioning protocol for the regional distribution study included series of coronal sections covering the entire brain (n = 3–6) with an interspace of 300 μm. Consecutive sections were used for ISHH, receptor autoradiography with [^{3}H]GR125743 and [^{3}H]alniditan. One additional brain was used for horizontal sections; six additional brains were sectioned in the coronal plane at the level of globus pallidus and substantia nigra and used to generate inhibition curves and concentration binding curves (see below) and for autoradiography of agonist-stimulated [^{35}S]GTPγS binding.

Human brains were obtained from the University of Antwerp. Tissue blocks (1 cm thick) were cut and frozen in dry ice-cooled 2-methylbutane (−40°C). Frontal sections (20 μm thick) were cut and thaw-mounted on adhesive microscope slides. The sections were kept at −70°C until use.

Quantitative ISHH. ISHH was performed with [^{35}S]UTP-labeled cRNA probes as described previously (Bonaventure et al., 1998b). A segment of DNA encoding the h5-HT$_{1B}$ receptor (full length; Leysen et al., 1996) was subcloned into pRcCMV. Riboprobes were produced using a SP6 (antisense) or T7 (sense) transcription system in a standard labeling reaction mixture (Bonaventure et al., 1998b). Brain sections were thawed and fixed in paraformaldehyde, acetylated, dehydrated, and delipidated. The fixed sections were hybridized with 1×10^6 cpm [^{35}S]UTP-labeled riboprobe per section. The diluted probe was applied to sections and hybridized at 50°C overnight in a humid chamber. After stringency washes, sections were exposed to Hyperfilm-^{3}H for 4 weeks. ^{14}C-standard strips previously cross-calibrated to ^{35}S were coexposed to allow densitometry. Films were developed manually in Kodak D-19 and fixed with Kodak Readymatic. The following control experiments were performed to determine the specificity of the hybridization signal: ISHH with sense probe and RNase treatment before hybridization with antisense probe.

Receptor Autoradiography. Sections were thawed and dried under a cold air stream and then preincubated three times for 5 min in 50 mM Tris·HCl buffer, pH 7.4, at an ambient temperature by immersing the sections on the microscope slides into a 400-ml jar. Next, they were incubated (drop incubation, 150 μl placed on each section) for 60 min at an ambient temperature in medium containing 4 nM [^{3}H]alniditan or 2 nM [^{3}H]GR125743, 50 mM Tris·HCl buffer, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% BSA, 0.01% ascorbic acid, and 2 μM pargyline (Bonaventure et al., 1998b). To measure [^{3}H]alniditan binding, 100 nM 8-OH-DPAT was added to occlude the 5-HT$_{1A}$ receptors. For concentration binding curves, [^{3}H]GR125743 was used at 0.1, 0.5, 1, 2, 4, 8, and 10 nM. Inhibition of [^{3}H]GR125743 binding by alniditan, 8-OH-DPAT, rauwolscine, CP93129, RU24969, pindolol, propranolol, sumatriptan, and 5-HT (using 15 concentrations per compound, within a range of 10^{-11} to 10^{-4} M) was performed on mouse brain sections at the level of the globus pallidus and substantia nigra and on human substantia nigra sections. Nonspecific binding was measured in the presence of 10 μM 5-HT. After incubation, the excess of radioligand was washed off by immersing the microscope slides in a jar (five times for 1 min) with Tris·HCl buffer, pH 7.4, at 4°C followed by a quick rinse in water and drying under a cold air stream. The sections and standard tritiated plastic microscales were placed in a light-tight cassette and covered with a light-sensitive Hyperfilm-^{3}H. After 6 weeks' exposure, they were developed manually in Kodak D-19 developer for 2 min and fixed with Kodak Readymatic for 3 min.

Quantitative Autoradiography of Agonist-Stimulated [^{35}S]GTPγS Binding. [^{35}S]GTPγS binding was visualized using the method described by Waeber and Moskowitz (1997) with slight modifications. Briefly, the tissue sections, brought to an ambient temperature 15 min before the experiments, were incubated for 30 min at an ambient temperature in 50 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 0.01% BSA, and 0.2 mM dithiothreitol; they were incubated for an additional 15 min in the same fresh buffer supplemented with 2 mM GDP. Agonist-stimulated binding was measured by incubating the sections for 60 min at 30°C in buffer containing 2 mM GDP, 0.04 nM [^{35}S]GTPγS, and 10 μM agonist (alniditan, CP93129, 5-HT, or 5-CT). Basal activity was determined in the absence of agonist. Nonspecific binding was assessed by including 10 μM unlabeled GTPγS in the incubation buffer. Slices were washed twice for 3 min in ice-cold 50 mM HEPES buffer, pH 7.0, dipped briefly in ice-cold distilled water, dried under a cold air stream, and exposed to Hyperfilm-βmax for 72 h. ^{14}C standards strips previously cross-calibrated to ^{35}S standards were coexposed to allow densitometry. Films were developed manually in Kodak D-19 (2 min) and fixed with Kodak Readymatic (3 min).

Data Analysis of ISHH, Receptor Autoradiography, and Agonist-Stimulated [^{35}S]GTPγS Binding. Autoradiograms of ISHH, radioligand, or [^{35}S]GTPγS binding were analyzed and quantified using an MCID-M4 3.0 image analysis system (Imaging Research, St-Catharines, Ontario, Canada). Optical densities in the anatomical regions of interest were transformed into levels of bound radioactivity after calibration of the image analyzer with gray values generated by the coexposed standards. The hybridization signal was expressed as dpm/mg tissue.

Radioligand binding signal in the absence and presence of competitors was expressed in fmol/mg wet weight tissue equivalent. Nonspecific ligand binding was determined in adjacent sections incubated with 10 μM 5-HT.

Ligand concentration binding curves and sigmoidal inhibition curves were generated and fitted by nonlinear regression analysis using Prism software (GraphPAD; San Diego, CA). The B_{max}, K_D, and pIC$_{50}$ ($-\log$ IC$_{50}$) values were derived from the curve calculation. The IC$_{50}$ is the concentration producing 50% inhibition of specific radioligand binding. Agonist-induced [^{35}S]GTPγS was expressed as

percentage of basal binding (% stimulation = 100 × [stimulated − basal]/basal).

In Vitro Electrically Evoked [^{3}H]5-HT Release Experiments. To save mice, the animals used for the in vitro release studies had been used previously in behavioral tests. Before sacrifice, both transgenic and wild-type mice had been drug free for at least 21 days.

Female mice were decapitated, and the brain was immediately removed from the skull and put on ice. Cerebral cortex, hippocampus, and striatum were quickly dissected and stored in ice-cold Krebs' buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 1.7 mM $CaCl_2$, 0.57 mM ascorbate, 1 mM EGTA, and 0.01 mM pargyline saturated with 95% O_2/5% CO_2. The tissue was chopped into 300 × 300-μm slices and washed with Krebs' buffer. After a 15-min preincubation in continuously bubbled Krebs' buffer at 37°C, the tissue from each brain area was incubated for 20 min at 37°C in 2 ml of Krebs' buffer containing 20 nM [^{3}H]5-HT. After three washing steps, the tissue slices were transferred to 200-μl tissue chambers of two superfusion systems (SF2000; Brandell). The tissue was continuously superfused with 95% O_2/5% CO_2 saturated Krebs' buffer containing 10 μM paroxetine at 37°C at a rate of 0.4 ml/min with the direction of the flow against gravity. After 50 min, 18 fractions of 4 min were collected. At 8 and 48 min after the start of fraction collection, the tissue was electrically stimulated (3 Hz, 30 mA, 2-ms pulses; hippocampus and striatum for 2 min, cerebral cortex for 4 min) with a Brandell electrical stimulator (ES220). The first (at 8 min) and second (at 48 min) stimulus trains were named S1 and S2, respectively. Where appropriate, the antagonist was added to the superfusion buffer 25 min before S2, and the agonist was added 21 min before S2. At the end of the experiment, the tissue in each chamber was collected and lysed in 800 μl of 5 mM HCl/50% ethanol. The tritium overflow in each fraction was counted in a liquid scintillation counter (Packard). Fractional release was calculated per collected fraction by dividing the amount of tritium collected in the fraction by the total amount of tritium present in the tissue at the start of collection of the fraction. Electrically induced release by S1 and S2 was calculated by subtracting the basal release from the elevated fractions during the stimulations. Drug effects were calculated by dividing S2 (in the presence of compounds) by S1 (in the presence of buffer alone); thus each chamber served as its own control. Each drug condition was tested in quadruplicate. Mean values of S2/S1 ratios from at least four experiments per drug condition per brain area were compared with the "no drug" control S2/S1 ratio using a Student's t test.

In Vivo Functional Studies: Locomotor Activity and Hypothermia. To reduce the number of animals used, we resorted to a protocol in which the same animals received increasing doses of drugs.

Male mice were 10 weeks old at the time of testing (n = 5 per group). They were housed alone in a standard cage with food and water and kept on a 12/12 h light/dark cycle. The mice were tested between 12:00 noon and 4:00 PM during the light phase. Esophageal temperature was monitored by gently inserting the thermosensitive probe (1.0-mm diameter) of an electronic thermometer (Comark) to a constant depth of 4 cm for a period of 15 s until a stable reading was obtained. Locomotor activity was measured by placing individual animals into a circular open field (29-cm inner diameter) bordered by a transparent screen 30 min after injection. Lines on the floor divided the open field into four equivalent quadrants and the number of crossings was counted over 5 min.

All the mice studied were first given the vehicle (t = 0). Thirty minutes later, the mice were placed in the open field, and locomotor activity was measured over 5 min (t = 30–35 min). At t = 35 min, body temperature was monitored. The mice then received the second injection with either the first dose of RU24969 (n = 5) or vehicle (n = 5), and the same protocol (locomotor activity, t = 65–70 min; body temperature, t = 70 min) was applied. The same procedure was repeated for the following doses.

RU24969 was dissolved in saline and administered s.c. at doses of 0.16, 0.63, 2.5, and 10 mg/kg b.wt. in a volume of 10 ml/kg b.wt.

An additional experiment (n = 6, three males and three females per group) was performed in which a 5-HT_{1B} receptor antagonist (GR127935, 2.5 mg/kg s.c.) or a 5-HT_{1A} receptor antagonist (WAY100635, 2.5 mg/kg s.c.) was administered 30 min before RU24969 (2.5 mg/kg s.c.). Temperature was measured before antagonist administration (t = −30 min), before RU24969 administration (t = 0 min), and 30 min after agonist administration (t = 30 min). GR127935 and WAY100635 were dissolved in saline and administered s.c. at a concentration of 2.5 mg/kg b.wt. in a volume of 10 ml/kg b.wt.

Statistical Analysis. The results are presented as mean ± S.D. Wilcoxon matched pairs signed-ranks test (two-tailed) was used for comparing treated versus vehicle groups. The Mann-Whitney U test (two-tailed) was used for comparison of wild-type with transgenic animals and for comparing antagonist-treated versus vehicle group.

Results

Targeting of m5-HT_{1B} Receptor Gene by h5-HT_{1B} Receptor Gene

In the targeting construct, the mouse coding sequence was replaced by the human under the control of the mouse promoter and a necessary positive selection marker was placed downstream of the gene in a *Sau*I site, in antisense orientation to minimally affect expression of the 5-HT_{1B} gene (Fig. 1A). The replacement vector was linearized at the unique *Sal*I site and electroporated into ES cells. Cells surviving electroporation were grown in selective medium containing HYG, resulting in several thousands of colonies, of which 342 were isolated, expanded, and analyzed by genomic Southern blotting.

The first genotyping of these 342 colonies was by Southern blotting after restriction with *Kpn*I and hybridization with the L probe to reveal fragments of 3.4 kb from the wild-type and 11.2 kb from the targeted allele (Fig. 1, A and B). By this criterion, 18 targeted ES cell lines were retained and expanded. DNA from these 18 lines was then analyzed with all three probes (L, R, and HYG, Fig. 1), yielding four ES cell lines with the expected *Kpn*I restriction patterns. These lines were further authenticated by restriction with *Eco*RV and confirmed to contain a single copy of the PGK-HYG cassette (Fig. 1).

The four correctly targeted ES cell lines, representing an overall recombination frequency of 1.2%, were injected into C57Bl blastocysts and all resulted in coat color chimeric mice that transmitted the targeted gene through the germline. A total of 172 pups with brown coat color were analyzed by Southern blotting of tail-tip DNA, identifying 48 female and 43 male heterozygous mice (total of 53%) that were mated and used to establish homozygous offspring. In total, 154 pups resulting from matings of heterozygous animals were genotyped by Southern blotting of tail-tip DNA after restriction with *Eco*RV. This identified 46 wild-type mice (29.9%), 72 heterozygous mice (46.8%), and 36 homozygous targeted mice (23.3%), clearly establishing a normal Mendelian inheritance pattern.

Expression of 5-HT_{1B} Receptor mRNA

Total RNA isolated from cerebrum, cerebellum, spleen, and liver was analyzed by Northern blotting (Fig. 1C). The h5-HT_{1B} receptor cDNA probe revealed transcripts of about 2

to 2.4 kb in the cerebrum and cerebellum of heterozygous and homozygous targeted mice. The difference in size from those normally expressed in human tissue as described (Jin et al., 1992) results from the use of a different 3′-UTR in our construct, incorporating the SV40 poly(A)$^+$ signal from the pSG5 vector (see *Experimental Procedures*). No equivalent h5-HT$_{1B}$ receptor transcripts were detected in wild-type mice. Subsequent hybridization of the same blots with the m5-HT$_{1B}$ receptor cDNA probe revealed a 6-kb transcript as expected (Maroteaux et al., 1992) in wild-type and in heterozygous mice, whereas no transcript was detected in the homozygous h5-HT$_{1B}$ receptor recombinant mice. No transcripts were detected in the liver of these same animals with either probe (Fig. 1C).

Quantitative ISHH

Control experiments were carried out to determine the specificity of the hybridization. Incubation with sense probe or RNase pretreatment before hybridization with antisense probe did not yield a hybridization signal (result not shown). The [^{35}S]cRNA probe generated by in vitro transcription from full-length cDNA coding for the h5-HT$_{1B}$ receptor has been used for hybridization on transgenic and wild-type mouse brain sections. The high percentage of sequence identity (88%) between the h5-HT$_{1B}$ and m5-HT$_{1B}$ receptor gene allows a direct comparison using the same ^{35}S-labeled riboprobes between h5-HT$_{1B}$ and m5-HT$_{1B}$ receptor mRNA levels of expression in transgenic and wild-type mice, respectively.

The distribution patterns of h5-HT$_{1B}$ and m5-HT$_{1B}$ receptor mRNA in homozygous transgenic and wild-type mice, respectively, were identical (Table 1, Fig. 2). Also, the h5-HT$_{1B}$ and m5-HT$_{1B}$ receptor mRNA densities measured in transgenic and wild-type animals were comparable (Table 1).

The main sites of 5-HT$_{1B}$ receptor mRNA expression in both wild-type and transgenic mice were the caudate-putamen, the CA1 field of the hippocampal formation, and the Purkinje cell layer of the cerebellum (Fig. 2, Table 1). High 5-HT$_{1B}$ receptor mRNA levels could also be detected in the dorsal raphe nucleus (Table 1), nucleus accumbens, and olfactory tubercle (not quantified). Moderate expression was found in the lateral geniculate nucleus of the thalamus (Table 1) and throughout the cortical mantle (not quantified). No significant levels of 5-HT$_{1B}$ receptor mRNA were detected within the globus pallidus and the substantia nigra of transgenic and wild-type animals.

Anatomic Distribution of [^{3}H]GR125743 and [^{3}H]Alniditan Binding Sites in Transgenic and Wild-Type Mouse Brain

The distribution pattern of 5-HT$_{1B}$ binding sites in transgenic and wild-type mouse brain was compared by radioligand binding autoradiography in frontal sections. Two different radioligands were used: the antagonist [^{3}H]GR125743, recognizing m5-HT$_{1B}$ and h5-HT$_{1B}$ receptors, and the agonist [^{3}H]alniditan, recognizing h5-HT$_{1B}$ receptors selectively under the conditions used. Illustrations of receptor labeling in the ventral pallidum of wild-type and transgenic mouse are shown in Fig. 3. A list of quantified [^{3}H]GR125743 (2 nM)

TABLE 1

Distribution and quantification of 5-HT$_{1B}$ receptor mRNA in wild-type and transgenic mice using quantitative in situ hybridization histochemistry

Data are expressed in dpm/mg tissue equivalent (mean ± S.D.; *n* in parentheses) and of [^{3}H]GR125743 (2 nM) and [^{3}H]alniditan (4 nM) binding sites in transgenic or wild-type mouse brain sections detected by autoradiography. Values are expressed in fmol/mg tissue equivalent (mean ± S.D.; *n* in parentheses).

Anatomic Region	In Situ Hybridization		Receptor Autoradiography			
			[^{3}H]GR125743		[^{3}H]Alniditan	
	Wild-type	Transgenic	Wild-type	Transgenic	Wild-type	Transgenic
Basal ganglia						
Caudate-putamen	1476 ± 266 (6)	1553 ± 260 (6)	52 ± 7 (3)	30 ± 8 (3)	0 (3)	24 ± 8 (3)
Nucleus accumbens	N.D.	N.D.	44 ± 21 (3)	30 ± 8 (3)	0 (3)	25 ± 7 (3)
Globus pallidus	0 (3)	0 (3)	186 ± 15 (3)	125 ± 26 (3)	0 (3)	53 ± 14 (3)
Substantia nigra	0 (3)	0 (3)	214 ± 17 (3)	140 ± 17 (3)	0 (3)	70 ± 7 (3)
Hippocampal formation						
CA1 field	796 ± 154 (3)	851 ± 157 (3)	75 ± 17 (3)	46 ± 7 (3)	0 (3)	29 ± 10 (3)
Dorsal subiculum	485 ± 116 (3)	513 ± 125 (3)	87 ± 15 (3)	56 ± 3 (3)	0 (3)	35 ± 8 (3)
Thalamus						
Lateral geniculate nucleus	398 ± 112 (3)	403 ± 117 (3)	44 ± 21 (3)	19 ± 3 (3)	0 (3)	15 ± 3 (3)
Brainstem						
Interpeduncular nucleus	N.D.	N.D.	69 ± 17 (3)	38 ± 14 (3)	0 (3)	30 ± 3 (3)
Superior colliculus	N.D.	N.D.	104 ± 17 (3)	54 ± 21 (3)	0 (3)	45 ± 10 (3)
Central gray	N.D.	N.D.	72 ± 9 (3)	35 ± 10 (3)	0 (3)	25 ± 5 (3)
Dorsal raphe	666 ± 169 (3)	580 ± 166 (3)	0 (3)	0 (3)	0 (3)	0 (3)
Cerebellum						
Purkinje cell layer	1185 ± 199 (3)	1042 ± 121 (3)	0 (3)	0 (3)	0 (3)	0 (3)

0, not detectable; ND, not determined.

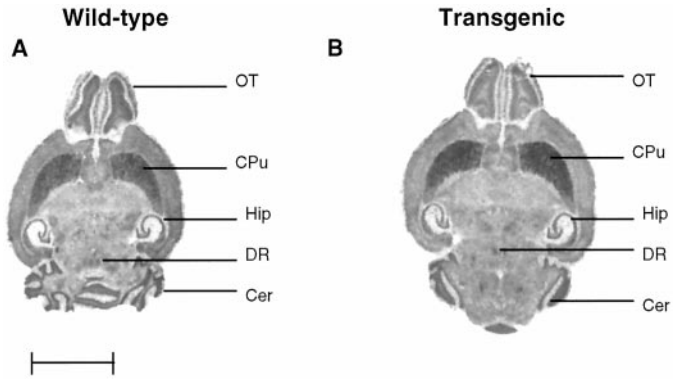

Fig. 2. Comparison of the anatomic distribution of m5-HT_{1B} and h5-HT_{1B} receptor mRNA in autoradiograms of a horizontal section of the wild-type (A) and transgenic (B) mouse brain, respectively. OT, olfactory tubercle; CPu, caudate putamen; Hip, hippocampal formation; DR, dorsal raphe nucleus; Cer, cerebellum. Note that the distribution patterns and densities are completely similar in transgenic and wild-type mouse brain. Scale bar, 5 mm.

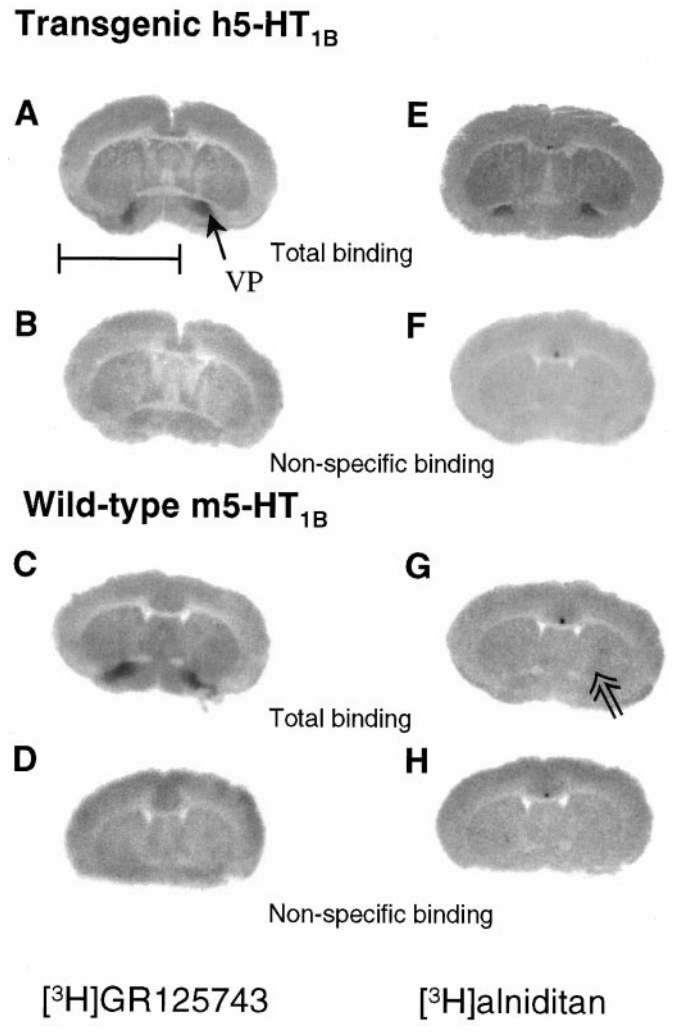

Fig. 3. [^{3}H]GR125743 (2 nM) (A–D) and [^{3}H]alniditan (4 nM) (E–H) binding in ventral pallidum of transgenic (A, B, E, and F) and wild-type mice (C, D, G, and H) visualized by receptor autoradiography (frontal sections). B, D, F, and H, nonspecific binding in the presence of 10 μM 5-HT. VP, ventral pallidum. Note the absence of [^{3}H]alniditan labeling in wild-type mouse ventral pallidum (G, open arrow). Scale bar, 5 mm.

and [^{3}H]alniditan (4 nM) binding sites in wild-type and transgenic mouse brain areas is presented in Table 1.

The pattern of [^{3}H]GR125743 binding sites throughout the transgenic and wild-type mouse brain was completely similar (Table 1, Fig. 3). Consistently, higher [^{3}H]GR125743 binding densities were measured in wild-type mouse brain, which is confirmed by the ligand concentration binding curves (see below). In both wild-type and transgenic animals, the highest densities were found in the two output structures of the basal ganglia (substantia nigra and globus pallidus). Medium-dense labeling was observed in the superficial layer of the superior colliculus, dorsal subiculum, CA1 field of the hippocampal formation, and central gray. Weak binding was found in the caudate-putamen, nucleus accumbens, interpeduncular nucleus, and lateral geniculate nucleus of the thalamus.

With [^{3}H]alniditan, the same distribution pattern as obtained with [^{3}H]GR125743 was found in transgenic mouse brain, but no specific labeling was detected in wild-type mice (Table 1, Fig. 3). The labeling intensity of [^{3}H]alniditan in transgenic mice was lower than the intensity measured with [^{3}H]GR125743 (Table 1).

Ligand Concentration Binding Curves

To compare the receptor density of the h5-HT_{1B} receptor expressed in transgenic mice and the m5-HT_{1B} receptor in wild-type mice, concentration binding curves of [^{3}H]GR125743 were performed on brain sections at two levels: the substantia nigra and the globus pallidus.

Transgenic and wild-type mice of the same age (10 weeks) were used in this study.

Derived B_{max} and K_D values are listed in Table 2. The nonspecific binding determined in the presence of 10 μM 5-HT was linear. The affinity of [^{3}H]GR125743 was comparable in wild-type and transgenic animals based on the overlap between the 95% confidence interval of K_D values (Table 2). The B_{max} values were higher in the substantia nigra than in the globus pallidus (Table 2). In both regions, higher B_{max} values were found in wild-type mice. The level of [^{3}H]GR125743 binding sites measured in transgenic mice reached approximately 70% of [^{3}H]GR125743 binding sites in wild-type mice.

Inhibition of [^{3}H]GR125743 Binding in Transgenic Mice, Wild-Type Mice, and Human Brain Sections

Further pharmacological characterization was performed by measuring the potency of several compounds (alniditan, rauwolscine, 8-OH-DPAT, CP93129, RU24969, pindolol, pro-

TABLE 2

Binding parameters derived from ligand concentration binding curves [^{3}H]GR125743 in substantia nigra and globus pallidus of wild-type and transgenic mice

Parameters were obtained by computerized curve-fitting (GraphPad Prism software) to the quantified labeling intensity in the autoradiograms. Mean K_D (nM, 95% confidence interval in parentheses) and B_{max} (fmol/mg tissue equivalent, 95% confidence interval) values are presented (n = 6).

Anatomical Region	Wild-Type		Transgenic	
	K_D	B_{max}	K_D	B_{max}
Substantia nigra	0.39 (0.19–0.59)	239 (215–263)	0.29 (0.08–0.50)	164 (142–185)
Globus pallidus	0.43 (0.15–0.70)	205 (179–232)	0.42 (0.13–0.72)	138 (118–158)

pranolol, sumatriptan, and 5-HT) to inhibit specific [^{3}H]GR125743 binding in wild-type and transgenic mouse and in human brain sections using quantitative autoradiography.

Inhibition curves were generated from measurements in substantia nigra and globus pallidus. The pIC$_{50}$ values are listed in Table 3.

Alniditan, rauwolscine, and 8-OH-DPAT displayed a higher affinity for the h5-HT$_{1B}$ receptor than for the m5-HT$_{1B}$ receptor (Table 3). CP93129, RU24969, pindolol, and propranolol showed a higher affinity for the mouse receptor (Table 3). The absence of inhibition of [^{3}H]GR125743 binding in the transgenic mouse substantia nigra by CP93129 is illustrated in Fig. 4. The affinities of sumatriptan and 5-HT were similar for the transgenic and wild-type mouse receptors (Table 3). The pIC$_{50}$ values derived from the inhibition curves in transgenic mouse and human substantia nigra were very similar; a highly significant correlation between pIC$_{50}$ values in both tissues was obtained (Table 3; Fig. 5A; r Pearson = .98; $p < .0001$). In contrast, no significant correlation was found between the binding affinities of the compounds for the m5-HT$_{1B}$ receptor in wild-type animals and the h5-HT$_{1B}$ receptor expressed in transgenic animals ($r = .18$, $p > .05$, Fig. 5B) or the h5-HT$_{1B}$ receptor in human substantia nigra ($r = .05$, $p > .05$, Fig. 5C).

Quantitative Autoradiography of Agonist (5-HT, 5-CT, CP93129, and Alniditan)-Stimulated [^{35}S]GTPγS Binding in Transgenic and Wild-Type Mouse Brain Sections

The functional coupling of the h5-HT$_{1B}$ receptor expressed in transgenic animals to G protein activation was investigated and compared with that of the mouse receptor by using quantitative autoradiography of agonist-stimulated [^{35}S]GTPγS binding. As previously reported (Waeber and Moskowitz, 1997), optimal agonist stimulation was observed in the presence of 10 μM agonist and 2 mM GDP at 30°C. Basal activity was determined in the absence of agonist and nonspecific binding in the presence of 10 μM GTPγS. The specificity of the agonist-induced signal was demonstrated by inhibiting the labeling with a 5-HT$_{1B}$ receptor antagonist (GR127935, results not shown). Percentages of agonist-induced stimulation of [^{35}S]GTPγS binding are reported in Table 4; autoradiograms are shown in Fig. 6. Both 5-HT and 5-CT stimulated [^{35}S]GTPγS binding in the substantia nigra and globus pallidus of wild-type and transgenic animals, with the highest stimulation in substantia nigra. The percentage of stimulation measured in the presence of 5-CT was higher than that in the presence of 5-HT (Table 4) in both wild-type and transgenic mice. For both agonists (i.e., 5-HT and 5-CT), the percentage of stimulation observed in wild-type mice was higher than that in transgenic animals. The selective rodent 5-HT$_{1B}$ receptor agonist CP93129 was effective in wild-type but not in transgenic mice (Table 4, Fig. 6, E and F). Conversely, alniditan (a selective h5-HT$_{1B}$ agonist) stimulated [^{35}S]GTPγS binding in transgenic but not in wild-type animals (Table 4, Fig. 6, G and H).

Modulation of [^{3}H]5-HT Release: Effects on Basal and Electrically Evoked Release of Mouse Strain and Pharmacological Modulation

Basal and electrically induced overflow of [^{3}H]5-HT from cerebral cortex, hippocampal, and striatal slices were studied in both wild-type and transgenic mice to evaluate the functional activity of the 5-HT$_{1B}$ receptor in the two strains. The effect on 5-HT$_{1B}$ receptors by exogenously applied agonist or antagonist was measured on basal and electrically stimulated ^{3}H overflow. An appropriate agonist was used for the strains: 1 μM RU24969 for the wild-type mice and 1 μM alniditan for the transgenic mice. The applied concentrations were 30-fold above their pIC$_{50}$ values to inhibit [^{3}H]GR125743 binding to 5-HT$_{1B}$ receptors in brain sections of wild-type and transgenic mice.

Electrically stimulated ^{3}H release in the absence of added drugs was lower in the hippocampus of wild-type than in that of transgenic mice (Table 5, $p < .0001$, Student's t test); in the other brain areas, no difference was seen between the two strains. Also, the evoked release compared with basal was significantly bigger in transgenic than in wild-type mouse hippocampus (Table 5, $p < .0001$, Student's t test). In none of the investigated brain areas of either mouse strain was a significant difference in the control S2/S1 ratios observed, measured in the absence of added drugs.

Neither agonist had a statistically significant effect on basal (fraction 12/fraction 2 ratio) or induced release (S2/S1 ratio) (Fig. 7). The use of alniditan (1 μM) also failed to affect [^{3}H]5-HT overflow in the three brain areas studied of the wild-type mice (data not shown). The m5-HT$_{1B}$ and h5-HT$_{1B}$ receptor antagonist SB224289, however, was able to significantly increase the S2/S1 ratio in wild-type cerebral cortex and hippocampus but not in striatum. In cerebral cortex of wild-type mice, SB224289 had a tendency to increase basal release (fraction 12/fraction 2 = 113 ± 2.1% in control and 121 ± 4.2% in the presence of SB224289), but this effect on basal release did not reach statistical significance. In the transgenic mice, SB224289

TABLE 3

Potency of compounds (pIC$_{50}$ values; 95% confidence interval in parentheses) for inhibition of [^{3}H]GR125743 (2 nM) binding in wild-type or transgenic mouse or in human brain sections, measured using quantitative receptor autoradiography (n = 3)

	Wild-Type		Transgenic		Human Substantia Nigra
	Globus pallidus	Substantia nigra	Globus pallidus	Substantia nigra	
Alniditan	5.74 (5.43–6.05)	5.55 (5.28–5.82)	8.32 (8.00–8.63)	8.22 (8.09–8.36)	8.52 (8.35–8.70)
Rauwolscine	<5	<5	6.59 (6.40–6.77)	6.28 (5.99–6.57)	6.83 (6.35–7.32)
8-OH-DPAT	<5	<5	6.08 (5.94–6.22)	6.08 (5.94–6.22)	6.18 (5.97–6.40)
CP93129	7.52 (7.36–7.68)	7.61 (7.38–7.85)	5.27 (5.02–5.52)	<5	5.05 (4.81–5.29)
RU24969	8.15 (7.95–8.36)	8.22 (8.13–8.31)	7.29 (7.11–7.47)	7.33 (7.15–7.51)	7.06 (6.76–7.36)
Pindolol	6.67 (6.42–6.92)	N.D.	4.90 (4.72–5.09)	N.D.	4.86 (4.46–5.25)
Propranolol	6.26 (5.93–6.59)	6.11 (5.82–6.41)	4.56 (4.29–4.82)	4.58 (4.44–4.73)	4.94 (4.92–4.97)
Sumatriptan	6.98 (6.66–7.30)	6.98 (6.78–7.19)	6.82 (6.36–7.28)	N.D.	6.74 (6.47–7.02)
5-HT	8.44 (8.20–8.69)	8.47 (8.22–8.72)	8.28 (8.18–8.38)	8.40 (8.33–8.46)	8.18 (8.03–8.34)

N.D., not determined.

did not affect basal or induced [^{3}H]5-HT overflow in any of the brain areas investigated (Fig. 7).

In Vivo Functional Studies: Locomotor Activity and Hypothermia

The in vivo effect of a 5-HT$_{1B}$ agonist was investigated in both the transgenic and the wild-type mice on two parameters, locomotor activity and hypothermia, which are reported to be modulated by 5-HT$_{1B}$ receptor activation (Ramboz et al., 1996; Hagan et al., 1997). The 5-HT$_{1B}$ agonist RU24969 was used because of its ability to penetrate the blood-brain barrier.

Locomotor Activity. In animals administered a single injection of vehicle, locomotor activity after 30 min was comparable in wild-type and transgenic mice ($p > .05$, two-tailed Mann-Whitney U test; Fig. 8). No statistically significant difference was observed in habituation (seen as a decrease in locomotor activity on repeated testing with vehicle injections) between transgenic and wild-type mice (Fig. 8, $p > .05$, two-tailed Mann-Whitney U test). RU24969 caused a dose-related increase in locomotor activity in wild-type mice that reached significance from 0.63 mg/kg on ($p < .05$, two-tailed Wilcoxon PPSR test; Fig. 8A) and consisted of distinctive thigmotactic circling. No increase in locomotor activity or thigmotactic circling was observed in the transgenic mice (Fig. 8B).

Hypothermia. RU24969 caused a dose-related drop in wild-type mouse body temperature that reached significance from 0.63 mg/kg on (Fig. 9A). A less pronounced drop was observed in transgenic mouse body temperature, which reached significance from 2.5 mg/kg on (Fig. 9B). Body temperature was not significantly influenced by the vehicle, as shown in Fig. 9. After a single injection of RU 24969 at 2.5 mg/kg, a significant drop in body temperature was observed in wild-type but not in transgenic mice (Fig. 10). The selec-

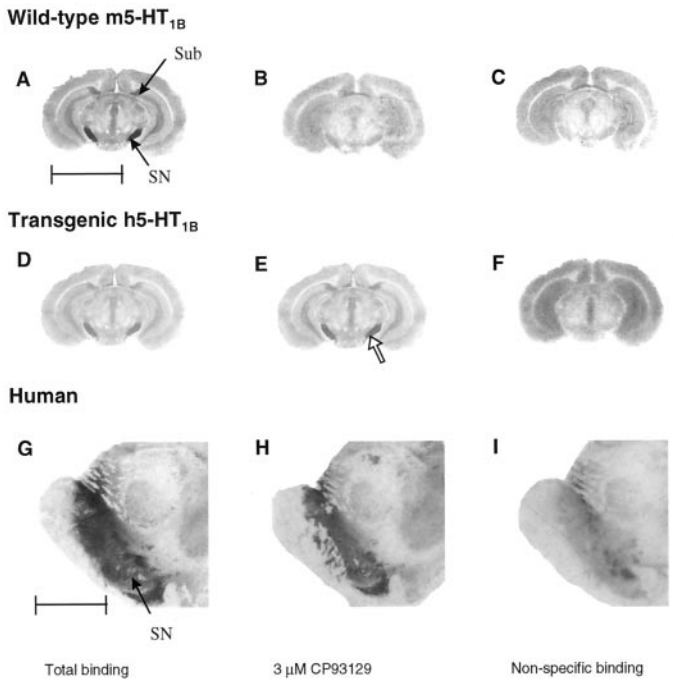

Fig. 4. Inhibition of [^{3}H]GR125743 binding by CP93129 (3 μM) in wild-type (A–C) or transgenic (D–F) mice and in human substantia nigra (G–I) visualized by receptor autoradiography (frontal sections). A, D, and G, total binding. B, E, and H, binding in the presence of 3 μM CP93129. C, F, and I, nonspecific binding in the presence of 10 μM 5-HT. SN, substantia nigra; Sub, subiculum. Note the absence of inhibition of [^{3}H]GR125743 in the transgenic mouse substantia nigra (B, open arrow) and in human brain. Scale bar, 5 mm.

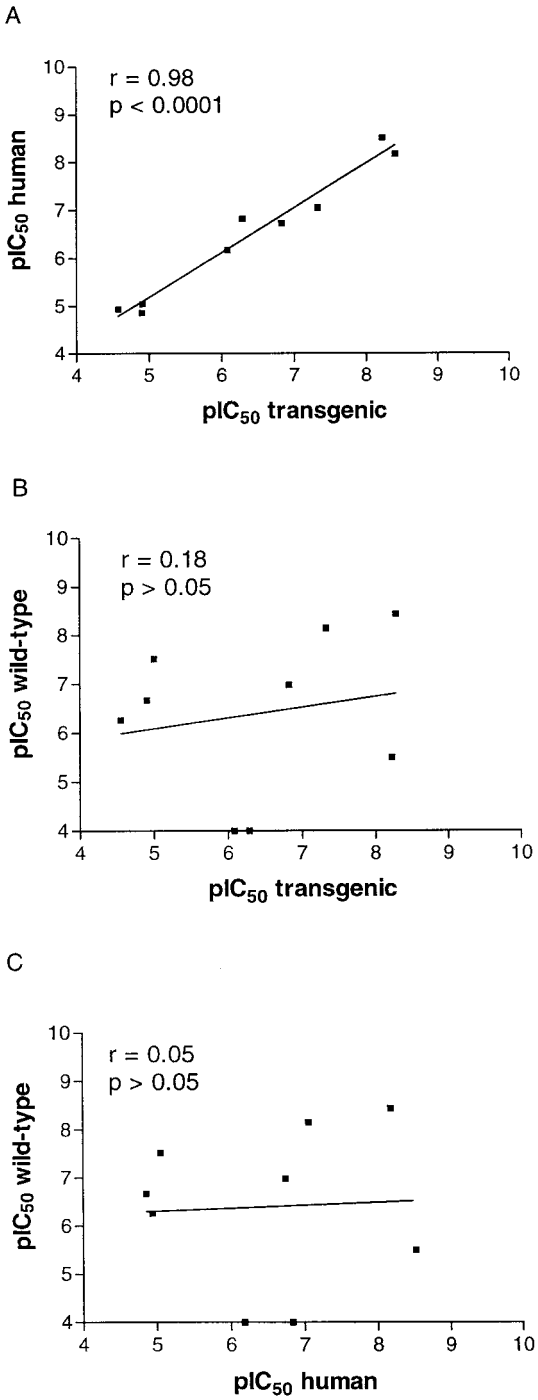

Fig. 5. Correlation between pIC$_{50}$ values of compounds (see Table 3) for (A) the 5-HT$_{1B}$ receptor in human substantia nigra and the 5-HT$_{1B}$ receptor in transgenic mouse substantia nigra, (B) the 5-HT$_{1B}$ receptor in wild-type mouse substantia nigra and the 5-HT$_{1B}$ receptor expressed in transgenic mouse substantia nigra, and (C) the 5-HT$_{1B}$ receptor from wild-type mouse substantia nigra and the 5-HT$_{1B}$ receptor from human substantia nigra. The correlation coefficient (r Pearson) is listed in each panel. Note the high correlation coefficient between the pIC$_{50}$ values for the h5-HT$_{1B}$ receptor expressed in transgenic mouse and the human substantia nigra.

tive 5-HT$_{1A}$ antagonist WAY100635 (2.5 mg/kg) but not the selective 5-HT$_{1B}$ receptor antagonist GR127935 (2.5 mg/kg) significantly blocked the hypothermic effect of RU24969 induced in wild-type mice ($p < .05$ and $p > .05$, respectively, two-tailed Mann-Whitney U test) (Fig. 10). GR127935 and WAY100635 per se did not affect body temperature in comparison with vehicle ($p > .05$, two-tailed Mann-Whitney U test).

TABLE 4

Quantitative autoradiography of agonist-stimulated [^{35}S]GTPγS binding in transgenic and wild-type mouse brain

Agonist-stimulated [^{35}S]GTPγS binding was expressed as a percentage of basal activity (n = 3; mean ± S.D.).

	Wild-Type		Transgenic	
	Globus pallidus	Substantia nigra	Globus pallidus	Substantia nigra
5-HT	84 ± 55	195 ± 64	58 ± 28	114 ± 15
5-CT	135 ± 46	203 ± 92	62 ± 5	175 ± 41
CP93129	94 ± 29	155 ± 35	5 ± 8	5 ± 7
Alniditan	0 ± 2	4 ± 3	28 ± 5	97 ± 31

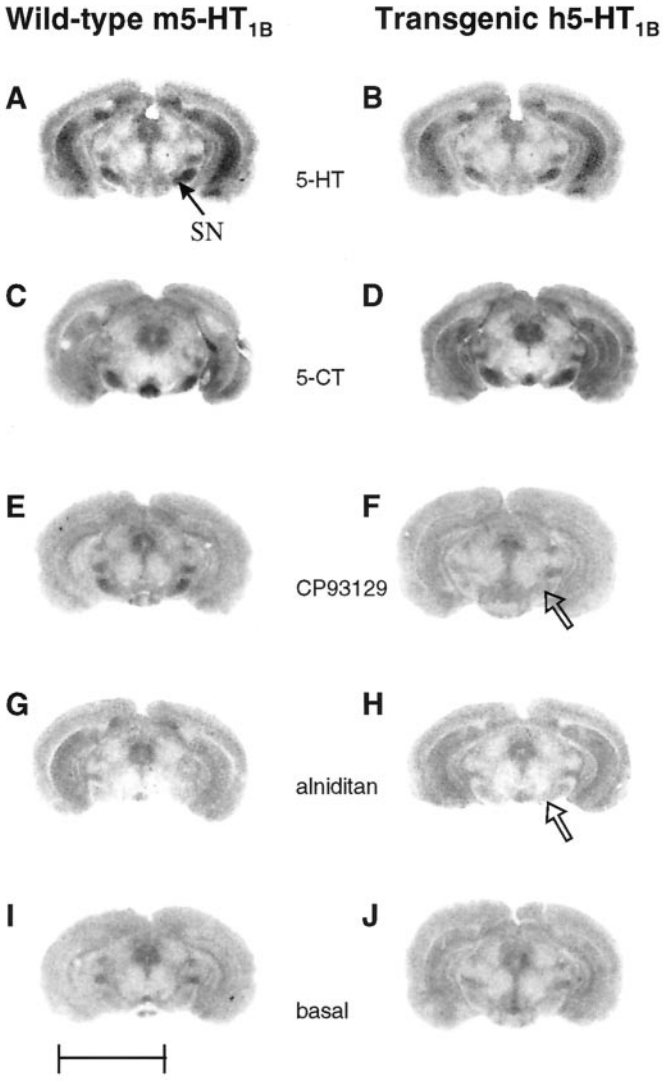

Fig. 6. Effect of agonists on [^{35}S]GTPγS binding in wild-type (A, C, E, G, and I) or transgenic (B, D, F, H, and J) mouse substantia nigra visualized by autoradiography (frontal sections). A and B, stimulated by 10 μM 5-HT. C and D, stimulated by 10 μM 5-CT. E and F, stimulated by 10 μM CP93129. G and H, stimulated by 10 μM alniditan. I and J, basal activity. SN, substantia nigra. Note the absence of stimulation of CP93129 in transgenic mouse substantia nigra and the stimulation of [^{35}S]GTPγS binding by alniditan in transgenic mouse substantia nigra (open arrow, F and H). Scale bar, 5 mm.

Discussion

An animal model to study the h5-HT$_{1B}$ receptor in vivo was generated by a "knock-in" method; by humanizing the mouse locus, mice were generated that express the h5-HT$_{1B}$ receptor instead of its murine counterpart from the same genetic locus. So far, functional studies of 5-HT$_{1B}$ receptors of relevance to human application have been hampered by the differences in the pharmacology between human and rodent receptors.

The "humanized" mice described in the present study developed and lived apparently normally.

Localization and Densities of h5-HT$_{1B}$ Receptor mRNA and Binding Sites Expressed in Transgenic Mouse Brain. The anatomic patterns and relative densities of h5-HT$_{1B}$ and m5-HT$_{1B}$ receptor mRNA throughout the brain of transgenic and wild-type mouse were completely similar; therefore, there is no indication that the spatial (and temporal) regulation of transcription from the 5-HT$_{1B}$ gene is altered in transgenic animals (Table 1, Fig. 2, A and B). The anatomic patterns observed in this study also parallel a previous ISHH study in wild-type mouse brain (Boschert et al., 1994).

The radioligand [^{3}H]GR125743 has been shown to be suitable for the labeling of human (Domenech et al., 1997), monkey, guinea pig, and rodent 5-HT$_{1B}$ receptors (Mengod et al., 1996; Audinot et al., 1997). It recognizes both high- and low-affinity states of the 5-HT$_{1B}$ receptors. [^{3}H]Alniditan labels human, calf, and guinea pig 5-HT$_{1B}$ receptors (Leysen et al., 1996). Alniditan is an agonist at the h5-HT$_{1B}$ receptor and recognizes preferentially the high-affinity state of the h5-HT$_{1B}$ receptor (Leysen et al., 1996). It is important to note that the two radioligands used in the present receptor autoradiography studies (i.e., [^{3}H]GR125743 and [^{3}H]alniditan) also bind to the 5-HT$_{1D}$ receptor. Because for all ligands tested so far, no species differences between mouse and human 5-HT$_{1D}$ receptors have been reported, it is safe to state that 5-HT$_{1D}$ receptors do not interfere with the conclusions of the present mapping study. Furthermore, 5-HT$_{1D}$ binding sites account for a low percentage of the total of 5-HT$_{1B}$ plus 5-HT$_{1D}$ binding sites (Boschert et al., 1994; Bonaventure et al., 1998b).

The distribution of [^{3}H]alniditan binding sites in transgenic mouse brain demonstrates the presence of h5-HT$_{1B}$ receptors because [^{3}H]alniditan does not bind to the m5-HT$_{1B}$ receptor at nanomolar concentration (Table 1; Fig. 3) (Leysen et al., 1996). The distribution pattern of [^{3}H]GR125743 and [^{3}H]alniditan binding sites observed in the present study (Table 1) parallels those of previous localization studies performed in wild-type mouse brain (Boschert et al., 1994; Saudou et al., 1994) and is similar to the distribution found in other species (Bruinvels et al., 1993; Bonaventure et al., 1998b).

The B_{max} values derived from the concentration binding studies of [^{3}H]GR125743 show a difference in expression levels between the h5-HT$_{1B}$ receptor in transgenic mice and

the m5-HT_{1B} receptor expressed in wild-type mice (approximately 30% lower in transgenic mice; see Table 2).

Pharmacological Characterization of h5-HT_{1B} Receptors Expressed in Transgenic Mouse Brain. The pIC_{50} values derived from the radioligand binding inhibition study with various compounds in transgenic mouse and human substantia nigra brain sections were identical (Table 3). Inhibition of [^{3}H]GR125743 binding by nine compounds was investigated. The pIC_{50} values that were determined in this study using autoradiography on brain sections of transgenic mice, human substantia nigra, and wild-type mice are in agreement with binding affinities determined on membrane preparations of cloned human and rodent 5-HT_{1B} receptor expressed in cells, respectively (Hamblin et al., 1992; Bach et al., 1993; Leysen et al., 1996; Zgombick et al., 1997). The present pharmacological characterization demonstrates that after being expressed in the mouse brain, the human receptor shows the same pharmacological profile as the 5-HT_{1B} receptor in human brain, which is indeed different from the pharmacological profile of the 5-HT_{1B} receptor in mouse brain. The present study is also the first autoradiographic characterization of [^{3}H]GR125743 binding sites in human tissue.

Functional Coupling of h5-HT_{1B} Receptors Expressed in Transgenic Mouse Brain to G Proteins. The observed stimulation of [^{35}S]GTPγS binding by 5-CT, 5-HT, and alniditan in transgenic mice shows that the coupling between the h5-HT_{1B} receptor and mouse G protein is possible. The higher percentage of stimulation measured with 5-HT and 5-CT in wild-type animals is probably a consequence of the higher receptor densities observed in wild-type mice; however, it could also reflect a less efficient coupling between a human receptor and a mouse G protein. The higher percentages of stimulation observed in substantia nigra compared with globus pallidus are probably the consequence of a higher receptor density in the former brain area.

Absence of Modulation of Electrically Evoked [^{3}H]5-HT Release by SB224289 in Slices of Transgenic Mice. The function of the 5-HT_{1B} receptor was measured in vitro by studying its effects on [^{3}H]5-HT release from tissue slices of cerebral cortex, hippocampus, and striatum. The ability of the selective 5-HT_{1B} receptor antagonist SB224289 to potentiate [^{3}H]5-HT release from electrically stimulated guinea pig cerebral cortical slices has recently been shown (Selkirk et al., 1998; M.H.M.B., I. Lenaerts and J.E.L., unpublished observations). Here, we demonstrate that SB224289 was also able to enhance electrically stimulated [^{3}H]5-HT release from cerebral cortex and hippocampus in the wild-type mouse (Fig. 7). It did so in the absence of exogenously added agonists, indicating that endogenous 5-HT already exerts an inhibiting influence on the stimulated release. Such a tonic inhibition by endogenous 5-HT may explain the observation that agonists were not able to further inhibit the electrically induced [^{3}H]5-HT release in the wild-type mice. In the transgenic mice, basal and electrically stimulated [^{3}H]5-HT release could not be modulated; neither the 5-HT_{1B} agonist (alniditan) nor the 5-HT_{1B} antagonist (SB224289) had an effect on the release from the cerebral cortex

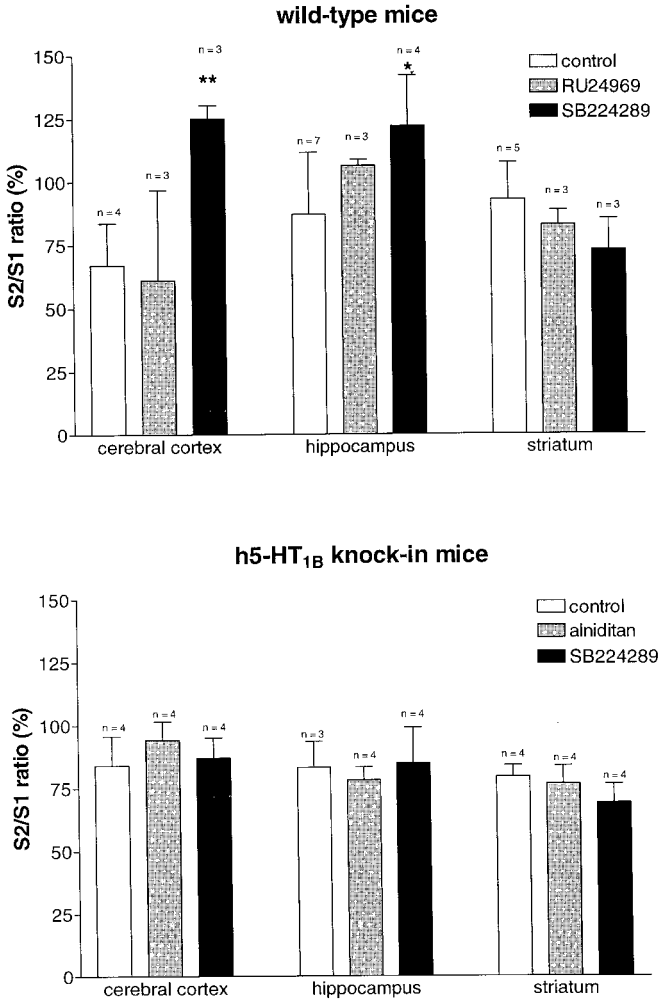

Fig. 7. Drug effects on electrically evoked [^{3}H]5-HT release from cerebral cortex, hippocampus, and striatum of wild-type and transgenic mice. Superfusion with 1 μM concentration of the drugs started 25 min (SB224289) and 21 min (RU24969) before S2; controls were simultaneously superfused with Krebs' buffer only. All values are mean ± S.D. of four to seven experiments. The difference between drug treated and controls was statistically significant: *$p < .05$, **$p < .01$, Student's t test.

TABLE 5

Strain effects on basal and non-drug-modulated in vitro [^{3}H]5-HT release from wild-type and transgenic mouse brain regions

Values are means ± S.D. of individual chambers (n in parentheses).

	Cerebral Cortex		Hippocampus		Striatum	
	Wild-type	Transgenic	Wild-type	Transgenic	Wild-type	Transgenic
Basal release (Fractional release / min)	0.72 ± 0.11 (4)	0.84 ± 0.10 (4)	0.87 ± 0.11 (7)	0.89 ± 0.08 (4)	0.76 ± 0.04 (5)	0.84 ± 0.11 (4)
S1 (Induced ^{3}H outflow)	7.42 ± 0.96 (4)	7.87 ± 0.28 (4)	3.15 ± 0.32 (7)	5.24 ± 0.24* (4)	7.82 ± 1.19 (5)	8.45 ± 0.73 (4)
S1/basal release	10.83 ± 2.66 (4)	9.86 ± 1.85 (4)	3.76 ± 0.40 (7)	6.05 ± 0.84* (4)	10.95 ± 1.61 (5)	10.86 ± 0.69 (4)

* Statistically significant difference between wild-type and transgenics $p < .001$.

or hippocampus slices, which is in contrast to the observations in brain slices of wild-type mice.

The striatum contains a high number of 5-HT$_{1B}$ receptors, as also shown in the present study. However, a modulation of [^{3}H]5-HT release by 5-HT$_{1B}$ receptors has never been demonstrated in this brain area. We took it along as a control area, and we indeed did not see any effects of 5-HT$_{1B}$ receptor stimulation or blockade on evoked [^{3}H]5-HT release.

A difference in regulatory mechanisms of [^{3}H]5-HT release between wild-type and transgenic mice was also observed in comparing the responses to electrical stimulation in the absence of added drugs. In the hippocampus, a 61% higher response to the first stimulation (S1) was observed in the transgenic compared with wild-type mice (Table 5). This may be due to an inhibitory control present in the wild-type hippocampus, which is either reduced or absent in the transgenic mice; however, in the cerebral cortex, this difference between wild-type and transgenic mice was not observed. The reason for this is not clear.

Together, these observations suggest that the h5-HT$_{1B}$ receptor expressed in mouse brain is not functional in vitro as a 5-HT release-regulating autoreceptor.

In Vivo Functional Responses to RU24969 in Transgenic Mice. The 5-HT$_{1B}$ agonist RU24969 did not induce hyperlocomotion in the transgenic mice. It is noteworthy that

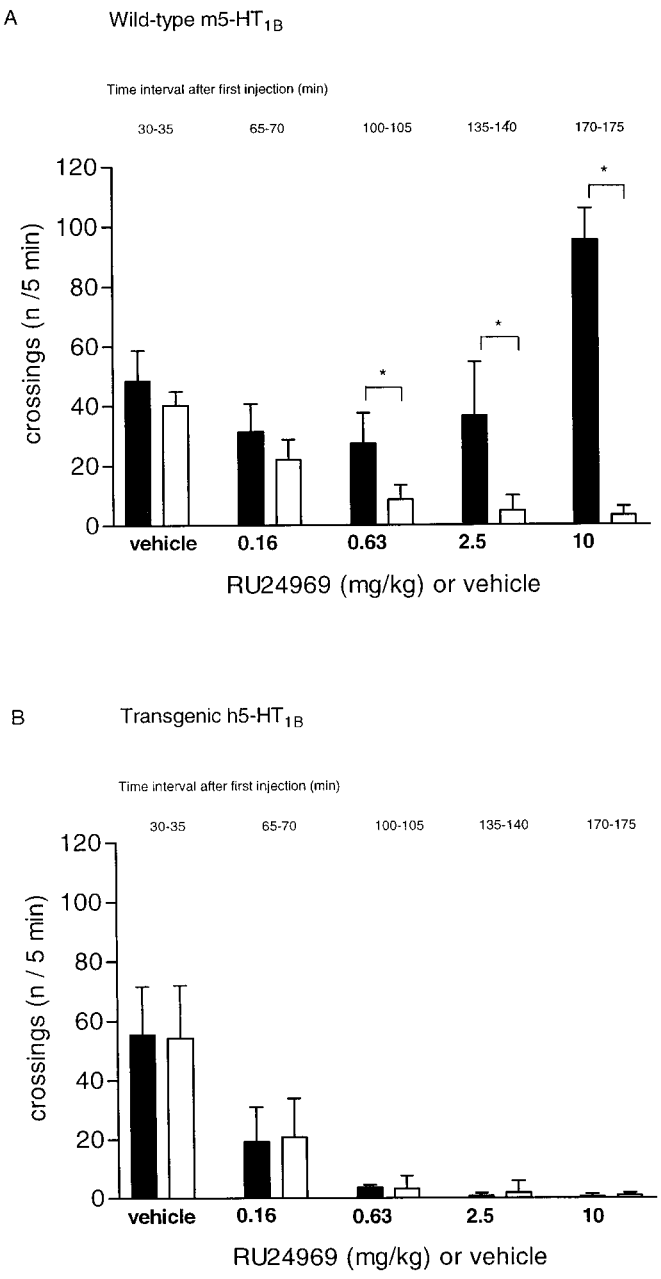

Fig. 8. Locomotor activity of wild-type (A) and transgenic (B) mice after s.c. injection of increasing doses of RU24969 (solid columns) or vehicle (open columns). The bars represent 5-min cumulative locomotor activity counts (crossings, mean ± S.D.; $n = 5$). Note the dose-related increase in locomotor activity in wild-type mice that reached significance at 0.63 mg/kg and the absence of effect in transgenic mice. *$p < .05$, two-tailed Wilcoxon MPSR test.

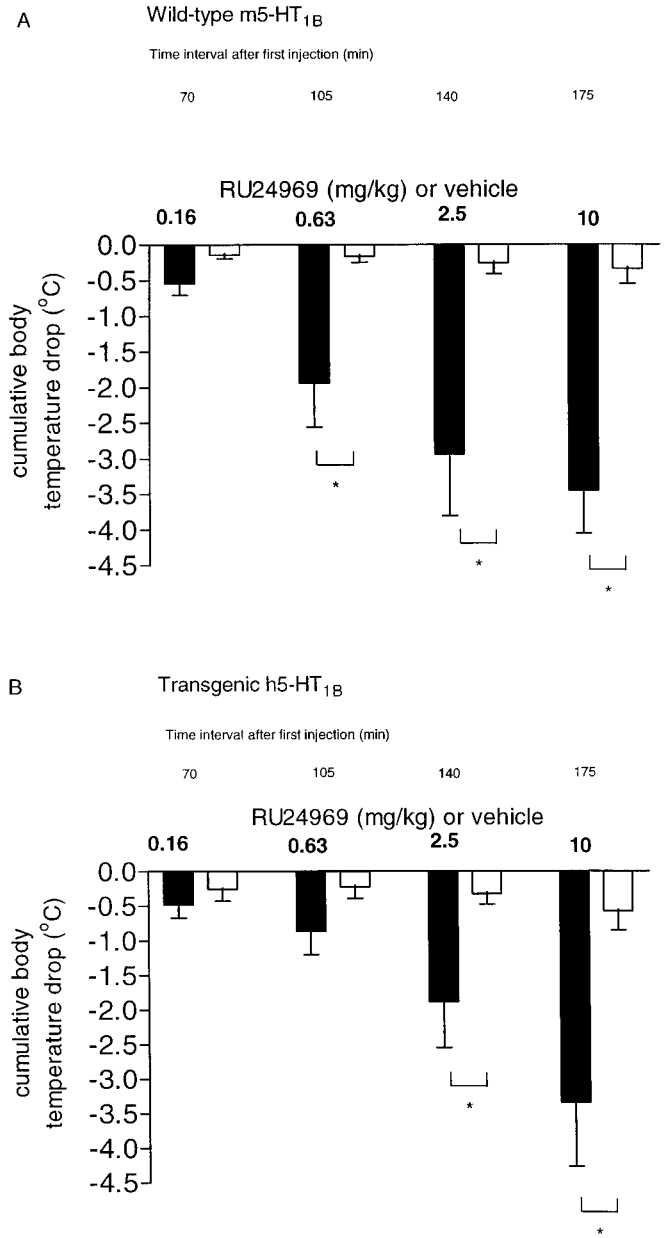

Fig. 9. Drop in esophageal temperature of wild-type (A) and transgenic (B) mice after s.c. injection of increasing doses of RU24969 (solid columns) or vehicle (open columns) (mean ± S.D.; $n = 5$). A, note that the drop in body temperature of wild-type mice occurs at lower dose than in transgenic mice (B). *$p < .05$, two-tailed Wilcoxon MPSR test.

RU24969 displays a 10 times lower affinity for h5-HT_{1B} receptor (Table 3; pIC_{50} = 7.29) than for m5-HT_{1B} receptor (Table 3; pIC_{50} = 8.15). We attempted to overcome this problem by increasing the doses of RU24969. Even at higher doses of RU24969 (20 and 40 mg/kg), no increase in locomotor activity was observed in transgenic animals, but at 40 mg/kg, the hyperlocomotion also was not present in wild-type mice, probably due to nonspecific sedative effects of the drug at a high dosage (unpublished observation). To the best of our knowledge, the mechanism of action by which RU24969 increases locomotor activity is far from clear (Tricklebank et al., 1986). Moreover, RU24969 is the only 5-HT_{1B} receptor agonist that was reported to increase locomotor activity; experiments with various other compounds failed.

RU24969 caused hypothermia in both wild-type and transgenic mice, but the drug was less potent in the latter. However, the maximal effect at 10 mg/kg was comparable in both strains (Fig. 9). In our hands, even at high doses (20 mg/kg s.c.), the 5-HT_{1B} agonists zolmatriptan and naratriptan, which are known to penetrate into the brain (Goadsby and Knight, 1997), did not induce hypothermia in either wild-type and transgenic mice (unpublished observations).

An important observation of the present study is that the selective 5-HT_{1A} receptor antagonist WAY100635, and not the selective 5-HT_{1B} receptor antagonist GR127935, blocked the hypothermia induced by RU24969 in wild-type mice (Fig. 10). Based on this observation, it can be concluded that hypothermia is mediated mainly by the 5-HT_{1A} receptors. However, the reduced potency of RU24969 on body temperature observed in transgenic mice could indicate that hypothermia induced by RU24969 may result from an interaction between 5-HT_{1A} and 5-HT_{1B} receptors.

Conclusions. The present in vitro and in vivo characterization studies demonstrate a complete replacement of the mouse receptor by its human receptor homolog and a functional coupling to G proteins; however, we could not reliably demonstrate downstream functional effects.

The lower level (by 30%) of the 5-HT_{1B} receptor in these "humanized" mice may hamper the functioning of the receptor. The threshold necessary for triggering physiological effects might not be reached. This would be in line with the theory on the existence of a very steep receptor "concentration"-versus-response curve (Koshland, 1998). In addition, the shortage of suitable tools (no potent and selective centrally active h5-HT_{1B} receptor agonists) complicates the in vivo investigations. The brain-penetrating nonselective 5-HT_{1B} agonist RU24969 displays a 10-fold lower affinity for h5-HT_{1B} receptor than for m5-HT_{1B} receptor.

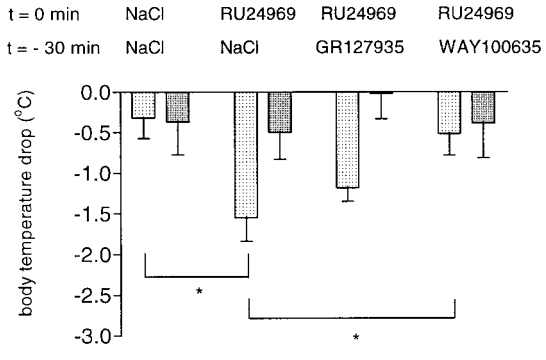

Fig. 10. Drop in esophageal temperature after administration of a 5-HT_{1B} antagonist (GR127934, 2.5 mg/kg) or a 5-HT_{1A} antagonist (WAY100635, 2.5 mg/kg) before the administration of RU24969 (2.5 mg/kg) (mean ± S.D.; n = 6). Note that WAY100635, but not GR127934, completely blocked the hypothermia induced by RU24969 in wild-type mice. *$p < .05$, two-tailed Mann-Whitney U test. Dotted columns, wild-type mice; gray columns, transgenic mice.

Other hypotheses can be put forward in trying to explain the problem of restoring functional response in these "humanized" mice. Desensitization of the h5-HT_{1B} receptor expressed in the transgenic mice could occur. However, the 5-HT_{1B} receptor has been expressed in a mouse cell line [LM (tk^-) fibroblasts] (Weinshank et al., 1992), and it was found to function normally. A difference in subcellular localization between the h5-HT_{1B} and m5-HT_{1B} receptor resulting from a difference in addressing processes could be an explanation.

Our study shows that the introduction of a human gene into an animal gene results in the expression of the corresponding "humanized" protein, which reveals some functional features; however, full-blown activity of the "humanized" protein is not evident.

Acknowledgments

The expert technical assistance of Monique Berben, Jozef Vermeire, Ilse Lenaerts, Paula te Riele, Lou Stas, and Nathalie Caluwaerts is gratefully acknowledged. We are grateful to Dr. Mirek Jurzak and Dr. Katty Josson for advice and stimulating discussions.

References

Adham N, Romanienko P, Hartig P, Weinshank R and Branchek T (1992) The rat 5-hydroxytryptamine$_{1B}$ receptor is the species homologue of the human 5-hydroxytryptamine$_{1D\beta}$ receptor. *Mol Pharmacol* **41:**1–7.

Audinot V, Lochon S, Newman-Tancredi A, Lavielle G and Millan M (1997) Binding profile of the novel 5-$HT_{1B/1D}$ receptor antagonist, [^{3}H]GR125743, in guinea-pig brain: A comparison with [^{3}H]-carboxamidotryptamine. *Eur J Pharmacol* **327:** 247–256.

Bach A, Unger L, Sprengel R, Mengod G, Palacios J, Seeburg P and Voigt M (1993) Structure, functional expression and spatial distribution of a cloned cDNA encoding a rat 5-$HT_{1D\text{-like}}$ receptor. *J Recept Res* **13:**479–502.

Bonaventure P, Schotte A, Cras P and Leysen JE (1997) Autoradiographic mapping of 5-HT_{1B} and 5-HT_{1D} receptors in human brain using [^{3}H]alniditan, a new radioligand. *Recept Channels* **5:**225–230.

Bonaventure P, Voorn P, Luyten WHML, Jurzak M, Schotte A and Leysen JE (1998b) Detailed mapping of serotonin 5-HT_{1B} and 5-HT_{1D} receptor mRNA and ligand binding sites in guinea pig brain and trigeminal ganglion: Clues for function. *Neuroscience* **82:**469–484.

Bonaventure P, Voorn P, Luyten WHML and Leysen JE (1998a) 5HT_{1B} and 5HT_{1D} receptor mRNA differential co-localization with peptide mRNA in the guinea pig trigeminal ganglion. *Neuroreport* **9:**641–645.

Boschert U, Amara D, Segu L and Hen R (1994) The mouse 5-hydroxytryptamine1B receptor is localized predominantly on axon terminals. *Neuroscience* **58:**167–182.

Bruinvels AT, Palacios JM and Hoyer D (1993) Autoradiographic characterization and localization of 5-HT_{1D} compared with 5-HT_{1B} binding sites in rat brain. *Naunyn-Schmeidelberg's Arch Pharmakol* **347:**569–582.

Buzzi MG, Carter WB, Shimizu T, Heath H and Moskowitz MA (1991) Dihydroergotamine and sumatriptan attenuate levels of CGRP in plasma in rat superior sagittal sinus during electrical stimulation of the trigeminal ganglion. *Neuropharmacology* **30:**1193–1200.

Domenech T, Beleta J and Palacios JM (1997) Characterization of human serotonin 1D and 1B receptors using [^{3}H]GR125743, a novel radiolabelled serotonin 5$HT_{1B/1D}$ receptor antagonist. *Naunyn-Schmeidelberg's Arch Pharmakol* **356:**328–334.

Goadsby P and Knight Y (1997) Direct evidence for central sites of action of zolmatriptan (311C90): An autoradiographic study in cat. *Cephalalgia* **17:**153–158.

Hagan J, Slade P, Gaster L, Jeffrey P, Hatcher J and Middlemiss D (1997) Stimulation of 5-HT_{1B} receptors causes hypothermia in the guinea pig. *Eur J Pharmacol* **331:**169–174.

Halazy S, Lamothe M and Jorand-Lebrun C (1997) 5-$HT_{1B/1D}$ antagonists and depression. *Exp Opin Ther Patents* **7:**339–352.

Hamblin MW, McGuffin RW, Metcalf MA, Dorsa DM and Merchant KM (1992) Distinct 5HT_{1B} and 5HT_{1D} serotonin receptors in rats: Structural and pharmacological comparison of the two cloned receptors. *Mol Cell Neurosci* **3:**578–587.

Hen R (1992) Of mice and flies: Commonalities among 5-HT receptors. *Trends Pharmacol Sci* **13:**160–165.

Hooper M, Hardy K, Handyside A, Hunter S and Monk M (1987) HPRT deficient (Lesh-Nyhan) mouse embryos derived from germ-line colonization by cultured cells. *Nature (Lond)* **236:**292–295.

Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR

and Humphrey PPA (1994) International Union of Pharmacology classification of receptors for 5-hydroxytryptamine. *Pharmacol Rev* **46:**157–203.
Hoyer D and Middlemiss DN (1989) Species differences in the pharmacology of terminal 5-HT autoreceptors in mammalian brain. *Trends Pharmacol Sci* **10:**130–132.
Jin H, Oksenberg D, Ashkanazi A, Peroutka S, Duncan A, Rozmahel R, Mengod G, Palacios J and O'Dowd B (1992) Characterization of the human 5-hydroxytryptamine1B receptor. *J Biol Chem* **267:**5735–5738.
Koshland DE (1998) The area of pathway quantification. *Science (Wash DC)* **280:**852–853.
Langlois X, Gerard C, Darmon M, Chauveau J, Hamon M and El Mestikawy S (1995) Immunolabeling of central serotonin 5HT$_{1D\beta}$ receptors in the rat, mouse, and guinea pig with anti-peptide antiserum. *J Neurochem* **65:**2671–2681.
Leysen JE, Gommeren W, Heylen L, Luyten WHLM, Van De Weyer I, Vanhoenacker P, Haegeman G, Schotte A, Van Gompel P, Wouters R and Lesage A (1996) Alniditan: A new 5-HT$_{1D}$ agonist and migraine abortive agent: Ligand binding properties of human 5-HT$_{1D\alpha}$, human 5-HT$_{1D\beta}$, and calf 5-HT$_{1D}$ receptor investigated with [^{3}H]5-HT and [^{3}H]alniditan. *Mol Pharmacol* **50:**1567–1580.
Lorent K, Overbergh L, Delabie J, Van Leuven F and Van den Berghe H (1994) Distribution of mRNA coding for alpha-2-macroglobulin, the murinoglobulins, the alpha-2-macroglobulin receptor and the alpha-2-macroglobulin receptor associated protein during mouse embryogenesis and in adult tissues. *Differentiation* **55:**213–223.
Maroteaux L, Saudou F, Amlaiky N, Boschert U, Plassat JL and Hen R (1992) Mouse 5-HT$_{1B}$ serotonin receptor: Cloning, functional expression, and localization in motor control center. *Proc Natl Acad Sci USA* **89:**3020–3024.
Mengod G, Raurich R, Palacios JM and Cortes R (1996) High and low affinity serotonin 5-HT$_{1D}$ receptor sites in brain: Characterization using a new radiolabeled antagonist. *Soc Neurosci Abstr* **22:**1330.
Metcalf MA, McGuffin RW and Hamblin MW (1992) Conversion of the human 5-HT$_{1D\beta}$ serotonin receptor to the rat 5-HT$_{1B}$ ligand-binding phenotype by Thr355Asn site-directed mutagenesis. *Biochem Pharmacol* **44:**1917–1920.
Middlemiss DN, Bremer ME and Smith SM (1988) A pharmacological analysis of the 5-HT receptor mediating inhibition of 5-HT release in the guinea-pig frontal cortex. *Eur J Pharmacol* **157:**101–107.
Molderings GJ, Werner K, Likungu J and Gothert M (1990) Inhibition of noradrenaline release from the sympathetic nerves of the human saphenous vein via presynaptic 5-HT receptor similar to the 5-HT$_{1D}$ subtype. *Naunyn-Schmiedeberg's Arch Pharmacol* **342:**371–377.
Moskowitz MA (1992) Neurogenic versus vascular mechanisms of sumatriptan and ergot alkaloids in migraine. *Trends Pharmacol Sci* **13:**307–311.
Oksenberg D, Marsters S, O'Dowd B, Jin H, Havlik S, Peroutka SJ and Ashkenazi AA (1992) Single amino-acid difference confers major pharmacological variation between human and rodent 5HT$_{1B}$ receptors. *Nature (Lond)* **360:**161–163.
Parker EM, Grisel DA, Iben LG and Shapiro RA (1993) A single amino acid difference accounts for the pharmacological distinctions between the rat and human 5-hydroxytryptamine receptors. *J Neurochem* **60:**380–383.
Raiteri M, Maura G, Bonanno O and Pittaluga A (1986) Differential pharmacology and function of two 5-HT$_1$ receptors modulating transmitter release in rat cerebellum. *J Pharmacol Exp Ther* **237:**644–648.
Ramboz S, Saudou F, Amara DA, Belzung C, Segu L, Misslin R, Buhot MC and Hen R (1996) 5-HT$_{1B}$ receptor knock-out: Behavioural consequences. *Behav Brain Res* **73:**305–312.
Saudou F, Amara DA, Dierich A, LeMeur M, Ramboz S, Segu L, Buhot MC and Hen R (1994) Enhanced aggressive behavior in mice lacking 5-HT$_{1B}$ receptor. *Science (Wash DC)* **265:**1875–1878.
Saudou F and Hen R (1994) 5-Hydroxytryptamine receptor subtypes in vertebrates and invertebrates. *Neurochem Int* **25:**503–532.
Selkirk JV, Scott C, Ho M, Burton MJ, Watson J, Gaster LM, Collin L, Jones BJ, Middlemiss DN and Price GW (1998) SB-224289: A novel selective (human) 5-HT$_{1B}$ receptor antagonist with negative intrinsic activity. *Br J Pharmacol* **125:**202–208.
Tricklebank MD, Middlemiss DN and Neill J (1986) Pharmacological analysis of the behavioral and thermoregulatory effects of the putative 5-HT$_1$ receptor agonist, RU24969, in the rat. *Neuropharmacology* **25:**877–886.
Umans L, Serneels l, Overbergh l, Lorent K, Van Leuven F and Van den Berghe H (1995) Targeted inactivation of the mouse alpha 2-macroglobulin gene. *J Biol Chem* **270:**19778–19785.
Waeber C, Dietl MM, Hoyer D and Palacios JM (1989) 5HT$_1$ receptors in the vertebrate brain: Regional distribution examined by autoradiography. *Naunyn-Schmiedeberg's Arch Pharmacol* **340:**486–494.
Waeber C and Moskowitz MA (1997) 5-Hydroxytryptamine$_{1A}$ and 5-hydroxytryptamine$_{1B}$ receptors stimulate [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate binding to rodent brain sections as visualized by in vitro autoradiography. *Mol Pharmacol* **52:**623–631.
Weinshank RL, Zgombick JM, Macchi MJ, Branchek TA and Hartig PR (1992) Human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-HT$_{1D\alpha}$ and 5HT$_{1D\beta}$. *Proc Natl Acad Sci USA* **89:**3630–3634.
Zgombick J, Bard J, Kucharewicz D, Urquhart D, Weinshank R and Branchek T (1997) Molecular cloning and pharmacological characterization of guinea pig 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. *Neuropharmacology* **36:**513–524.

Send reprint requests to: Dr. Pascal Bonaventure, R.W. Johnson Pharmaceutical Research Institute, 3535 General Atomic Court, Suite 100, San Diego, CA. E-mail: pbonave1@prius.jnj.com