Taking advantage of three cellular systems, we established that 5-HT<sub>2B</sub> receptors are coupled with NO signaling pathways. In the 1C11 serotonergic cell line and <i>Mastomys natalensis</i> carcinoid cells, which naturally express the 5-HT<sub>2B</sub> receptor, as well as in transfected LMTK<sup>−</sup> fibroblasts, stimulation of the 5-HT<sub>2B</sub> receptor triggers intracellular cGMP production through dual activation of constitutive nitric-oxide synthase (cNOS) and inducible NOS (iNOS). The group I PDZ motif at the C terminus of the 5-HT<sub>2B</sub> receptor is required for recruitment of the cNOS and iNOS transduction pathways. Indeed, the 5-HT<sub>2B</sub> receptor-mediated NO coupling is abolished not only upon introduction of a competitor C-terminal 5-HT<sub>2B</sub> peptide in the three cell types but also in LMTK<sup>−</sup> fibroblasts expressing a receptor C-terminally truncated or harboring a point mutation within the PDZ domain. The occurrence of a direct functional coupling between the receptor and cNOS activity is supported by highly significant correlations between the binding constants of drugs on the receptor and their effects on cNOS activity. The 5-HT<sub>2B</sub>/iNOS coupling mechanisms appear more complex because neutralization of endogenous G<sub>α13</sub> by specific antibodies cancels the cellular iNOS response while not interfering with cNOS activities. These findings may shed light on the PDZ domain's multifaceted role in downstream transduction pathways of a G protein-coupled receptor.

The present work provides biochemical evidence for a functional link between NO signaling and the 5-HT<sub>2B</sub> Receptor. NO synthase (NOS), the enzyme responsible for NO synthesis, occurs as three distinct isoforms (11, 12). Two isoforms are constitutive (cNOS), and their activities are dependent upon the calcium-calmodulin complex. One is expressed in some neurons (13) and in skeletal muscles (14). It corresponds to the neuronal isoform (NOS-1). The other is the endothelial isoform (NOS-3), abundant in endothelial and epithelial cells (15). The third NOS isoform is calcium-independent and inducible (iNOS or NOS-2) (11). It responds to endotoxins of Gram-negative bacteria or inflammatory cytokines in a wide variety of cells (most notably in macrophages, glia, or vascular cells). Here, we show that both cNOS and iNOS contribute to NO production upon 5-HT<sub>2B</sub> receptor activation in the murine serotonergic neuronal differentiation of 1C11 cells, referred to as 1C11/e<sup>1</sup>H<sub>T</sub> (4, 5). In both cases, the receptor couples with PIP<sub>2</sub> hydrolysis, similarly to the two other members (5-HT<sub>2A</sub> and 5-HT<sub>2C</sub>) of the 5-HT<sub>2</sub> receptor family. In contrast, the 5-HT<sub>2B</sub> receptor stimulation fails to stimulate PIP<sub>2</sub> hydrolysis in rat fundus (6) and in rat vasculature (7). Signal transduction processes involving 5-HT<sub>2B</sub> receptors and distinct from PIP<sub>2</sub> hydrolysis might thus occur. Accordingly, 5-HT<sub>2B</sub>-dependent activations of both the ras-mitogen-activated protein kinase cascade (8) and phospholipase A<sub>2</sub> (5) have already been reported. The possibility that NO, a major messenger molecule in the cardiovascular, immune, and nervous systems (see Ref. 9 for review), may behave as a signaling effector associated to the 5-HT<sub>2B</sub> receptor, is suggested by data obtained in rat vasculature (7), rat fundus (6), and human endothelial cells (10).

The present work provides biochemical evidence for a functional link between NO signaling and the 5-HT<sub>2B</sub> Receptor. NO synthase (NOS), the enzyme responsible for NO synthesis, occurs as three distinct isoforms (11, 12). Two isoforms are constitutive (cNOS), and their activities are dependent upon the calcium-calmodulin complex. One is expressed in some neurons (13) and in skeletal muscles (14). It corresponds to the neuronal isoform (NOS-1). The other is the endothelial isoform (NOS-3), abundant in endothelial and epithelial cells (15). The third NOS isoform is calcium-independent and inducible (iNOS or NOS-2) (11). It responds to endotoxins of Gram-negative bacteria or inflammatory cytokines in a wide variety of cells (most notably in macrophages, glia, or vascular cells). Here, we show that both cNOS and iNOS contribute to NO production upon 5-HT<sub>2B</sub> receptor activation in the murine serotonergic 1C11/e<sup>1</sup>H<sub>T</sub> cells (4) or in <i>Mastomys natalensis</i> carcinoid tumor primary cultured cells (16). These two in vitro systems, which naturally express 5-HT<sub>2B</sub> receptors within a complete serotonergic phenotype, allow NO couplings to be studied under conditions close to in vivo physiological situations.

The PDZ domains present in a diverse family of structural proteins and enzymes as cNOS appear to be important elements in protein-protein interactions at the plasma mem-

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**PDZ-dependent Activation of Nitric-oxide Synthases by the Serotonin 2B Receptor**

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brane. Interactions between proteins containing either PDZ domains or PDZ recognition motifs are assumed to mediate formation of macromolecular signaling complexes (see Ref. 17 for review). For instance, in rat cerebellum and forebrain (18), the resulting multiprotein clusters contribute to the functional coupling of the NMDA receptor to NOS-1 activity. PDZ target motifs typically consist of 4–11 amino acid residues at the extreme C terminus of the target protein (17). Interestingly, a group I PDZ motif, VSYI(D/E), is present at the C terminus of the 5-HT$_{2B}$ receptor. This led us to examine the role of this motif in NO signaling pathways.

In mouse LMTK$^-$ fibroblasts stably transfected with the full-length cDNA encoding the mouse 5-HT$_{2B}$ receptor (LM6 cells), a 5-HT$_{2B}$-NO coupling mediated by cNOS and iNOS is observed. Instead, if the transfected cDNA expresses a C-truncated form of the receptor (LM5 cells), NO production is fully abolished. An involvement of the 5-HT$_{2B}$ PDZ target domain in the coupling to cNOS and iNOS could be further indicated upon introduction, in the three cell types studied, of a 20-amino acid competitor peptide whose sequence corresponds to the C terminus of the 5-HT$_{2B}$ receptor. In all cases, the peptide abolished the 5-HT$_{2B}$-dependent NO production. The observation that the 5-HT$_{2B}$-NOSs coupling is canceled in LMTK$^-$ fibroblasts expressing a 5-HT$_{2B}$ receptor with a point mutation in the VSYI(D/E) motif (LMTK$^-$VSSI) motif confirms the implication of the PDZ domain of the receptor in its coupling to cNOS and iNOS.

The ability of G proteins to directly regulate NO production remains largely to be elucidated. One recent report indicates a regulation of iNOS expression by G$\alpha$$q/11$ in an epithelial renal cell line (19) and suggests that proinflammatory agents such as thrombin or thromboxane might induce cellular responses similar to those initiated by bacterial endotoxins or cytokines. In the present study, we observed that introduction of anti-G$\alpha_{13}$ antibodies in either LM6, 1C11$^{\text{MAMT}}$ or Mastomys permeabilized cells systematically blocked the 5-HT$_{2B}$ receptor-mediated NO activation. Altogether, these findings highlight novel GPCR-dependent regulations of NO production.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dibutyryl cAMP and cyclohexane carboxylic acid were from Sigma-Aldrich. Neurochemicals were from RBI (Natick, MA). All other chemicals, of the purest grade available, were from classical commercial suppliers. The 5-nucleotide 4-iodohep-tadecanucleotide, and 1$^\text{cH}$[Citrulline (1.73 GBq/mlmol), and 1$^\text{cH}$[Citrulline (1.73 GBq/mlmol) were from NEN Life Science Products. The *Thermus aquaticus* polymerase was from Perkin-Elmer. The peptide sequence is shortTTTLEENDDGKAAEQVSYI corresponding to the 20 amino acids at the C terminus of the mouse 5-HT$_{2B}$ receptor, as well as peptides harboring mutated versions of the VSYI motif were synthesized and purified by Dr. Marx (Boehringer Mannheim, Germany). Rabbit antiserum against rodent Gas (C-18), Go (C-20), and G$_{\alpha q}$ (C-19) and G$_{\alpha 12}$ (A-20) as well as the corresponding blocking peptide are from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Cultures**—To avoid any interfering iNOS induction because of cytokines or undefined components of the media, all cell types were grown, as previously reported (5) in a serum-free medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1) with 5 mM insulin, 5 mM glutamine, 30 mM sodium, 20 mM procaterol, and 100 mM putrescine). *M. natalensis* carcinoid tumor primary cultured cells were obtained as previously reported (16). 1C11 progenitor cells are grown and induced to differentiate toward the serotonergic pathway in the presence of 1 mM dibutyryl cAMP and 0.05% cyclohexane carboxylic acid (20). Experiments were performed on 1C11 serotonergic cells 2 days after addition of the inducers (1C11$^{\text{MAMT}}$, cells). Mouse LMTK$^-$ fibroblasts, lacking 5-HT receptors and thus nonresponsive to DOI stimulation, were stably transfected with cDNA encoding either the full-length (LM6 cells) or a truncated (LM5 cells) mouse 5-HT$_{2B}$ receptor and cloned. The 5-HT$_{2B}$ receptor expressed in LM5 cells was deleted for 77 amino acid residues of the C-terminal domain from amino acid 403. LMTK$^-$ cells were also transfected with a cDNA encoding the full-length 5-HT$_{2B}$ receptor harboring a point mutation in the VSYI motif (LMTK$^-$VSSI).

**Cell Permeabilization**—The cells were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin and exposed to 1 hemolytic unit of alveolysin/10$^6$ cells at 37°C under agitation (21). Alveolysin was purified from *Mastomys* fibroblasts, lacking 5-HT receptors and thus nonresponsive to DOI stimulation (21, 22). One hemolytic unit is equivalent to ~1 ng of protein (193 pmol).

**Radioligand Binding Experiments**—Radioligand binding experiments were performed as previously detailed (4, 23). LMS6, 1C11$^{\text{MAMT}}$, and Mastomys cells expressed 178 ± 16, 107 ± 12, and 125 ± 14 fmol of 5-HT$_{2B}$ receptor/mg of protein, respectively, as measured by [3H]5-HT binding. LMS fibroblasts expressed 137 ± 7 and 124 ± 25 fmol of 5-HT$_{2B}$ receptor/mg of protein, respectively. It is noteworthy that the B$_{\text{max}}$ value measured for Mastomys cells may be underestimated and may not accurately reflect the number of 5-HT$_{2B}$ binding sites/cell because measurements are performed on primary cultures of carcinoid tumors. The presence of 5-HT$_{1\alpha}$ receptors onto 1C11 cells led us to use $^{[3H]}$DOI as 5-HT$_{2B}$ receptor radioligand instead of $^{[3H]}$serotonin.

**Determination of Intracellular cGMP Levels**—Cells were washed twice in fresh serum-free medium and incubated for various times at 37°C with 100 mM isobutylmethylxanthine and test agents. The reaction was stopped by aspiration of the medium followed by addition of 500 µl ice-cold 95% ethanol/5% formic acid (1:1, v/v). After 1 h at 4°C, the ethanol phase was collected and lyophilized. cGMP was quantified using an iodinated radioimmunoassay kit (cGMP RIA kit RPA 525, Amersham Pharmacia Biotech).

**Determination of Intracellular Inositol 1,4,5-Trisphosphate Levels**—Cells were washed twice with fresh serum-free medium and incubated for various times at 37°C with 100 mM DOI and test agents. The reaction was stopped by aspiration of the medium, followed by addition of 0.5 ml of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.40, 1.15% KCl (w/v), 1 mM EDTA, 5 mM glucose, 0.1 mM EDTA, 200 units/ml superoxide dismutase, 2 mg/ml pepstatin A, 10 mg/ltripenin inhibitor, and 44 mg/liter phenylmethylsulfonyl fluoride). Cells were scrapped and lysed by sonication. Cell cytosols were prepared by centrifugation at 2,000 × g for 10 min at 4°C followed by centrifugation of 10,000 × g for 15 min at 4°C. The pellets (≥80 µl) were incubated in the presence and absence of nitro-l-arginine (1 mM) for 30 min at 37°C with $^{[3H]}$Harginine (1 µM, 1 µCi), 10 mM NADPH, 15 µM (6R,5R,6S)-1,6,7,8-tetrahydro-6-l-biotinopiperidino, 1 µM flavine-adenine dinucleotide, and 1 µM calmodulin in 50 mM HEPES buffer (pH 7.40) containing 1 mM Mg-dithioiborhod, 1 mM EDTA, and 1.5 µM CaCl$_2$ (final volume, 150 µl). The incubations were quenched by the addition, for 5 min, of 1 ml of 100 mM EDTA, 100 mM HEPES buffer (pH 7.40) containing 10 mM EGTA and 500 µg of AG 50W-X8 (cation-ion Na$^+$) cation exchange resin, followed by a 5-min centrifugation at 10,000 × g. $^{[3H]}$Citru-line in the supernatant was quantified by liquid scintillation counting. Specific NOS activity was calculated as the nitro-l-arginine-sensitive formation of $^{[3H]}$citru-line/min/mg of protein. l- Citru-line was quantitatively recovered by this batch assay as determined by using 1$^\text{cH}$[Citru-line. In parallel experiments, cell cytosols were incubated in Ca$^{2+}$-free buffer to determine Ca$^{2+}$-independent NOS (iNOS) activities.

**Isolation of Total RNA and Reverse Transcription PCR of NOS Isoforms**—First-strand cDNA was synthesized from 1 µg of total RNA with oligo(T)$_{17}$ primer, using 200 units Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in a 20-µl reaction volume for 1 h at 37°C. Primer pairs specific for murine NOS isoforms were selected with the aid of the Oligo 4.0 Primer Design Software (National Biosciences Inc., Plymouth, Minn.). Polynucleotides in the primers are underlined. NOS-1 (accession number D14582, amplified product 2208–2807), 5'-GTCAATGTCCTGAGACCTGCTG-3' (sense), 5'-TCCATGAGCAATATTGGGAGAGAGAGGTC-3' (antisense); NOS-2 (accession number 87039, amplified product 877–2615), 5'-CTGACCGGGAGAGAGAGGTCG-3' (sense), 5'-GGTCGAGGAGAGAGGTCGAGAGGATCAGCTG-3' (antisense); NOS-3 (accession number U53142, amplified product 877–2615), 5'-GCTCGTGAGGAGAGAGGTCGAGAGGATCAGCTG-3' (antisense). PCR reactions were performed using the Gene-Amp kit (Perkin-Elmer) in a
50-μl volume containing 2 μl of the reverse transcription products (40 cycles, 60 °C). PCR products were analyzed on 2% agarose gels. NOS-2 mRNA steady state levels were monitored during 1 h upon 5-HT2B receptor activation by real-time RNA quantification (25).

**Site-directed Mutagenesis by Recombinant Circle PCR—**Point mutations were introduced into the wild-type mouse 5-HT2B R cDNA by oligonucleotide-directed mutagenesis as described previously (26). A single-stranded uracil-containing DNA template was generated in *Escherichia coli* CJ236 (dut-, ung-) (Invitrogen) using R408 helper phage. Using oligonucleotides encoding the mutations, the following mutations were introduced: m5-HT2B Y478S and R463del. Identity of the mutant cDNAs was confirmed by dideoxynucleotide sequencing using a Sequenase II kit (U.S. Biochemical Corp.). To verify the absence of additional mutations in the parental cDNAs, rescue constructs of all mutations were constructed and tested for recovery of NOS activities. In all cases, the rescue constructs demonstrated wild-type NOS activities—(data not shown), confirming that the disruption of NOS couplings observed for both mutants resulted from the mutations.

**RESULTS**

**Activation of the 5-HT2B Receptor Triggers Intracellular cGMP Accumulation**—Most of the transductional actions of NO are mediated through an activation of cytosolic guanylate cyclases with concomitant increase of cellular cGMP (27). Thus, we first examined whether exposure of 5-HT2B receptor-expressing cells (i.e. *Mastomys*, 1C11s<sup>5HT</sup>, and LM6 cells) to DOI, a specific agonist of 5-HT<sub>2</sub> receptors, triggered cGMP formation. All cells were grown in serum-free cultures to preclude signaling interferences mediated by cytokines or undefined serum components. As shown in Fig. 1A, saturation of the receptor with 100 nM DOI resulted in an accumulation of cGMP observable after 10 min in all three cell types. The increase in cGMP was lower in 1C11<sup>5HT</sup> and LM6 cells than in *Mastomys* cells. The elevated response of *Mastomys* cells can be related to the overexpression of 5-HT<sub>2B</sub> receptors in these carcinoid tumoral cells, as previously evidenced by anti-5-HT<sub>2B</sub> antibodies (8). It may also reflect cell type-specific features of the 5-HT<sub>2B</sub> receptor-mediated signaling responses. Indeed, as observed in rat fundus (6), *Mastomys* 5-HT<sub>2B</sub> receptors do not couple to PLC<sub>β</sub>, whereas DOI treatment (100 nM) activates PIP<sub>2</sub> hydrolysis in both LM6 and 1C11<sup>5HT</sup> cells (Fig. 1B).

The DOI-induced cGMP formation observed for the three cell types supports the idea of a 5-HT<sub>2B</sub>-NO coupling. We therefore decided to search for the occurrence of a DOI-mediated NOS activation.

**5-HT<sub>2B</sub> Receptors Are Coupled to Both cNOS and iNOS Activities**—To assess the contributions of Ca<sup>2+</sup>-dependent NOSs (cNOS) and/or Ca<sup>2+</sup>-independent iNOS to NO synthesis in the different cell systems, NOS activities were monitored in cell lysates in the presence or absence of calcium ions. In each cell investigated, activation of 5-HT<sub>2B</sub> receptors by 100 nM DOI resulted in a time-dependent increase in both cNOS (Fig. 2A) and iNOS (Fig. 2B) activities. cNOS activity becomes maximal 5–15 min after addition of the agonist. Noticeably, the enhancement of cNOS activity lasts longer in *Mastomys* cells than in the two other strains. This discrepancy is consistent with the high level of cGMP accumulating in *Mastomys* cells (Fig. 1A). In the absence of agonist stimulation, low levels of cNOS activity were measured in all three cell lysates but not in LMTK<sup>-</sup> untransfected cells. These levels might reflect a constitutive basal activity of the 5-HT<sub>2B</sub> receptor. Such a basal activity has already been observed for its PLC<sub>β</sub> (4, 23), ras-mitogen-activated protein kinase (8), and phospholipase A<sub>2</sub> (5) couplings.

It is noteworthy that DOI-mediated 5-HT<sub>2B</sub> stimulation also induces a rapid and transient increase of iNOS activity (Fig. 2B). Activation of iNOS is only very shortly delayed (lag is about 5 min or less) with respect to cNOS activation. This extremely rapid activation of iNOS is reproducibly observed in all three cell types. It contrasts with the iNOS activation by proinflammatory agents, for which the time of response ranges between 3 h (28) and up to several days. In all three cell types, iNOS mRNA steady state levels were assessed by real-time quantitative PCR and could be observed not to vary (data not shown). This suggests that 5-HT<sub>2B</sub>-mediated iNOS activation depends on post-transcriptional controls.

The distinct NOS isoforms expressed in each cell type were further characterized through reverse transcription PCR experiments. As shown in Fig. 3, all three cell types contain transcripts encoding iNOS (NOS-2) and either of the two cNOS (NOS-1 or NOS-3) isoforms. As expected, NOS-1 mRNA is present in 1C11<sup>5HT</sup> neural-like cells and in *Mastomys* neuroendocrine cells, whereas NOS-3 mRNA can only be detected in LM6 fibroblasts, which, like endothelial cells, have a mesodermal origin.

**Correlations between NOS Activities and 5-HT<sub>2B</sub> Receptor Binding Constants**—The functional relation between the 5-HT<sub>2B</sub> receptor and NOS activities could be further evidenced by examining the effects of a series of known agonists or antagonists on both DOI binding and NOS activities. Their binding to the receptor was assessed through competition with DOI,
Three independent experiments performed in duplicate.

1C11*/5HT (Fig. 4B) cells; NOS-3 was followed in LM6 fibroblasts; Fig. 4C). Such pharmacological correlations strongly support the occurrence of a direct functional coupling between the 5-HT<sub>2B</sub> receptor and cNOS activity.

With the same set of drugs, lower but still significant correlations (r<sub>p</sub> = 0.643, n = 8, p < 0.05) were found in the case of iNOS activity in either Mastomys (Fig. 4D) or 1C11*/5HT (Fig. 4E) serotonergic cells. Using LM6 fibroblasts, the correlation did not reach significance (Fig. 4F, r<sub>p</sub> = 0.571, n = 8, p > 0.05).

We conclude from these observations that coupling of the 5-HT<sub>2B</sub> receptor to the iNOS activity most likely follows more complex mechanisms than those involved in the cNOS coupling and that these mechanisms may vary with the cell context.

**Activation of cNOS and iNOS Requires the 5-HT<sub>2B</sub> Receptor C-terminal PDZ Motif**—In the central nervous system, the NMDA receptor-mediated stimulation of NOS-1 occurs via PDZ-PDZ interactions (18). Like the NR2 subunit of the NMDA receptor, the sequence of the 5-HT<sub>2B</sub> receptor polypeptide ends in a type I consensus target sequence for PDZ domain-containing proteins. This led us to search for a possible role of the C-terminal domain of the 5-HT<sub>2B</sub> receptor in its coupling to NO activities.

LMTK<sup>−</sup> fibroblasts were thus stably transfected with a 3′-terminal deleted version of the cDNA encoding the 5-HT<sub>2B</sub> receptor (LM5 cells). As shown in Fig. 5, upon expression of this truncated 5-HT<sub>2B</sub> receptor, DOI-dependent cNOS and iNOS activations were no longer observed. Although deletion of the C-terminal domain of the 5-HT<sub>2B</sub> receptor did not impair its functional coupling to PIP<sub>2</sub> hydrolysis (Fig. 1B), it completely abolished DOI-dependent cGMP formation (Fig. 1A). We therefore conclude that, at least in LMTK<sup>−</sup> fibroblasts, the C-terminal domain of the 5-HT<sub>2B</sub> receptor participates in the recruitment of both the cNOS and iNOS signaling pathways.

We finally examined whether a peptide, the sequence of which overlaps that of the VSYY(D/E) motif at the C terminus of the 5-HT<sub>2B</sub> receptor, could interfere with the NOS activities. As shown in Fig. 5, addition to permeabilized cells of a 20-amino acid peptide identical to the C terminus of the receptor prevents stimulation by DOI of cNOS and iNOS activities in all three cell types. Together, these results clearly establish that the C-terminal tail of the 5-HT<sub>2B</sub> receptor is critical for functional coupling to cNOS and iNOS.

The role of the VSYY(D/E) motif in 5-HT<sub>2B</sub> receptor-mediated NOSs activation was further tested by introducing in permeabilized cells various 20-amino acid C-terminal peptides harboring mutated versions of the VSYY(D/E) motif, i.e., VSYY(D/E), VSYF(D/E), VAYF(D/E), and GSYY(D/E). In all three cell types, DOI-induced stimulation of cNOS and iNOS activities still occurred in the presence of these peptides (Fig. 5). In parallel, LMTK<sup>−</sup> cells were transfected with a cDNA encoding a 5-HT<sub>2B</sub> receptor in which the tyrosine residue of the VSYY motif was substituted by a serine. DOI stimulation of the resulting LMTK<sup>−</sup>VSSY cells does not trigger NOSs activation (Fig. 5) while producing the same IP<sub>3</sub> response as in LM6 cells (data not shown). These data strongly suggest that the VSYY(D/E) motif of the 5-HT<sub>2B</sub> receptor represents the binding site for association with PDZ-containing complexes involved in 5-HT<sub>2B</sub>/NOSs couplings. For the first time, an involvement of PDZ interactions in a GPCR signaling pathway is evidenced.

**G<sub>α13</sub> Is Involved in the 5-HT<sub>2B</sub> Receptor-mediated Stimulation of iNOS**—The initial evidence for the involvement of heterotrimeric G protein in the regulation of NO production arose from transfection experiments of renal epithelial (MCT) cells by cDNAs encoding various G protein α chains (G<sub>αi2</sub>, G<sub>αo</sub>, G<sub>α1</sub>, and G<sub>α11</sub>). The only Go chain having a marked stimulatory effect on iNOS expression was G<sub>α13</sub> (19). To assess a possible role of

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**Fig. 2. 5-HT<sub>2B</sub> receptor-mediated activation of NOS activities.** Time-dependent stimulation of NOS activities in LM6, 1C11*/5HT, and Mastomys cells, by 100 nM DOI. A, calcium-dependent cNOS activity. B, calcium-independent iNOS activity. Data shown are the means ± S.E. of three independent experiments performed in duplicate. △, LM6; ▲, 1C11*/5HT; ◆, Mastomys.

**Fig. 3. Reverse transcription PCR analysis of NOS isoforms expression in LM6 (lane 1), 1C11*/5HT (lane 2), and Mastomys (lane 3) cells.**

and the corresponding IC<sub>50</sub> values were determined. K<sub>i</sub> values were deduced using the Cheng-Prusoff equation (29). The dose-response effect of agonists on NOS activities could be directly followed. In the case of antagonists, effects were estimated through the capacity of each drug to inhibit the DOI-dependent stimulation of NOS activities.

For the three cells studied, highly significant correlations (r<sub>p</sub> = 0.976, n = 8, p < 0.01) were found between the 5-HT<sub>2B</sub> receptor binding constants of the drugs, and the apparent equilibrium constants deduced from their effect on cNOS activities (NOS-1 was followed in Mastomys (Fig. 4A) and 1C11*/5HT (Fig. 4B) cells; NOS-3 was followed in LM6 fibroblasts; Fig. 4C). Such pharmacological correlations strongly support the occurrence of a direct functional coupling between the 5-HT<sub>2B</sub> receptor and cNOS activity.
Ga13 on 5-HT2B-mediated NO production, each of the three cellular systems were permeabilized and then incubated for 30 min with either anti-Ga13, anti-Gaq/11, anti-Gas, or anti-Gai antibodies, prior to the addition of 100 nM DOI. cNOS and iNOS activities were measured 10 min after the agonist treatment. As shown in Fig. 6 (A and B), the anti-Ga13 antibodies fully abolished the DOI-mediated iNOS activation in all cell types while having no effect on cNOS activities. As a control, anti-Ga13 antibodies preincubated overnight with the related blocking peptide no longer inhibit iNOS activity (Fig. 6 B). Furthermore, antibodies against Gaq/11, Gas, or Gai were without any effect on cNOS (data not shown). These antibodies slightly reduced the iNOS response in LM6 and Mastomys extracts. Shown are the apparent pKi or pKd values of drugs in each process. In the case of the agonists and the antibodies, their binding to the receptor was assessed through competition with DOI binding, and the corresponding IC50 value was determined. Kd values were deduced using the Cheng-Prusoff equation (29). The dose-response effect of agonists on NOS activity could be directly followed. In the case of antagonists, effects were estimated through the capacity of each drug to inhibit the DOI-dependent stimulation of NOS activities. Ten different concentrations of each competing drug were used: 2, tryptamine; 3, serotonin; 4, 5-carboxamidotryptamine; 6, 8-OH-DPAT; 9, pizotifen; 10, methylsergide; 14, spiperone; 17, mianserin. Reported values are the means of four independent experiments performed in triplicate.

**DISCUSSION**

The present study provides strong biochemical evidence for a functional coupling of 5-HT2B receptors to the NOS signaling pathways. In the three cell types assayed, i.e. Mastomys carcinoma tumor primary cultures, clonal neural-like 1C11*/5HT serotonergic cells and 5-HT2B receptor transfected LM6 fibroblasts, the 5-HT2B receptor agonist DOI (or 5-HT, data not shown) always triggers intracellular cGMP accumulation resulting from both cNOS and iNOS activation. Such a link between 5-HT and NO could already be suspected in view of the simultaneous involvement of these two agents in smooth muscle contractility (6, 30), vascular contraction (7, 31), and related migraine pathogenesis (32). Because 5-HT2B receptors are present in stomach, intestine, pulmonary smooth muscles, kidney, as well as in myocardium, vascular endothelium, and meningeal tissues (3, 33, 34), they are obvious candidates to trigger the NO response associated to 5-HT. However, a direct 5-HT2B receptor-NO coupling is difficult to evidence in vivo (35), because of the paucity of 5-HT2B-bearing cells in intact tissues, on the one hand, and the diversity of signals likely to mobilize NOSs responses, on the other hand.

Here, the occurrence of a direct coupling between 5-HT2B...
Fig. 5. Involvement of the VSYI(D/E) motif at the C-terminal PDZ containing domain of the 5-HT_{2B} receptor in coupling to eNOS and iNOS. 5-HT_{2B} receptor-mediated activation of cNOS (A) and iNOS (B) is impaired (i) in LM5 cells expressing a C-terminal truncated form of the receptor and (ii) in LMTK-VSSI cells expressing a 5-HT_{2B} receptor harboring a point mutation in the VSYI(D/E) motif. 5-HT_{2B} receptor-mediated activation of cNOS (A) and iNOS (B) is abolished upon introduction of a 20-amino acid C-terminal 5-HT_{2B} peptide in LM6, 1C11*/5HT, and Mastomys cells. 5-HT_{2B} receptor-mediated cNOS (A) and iNOS (B) activities are unaffected upon introduction in these three cell types, of 20-amino acid C-terminal peptides harboring mutated versions of the VSYI(D/E) motif: VSYV(D/E), VSYF(D/E), VAYI(D/E), and GSYI(D/E). Mutated peptide data correspond to the means of the values separately obtained for each of the four mutated peptides. In all cases (A and B), NOS activities were measured 10 min after the addition of 100 nM DOI. White bars, control; shaded bars, 20 amino acids; hatched bars, mutated peptides.

This work also establishes that the cytoplasmic VSYI(D/E) containing C-terminal domain of the 5-HT_{2B} receptor is involved in the recruitment of NOS activity. 5-HT_{2B}/NO coupling is fully abrogated either in LM5 fibroblasts stably transfected with a cDNA encoding a C-terminally truncated form of the 5-HT_{2B} receptor or by disrupting PDZ interactions in all three cell types. The latter effect is obtained through introduction of a competitor peptide corresponding to the 20 C-terminal amino acids of the 5-HT_{2B} receptor. Versions of this peptide harboring mutations in the VSYI(D/E) motif no longer abolish the 5-HT_{2B}-mediated PLCβ activation and involving a Ca^{2+}/calmodulin complex, cannot, however, be excluded. Indeed, interactions between the IP_{3} and NO signaling pathways have already been reported (36, 37).

Although interactions of GPCRs with PDZ-containing proteins have already been reported, notably for the β2-adrenergic receptor (see Ref. 39 for review), our study indicates for the first time that VSYI(D/E)/PDZ domain interactions play a role in the coupling of a GPCR (namely the 5-HT_{2B} receptor) to downstream transductional NOS activities. Such findings may represent a first step in the demonstration that NO participates, in a general manner, to the cellular responses obtained upon activation of 5-HT_{2B} receptors. In agreement with this idea, stimulations of 5-HT_{2A} and 5-HT_{2C} receptors have already been shown to trigger increase in cGMP concentration in C6 glioma cells (40) and in choroid plexus (41), respectively. Although an interaction of these two receptors with NOsyn has not yet been described, it is worthwhile to note that type I-PDZ recognition motifs also occur at the C termini of these two proteins (42). Moreover, the possibility that the recruitment of NOsyns by these 5-HT_{2A} receptors modifies their coupling with other macromolecular complexes partners has to be considered. Indeed, such networks might explain the 5-HT-mediated inhibition of the NMDA receptor/NO/cGMP pathway in rat cerebellum and the inhibition of NO synthesis in interleukin-1β-stimulated rat vascular smooth muscle, attributed to the 5-HT_{2C} (43) and the 5-HT_{2A} (37) receptors, respectively. Further work is necessary to reach general conclusions. However, the present finding of the 5-HT_{2B} receptor provides new insight for analyzing the physiological role of 5-HT_{2B} receptors whose localizations (choroid plexus, meningeal tissues, platelets, kidney, lung, etc.) mainly occur at sites of NO production.

PDZ interactions are also involved in the coupling of the 5-HT_{2B} receptor to the iNOS signaling pathway. Indeed, iNOS activation is impaired in LM5 and LMTK-VSSI cells as well as in the three cell types studied here after introduction of the C-terminal 5-HT_{2B} receptor peptide. Interestingly, the 5-HT_{2B} receptors and cNOS is strongly supported by the highly significant correlations found between the binding constants of agonists and antagonists on the receptor and the dose-response effects of the drugs on cNOS activity. cNOS activation occurs whether 5-HT_{2B} receptors couple (1C11*/5HT or LM6) or do not couple (Mastomys) to phosphoinositide hydrolysis. It is therefore likely that the recruitment of cNOS by 5-HT_{2B} receptors does not depend on PLCβ-dependent intracellular Ca^{2+} mobilization. An indirect effect of calcium ions on the length and/or the amplitude of the cNOS response, depending on 5-HT_{2B}-dependent intracellular Ca^{2+} mobilization. An indirect effect of calcium ions on the length and/or the amplitude of the cNOS response, depending on 5-HT_{2B}-mediated PLCβ activation and involving a Ca^{2+}/calmodulin complex, cannot, however, be excluded. Indeed, interactions between the IP_{3} and NO signaling pathways have already been reported (36, 37).

This work also establishes that the cytoplasmic VSYI(D/E) containing C-terminal domain of the 5-HT_{2B} receptor is involved in the recruitment of NOS activity. 5-HT_{2B}/NO coupling is fully abrogated either in LM5 fibroblasts stably transfected with a cDNA encoding a C-terminally truncated form of the 5-HT_{2B} receptor or by disrupting PDZ interactions in all three cell types. The latter effect is obtained through introduction of a competitor peptide corresponding to the 20 C-terminal amino acids of the 5-HT_{2B} receptor. Versions of this peptide harboring mutations in the VSYI(D/E) motif no longer abolish the 5-HT_{2B}-mediated PLCβ activation and involving a Ca^{2+}/calmodulin complex, cannot, however, be excluded. Indeed, interactions between the IP_{3} and NO signaling pathways have already been reported (36, 37).
receptor-mediated iNOS activation is extremely fast if compared with the length of the iNOS response normally observed during infection or inflammation. A 5-HT2B-dependent transcriptional regulation of iNOS activity can be excluded because the steady state level of iNOS transcripts is found constant during the cell response. Therefore the possibility that, upon DOI-mediated induction, iNOS is turned on from an inactive state by post-translational controls only, has to be considered (44, 45).

This study provides evidence that the α subunit of the Go_{13} protein plays a critical role in the mechanisms of 5-HT2B/iNOS coupling. In the three cell types studied, introduction of anti-Go_{13} antibodies fully inhibits iNOS activation, without interfering with the 5-HT2B receptor/cNOS signaling. Inhibition of other endogenous G proteins with the help of anti-Go_{11}, Go_{q}, or Go_{i} antibodies has no consequence on NOS activities. Thus, although the C-cytoplasmic domain of the 5-HT2B receptor plays a pivotal role in the recruitment of all three NOS isoforms, the mechanism that couples the receptor to iNOS differs from that which regulates cNOS. In addition, the relatively low significance of pharmacological correlations between the 5-HT2B receptor binding constants of drugs and the dose-re-

![Fig. 6](https://example.com/fig6.png)

**Fig. 6.** Go_{13} is involved in the 5-HT2B receptor-mediated iNOS but not cNOS-mediated coupling in LM6, IC11*5HT, and Mastomys cells. Anti-Go_{13} antibodies do not interfere with the 5-HT2B receptor-mediated cNOS activation (A) but selectively cancel iNOS activation (B) in the three cell types. The 5-HT2B receptor-mediated iNOS activation is unaffected upon incubation with anti-Go_{13} antibodies neutralized with the corresponding blocking peptide (B). Antibodies against Go_{q}, Go_{i}, or Go_{s} have no effect on the iNOS response in IC11*5HT cells and slightly reduce iNOS activation in LM6 and Mastomys cells (C). In all cases (A–C), NOS activities were measured 10 min after the addition of 100 nM DOI. White bars, control; shaded bars, anti-Go_{13}; hatched bars, anti-Go_{13} incubated with peptide.
sponse effect of the same drugs on iNOS activity (Fig. 4) suggests that the mechanism coupling the 5-HT$_{2B}$ receptor to iNOS is more complex than that acting on cNOS. For instance, instead of resulting from a direct 5-HT$_{2B}$ receptor/Go$_{13}$ coupling, iNOS activation might integrate cross-talk(s) with other 5-HT$_{2B}$ receptor transductional pathways.

Knowledge of the signaling pathways mediating 5-HT$_{2B}$ receptor physiological functions in vivo is of the utmost interest in view of the probable involvement of these receptors in pathological situations such as migraine (32) and hypertension (46). Nevertheless, these pathways may greatly depend on the cell type considered (47), and in vitro cell cultures appear to be an indispensable tool to progressively solve the complexity of 5-HT$_{2B}$-coupling mechanisms. For instance, the present study established that in addition to its role in phosphoinositol hydrolysis (4), p21ras mitogen-activated protein kinase signaling (8), and phospholipaseA$_2$/AA release (5), the 5-HT$_{2B}$ receptor also controls NO/cGMP production through dual activation of cNOS and iNOS. To eventually describe the contribution of such 5-HT-mediated signals to the control of serotonergic functions, corresponding intracellular targets will have to be identified. Hopefully, the availability of the 1C11 cell line, which exhibits all the couplings of the 5-HT$_{2B}$ Receptor described to date in the same integrated serotonergic phenotype, may help dissecting these pathways and their cross-talks.

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