Modified Receptor Internalization upon Coexpression of 5-HT\textsubscript{1B} Receptor and 5-HT\textsubscript{2B} Receptors

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ABSTRACT
Serotonin 5-HT\textsubscript{2B} receptors are often coexpressed with 5-HT\textsubscript{1B} receptors, and cross-talk between the two receptors has been reported in various cell types. However, many mechanistic details underlying 5-HT\textsubscript{1B} and 5-HT\textsubscript{2B} receptor cross-talk have not been elucidated. We hypothesized that 5-HT\textsubscript{2B} and 5-HT\textsubscript{1B} receptors each affect the others' signaling by modulating the others' trafficking. We thus examined the agonist stimulated internalization kinetics of fluorescent protein-tagged 5-HT\textsubscript{2B} and 5-HT\textsubscript{1B} receptors when expressed alone and upon coexpression in LMTK\textsubscript{6} murine fibroblasts. Time-lapse confocal microscopy and whole-cell radioligand binding analyses revealed that, when expressed alone, 5-HT\textsubscript{2B} and 5-HT\textsubscript{1B} receptors displayed distinct half-lives. Upon coexpression, serotonin-induced internalization of 5-HT\textsubscript{2B} receptors was accelerated 5-fold and was insensitive to a 5-HT\textsubscript{1B} receptor agonist. In contrast, coexpression did not render 5-HT\textsubscript{1B} receptor internalization sensitive to a 5-HT\textsubscript{2B} receptor agonist. The altered internalization kinetics of both receptors upon coexpression was probably not due to direct interaction because only low levels of colocalization were observed. Antibody knockdown experiments revealed that internalization of 5-HT\textsubscript{1B} receptors (expressed alone) was entirely clathrin-independent and Caveolin1-dependent, whereas that of 5-HT\textsubscript{2B} receptors (expressed alone) was Caveolin1-independent and clathrin-dependent. Upon coexpression, serotonin-induced 5-HT\textsubscript{2B} receptor internalization became partially Caveolin1-dependent, and serotonin-induced 5-HT\textsubscript{1B} receptor internalization became entirely Caveolin1-independent in a protein kinase C-dependent fashion. In conclusion, these data demonstrate that coexpression of 5-HT\textsubscript{1B} and 5-HT\textsubscript{2B} receptors influences the internalization pathways and kinetics of both receptors.
erosis of migraine headaches (Kalkman, 1994; Schaerling et al., 2003; Poissonnet et al., 2004), an idea supported by the efficacy of 5-HT$_{1B}$ receptor agonist-based antimigraine therapy [sumatriptan succinate (Imitrex), methysergide maleate (Sansert)]. The antimigraine efficacy of 5-HT$_{2B}$ receptor antagonists is less clear but suspected (Schmuck et al., 1996), although the underlying mechanisms remain to be identified.

A general feature of GPCRs is the existence of complex intracellular regulatory mechanisms that modulate receptor responsiveness. Receptor desensitization and down-regulation are well described for various individual GPCR subtypes and are important homeostatic mechanisms. Homologous desensitization involves the desensitization of a particular receptor subtype upon activation of that receptor subtype. In contrast, heterologous desensitization involves the desensitization of receptor subtype(s) upon stimulation of a different receptor subtype. In view of the increasing number of reports of GPCRs participating in complexes via dimerization or scaffolding proteins, cross-talk between receptor subtypes may represent an important additional regulatory mechanism at modulating sensitivity and/or signal transduction. Agonist-induced internalization of GPCRs uses a pathway determined by a kinase that phosphorylates the receptor: for the β1-adrenergic receptor, protein kinase A (PKA)-mediated phosphorylation directs internalization via caveolae, whereas GPCR kinase (GRK)-mediated phosphorylation directs internalization through clathrin-coated pits (Rapaciuolo et al., 2003). The recruitment, activation, and scaffolding of cytoplasmic signaling complexes occurs via two multifunctional adaptor and transducer molecules, β-arrestin-1 and -2 (Leffkowitz and Shenoy, 2005).

Among the described internalization mechanisms, 5-HT$_{1B}$-induced receptor desensitization has been reported for receptors closely related to 5-HT$_{2B}$ receptors (i.e., 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors). Desensitization of 5-HT$_{2A}$ receptor was shown to involve receptor internalization through caveolin1 (Cav1), a scaffolding protein enriched in caveolae, in a number of cell lines expressing exogenous 5-HT$_{2A}$ receptors, and rat brain synaptic membrane preparations (Bhatnagar et al., 2004). There is also evidence for functional interactions among 5-HT$_{2A}$ receptors and other plasma membrane microdomains: 5-HT$_{1B}$-induced 5-HT$_{2A}$ receptor desensitization can also involve receptor internalization through a clathrin- and dynamin-dependent process (Hanley and Hensler, 2002). Internalization and desensitization of 5-HT$_{2A}$ receptors in some cell types is β-arrestin-independent (Gray et al., 2003). A direct interaction between PSD-95 and the 5-HT$_{2A}$ receptor at a type I PSD-95, Dil, ZO-1 (PDZ)-binding domain at the C terminus regulates the receptor’s signal transduction and trafficking (Xia et al., 2003). For the 5-HT$_{2C}$ receptor, constitutively active edited isoform is spontaneously internalized in an agonist-independent manner via the activity of a GRK/β-arrestin (Marion et al., 2004).

Receptor oligomerization is a pivotal aspect of the structure and function of GPCRs that has also been shown to have implications for receptor trafficking, signaling, and pharmacology (George et al., 2002). Serotonin 5-HT$_{2C}$ receptors were shown to exist as constitutive homodimers on the plasma membrane of living cells using a confocal-based fluorescent resonance energy transfer (FRET) method (Herrick-Davis et al., 2004). Inactive 5-HT$_{2C}$ receptors can inhibit wild-type 5-HT$_{2C}$ receptor function by forming nonfunctional heterodimers expressed on the plasma membrane (Herrick-Davis et al., 2005). The 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes that share a high amino acid sequence identity have also been shown to exist as monomers and homodimers when expressed alone and when monomers and heterodimers when coexpressed (Xie et al., 1999). Heterodimerization between 5-HT$_{1}$ and 5-HT$_{2}$ receptors, and its functional consequences have yet to be investigated.

The mechanistic details of 5-HT$_{1B}$ receptors internalization have not yet been determined. Despite the coexpression of 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors in various tissues including endothelial and smooth muscle cells (Ullmer et al., 1995), and given the inhibitory effect of 5-HT$_{2B}$ receptors on 5-HT$_{1B}$ receptor signaling (Tournois et al., 1998), physical interaction between the two receptors seems plausible. The internalization of the 5-HT$_{2B}$ receptor is faster than that of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors (Porter et al., 2001; Schaerling et al., 2003; Deraet et al., 2005), although the mechanism underlying this distinction has not been uncovered. In this work, we investigated potential 5-HT$_{1B}$/2B receptor interactions by examining the colocalization and internalization kinetics of 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors expressed alone or together. Using cyan and yellow fluorescent protein (CFP and YFP) tagged receptors and confocal microscopy, we observed agonist-induced receptor endocytosis in real time. We also performed whole-cell radioligand binding studies as an additional means of measuring receptor internalization. Our results indicate that the stimulation of 5-HT$_{1B}$ receptors affects the internalization dynamics of 5-HT$_{2B}$ Receptors and vice versa, with the effect of 5-HT$_{1B}$ Receptors on 5-HT$_{2B}$ receptors being more pronounced than the effect of the latter receptor on the former. Furthermore, we used an antibody knockdown strategy to ascertain which pathways each receptor used for internalization. Our findings reveal that coexpression of 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors affects both the kinetics of receptor internalization and the internalization pathway employed compared with either receptor expressed alone.

**Materials and Methods**

**Reagents.** RS127445 was kindly provided by Roche (Mannheim, Germany); CP93129, BW72C96, H-89, G6 6850-Bisindolylmaleimide I, G6 6976, and all other chemicals were reagent grade, purchased from usual commercial sources. The radioactive compounds 1-(2,5-dimethoxy-4-[125I]iodophenyl)-2-aminopropane hydrochloride ([125I]IDI; 81.4 TBq/mmol) and [125I]serotonin-5-O-carboxymethylglycyl-ido-tyrosamine ([125I]GIT; 81.4 TBq/mmol) were purchased from PerkinElmer Life and Analytical Sciences. Antibodies that were already specificity-tested were used: rabbit antisera against rodent Cav1 (H-97), clathrin (H-300), GRK2,3 (H-222), and GRK5,6 (C-20) and goat antiserum against rodent β-arrestin-2 (D-18) and PKCe (C-15) (Santa Cruz Biotechnology; Santa Cruz, CA).

**Mutagenesis and 5-HT$_{1B}$ Receptor Constructs.** The fluorescent-tagged fusion proteins of the mouse 5-HT$_{1B}$ and 5-HT$_{1B}$ receptors were generated by PCR-based subcloning. The receptor coding regions were subcloned into pECFP, pEYFP, pPA-GFP (photo-activable GFP) vectors with the XFP fused to the N terminus of the receptors. The entire coding sequence of all constructs was verified by automated DNA sequencing.

**Cell Culture.** 5-HT$_{2A}$ and 5-HT$_{1B}$ receptor cDNAs were stably transfected into nontransformed murine fibroblast (LMTK) cells, which are devoid of endogenous 5-HT receptors because they do not exhibit a concentration-dependent rise in second messengers after...
5-HT stimulation (Manivet et al., 2000). LMTK– cell lines were routinely cultivated in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum and 40 μg/ml gentamicin. The stably expressing mouse 5-HT1B and 5-HT2B cell lines were generated by calcium phosphate transfection followed by selection with either G-418 or hygromycin and clonal isolation. Stable receptor-expressing lines were subcultured in serum free medium for at least 24 h before experiments. Stable cell lines with different combinations of receptor expression insured the absence of individual cell based effects.

**Radioligand Binding Experiments.** Radioligand binding experiments were performed using [125I]DOI or [125I]GTI either on intact cells or on membranes from stably transfected cells as previously detailed (Loric et al., 1995).

**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/10⁶ cells at 22°C under agitation as described previously (Manivet et al., 2000).

**Internalization Measurements by Confocal Microscopy.** For confocal microscopic studies of living cells, stable or transiently transfected cell lines were plated 36 h before the analysis on 35-mm glass-bottomed dishes (MatTek, Ashland, MA) and grown in a 5.4% CO2 incubator. Twelve hours before the experiment, cells were washed in Dulbecco’s modified Eagle’s medium without serum and maintained in this medium until the experiment. Confocal analysis were performed at 22°C unless otherwise mentioned in the text to extend the kinetic and 37°C was used to confirm the observed phenomenon at 22°C. Cells were visualized using a confocal microscope (SP2AOBS; Leica, Wetzlar, Germany) with laser excitation lines of 458 nm and 514 nm for CFP- and YFP-tagged receptors and transmitted light. Images of CFP and YFP emission were recorded simultaneously with the transmitted light images. The emission recording channels and the intensity of the excitation lasers were carefully chosen using single tag control linear unmixing technique (Leica) to avoid bleed-through. Images (in xyz mode) were recorded sequentially every 5 min in two different emission intervals (462–500 nm for CFP and 520–600 nm for YFP) with two different excitation wavelengths (458 and 514 nm). To ensure consistency among z-plane images during time-lapse study, we took at least five z-plane images and manually selected only one plane for the time-lapse analysis. To visualize receptor internalization after agonist treatment, time-lapse series were taken every 5 min over a 30-min period. To calculate the internalization kinetics of the receptors, we selected 10 regions of interest (ROIs) on the plasma membrane per cell and followed the relative intensity changes of these ROIs by time, including at least three to five individual cells per experiment. Data represent more than four independent experiments. The kinetic curves were corrected for bleaching by using the intensity of the whole cell as a normalization factor. PA-GFP was activated with a 405 nm diode laser line pulse lasting 10 to 15 sec and visualized between 495 and 515 nm using excitation at 488-nm.

**FRET Measured by Confocal Microscopy.** For the colocalization of 5-HT1B and 5-HT2B receptors in living cells, sensitized emission fluorescent resonance energy transfer (FRET) was used. Two additional channels (the FRET channel (excitation, 458 nm; emission, 520–600 nm) and a control channel (excitation, 514 nm; emission, 462–500 nm)) were recorded along with the CFP and YFP channels as described above. The bleed-through of CFP and the direct excitation of YFP by the 458 nm laser light were subtracted from the FRET channel signal. To estimate these artifacts, we used cells transfected with either CFP- or YFP-tagged receptors. Calculation of corrected FRET was carried out on a pixel-by-pixel basis for the entire image. Indeed, positive FRET signal could be obtained using tagged proteins known to interact in similar experimental set-ups (data not shown).

**Colocalization Calculation.** We considered two proteins to be colocalized if the observed signals of the two corresponding labels were nonzero at the same pixel. The quantitative estimate of colocalization is given as the colocalization coefficients (Manders et al., 1992). To quantify the colocalized fraction of each receptor pair, a threshold value for each channel was estimated and subtracted. Bleed-through in each of the two detecting channels was subtracted using the linear unmixing method (Leica).

**Data Analysis.** Binding data were analyzed using the iterative nonlinear regression model (Prism 2.0; GraphPad Software, San Diego, CA). This allowed the calculation of dissociation constants (Kd) and the number of sites (Bmax). All values represent the average of independent experiments ± S.E.M. (n = number of experiments as indicated in the text). Comparisons between groups were performed using Student’s unpaired t test or analysis of variance and a Fischer test. Significance was set at p < 0.05.

**Results**

Radioligand saturation binding assays were performed on membranes from stable, clonal cell lines expressing either 5-HT1B or 5-HT2B Receptors, bearing various N-terminal fluorescent protein tags as well as on cells coexpressing both receptors. The clones chosen for subsequent experiments were selected so as to approximate physiological receptor expression (Bmax of approximately 100 fmol/mg of protein) (Tables 1 and 2). In control experiments with nontagged receptors, we verified that none of the N-terminal fluorescent tags affected radioligand affinity (Tables 1 and 2). In addition, whole-cell radioligand binding experiments on clones expressing either fluorescent protein-tagged or nontagged receptors yielded a Bmax approximately 60% of that mea-

<table>
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<th>Table 1</th>
<th>Pharmacological properties of native or GFP-tagged receptors</th>
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<td><strong>Saturation binding assays were performed using [125I]DOI (5-HT1B receptor) or [125I]GTI (5-HT2B receptor) on membrane fraction of cell homogenate (Membranes) or intact cells (Whole Cells) of the different stable clonal cell lines expressing the 5-HT1B receptor and the 5-HT2B receptor alone with or without tag. Binding data were analyzed using the iterative nonlinear fitting software GraphPad Prism 2.0 to calculate dissociation constants (Kd) and maximum number of sites (Bmax). Insertion of the various GFPs at the N-terminal part of the receptor did not affect receptor affinity. Intact cell Bmax represents only about 60% of that of binding on membranes. Values are means ± S.E. of three independent determinations in triplicate.</strong></td>
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<td><strong>Membrane</strong></td>
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<td><strong>Bmax</strong></td>
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<td>5-HT1B-YFP clone 9</td>
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<td>5-HT1B clone 5</td>
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<td>5-HT1B clone 17</td>
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<tr>
<td>5-HT1B-YFP clone 22</td>
<td>28.8 ± 5.8</td>
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* P < 0.05 versus non-tagged receptors by Student’s t test.
Kinetcs of 5-HT-Induced Internalization of 5-HT2B and 5-HT1B Receptors. To establish a quantitative method to measure internalization kinetics of the tagged receptors, fluorescence intensity at the membrane was compared with that in the cytoplasm in cells treated with 100 nM 5-HT for 0 to 30 min. The time-dependent fluorescence intensity changes at the plasma membrane and in the cytoplasm both fit a one-phase exponential decay (decrease for the plasma membrane, increase for the cytoplasm) and had similar half-lives (5-HT2B receptor cytoplasm $t_{1/2} = 23.0 \pm 3.0$ min; membrane $t_{1/2} = 22.2 \pm 3.3$ min; 5-HT1B receptor cytoplasm $t_{1/2} = 9.8 \pm 2.2$ min, membrane $t_{1/2} = 10.7 \pm 1.4$ min). Because the signal-to-noise ratio was higher for the measurement of decreases in plasma membrane fluorescence, this was chosen to measure endocytosis kinetics at the plasma membrane (Fig. 1, A and B). To further confirm that we were measuring receptor endocytosis, we performed experiments with photoactivatable (PA) GFP-tagged 5-HT1B receptors and compared the rate of decrease in fluorescence intensity of the illuminated membrane region with that determined for plasma membrane YFP-tagged 5-HT1B receptors. As before, for both GFP- and YFP-tagged 5-HT1B receptors, the internalization kinetics fitted a one-phase exponential decay and were identical (5-HT1B-PA-GFP $t_{1/2} = 9.8 \pm 0.7$ min; 5-HT1B-YFP $t_{1/2} = 10.1 \pm 1.2$ min), further validating our image-based analysis of receptor internalization kinetics.

Similar confocal laser microscopy studies with various concentrations of 5-HT revealed a concentration dependence on internalization kinetics. At 100 nM, 5-HT-induced internalization of 5-HT1B receptors was twice as fast as that of 5-HT2B receptors; at higher concentrations of 5-HT, internalization of both receptors occurred at similar rate. Furthermore, the 5-HT-induced 5-HT2B receptor internalization rate increased twice as fast as that of 5-HT1B receptors as a function of 5-HT concentration (Fig. 1C).

Having established internalization kinetics for both receptors expressed alone, we set out to ascertain whether coexpression altered 5-HT-induced 5-HT1B and 5-HT2B receptor internalization rates. Coexpression with the 5-HT1B receptor resulted in a 5-fold increase in the rate of 5-HT-induced 5-HT2B receptor internalization (2B1H) ($t_{1/2} = 4.0 \pm 1.5$ min versus 23.0 $\pm$ 3.0) (Fig. 1, D and E). On the other hand, coexpression with 5-HT2B receptors had no effect on 5-HT-induced 5-HT1B receptor internalization rate ($1B2B$) ($t_{1/2} = 9.0 \pm 1.0$ min versus 9.8 $\pm$ 2.2) (Fig. 1, D and E). Kinetic whole-cell radioligand binding experiments corroborated our microscopy data, revealing the asymmetric effect of receptor coexpression on 5-HT-induced 5-HT1B and 5-HT2B receptor internalization rate (Fig. 1, E and F).

Agonist-Dependent 5-HT2B Receptor Internalization. To determine whether the effect of 5-HT1B receptors on 5-HT2B Receptor internalization kinetics involved activation of 5-HT2B receptors, we stimulated cells coexpressing both receptors in the absence and presence of the highly selective 5-HT2B receptor antagonist RS127445 (RS). In cells expressing 5-HT2B receptor alone, 100 nM RS completely blocked 5-HT-induced 5-HT2B receptor internalization. It was striking that RS had no effect on 5-HT-induced 5-HT2B Receptor internalization in cells coexpressing 5-HT1B receptors (Table 3). RS did not significantly affect 5-HT-induced 5-HT1B receptor internalization irrespective of 5-HT2B receptor coexpression.

To investigate whether modulation of internalization kinetics was agonist-dependent, we stimulated with the preferential 5-HT2B receptor agonist BW723C86 (BW). Treatment with 50 nM BW induced no 5-HT1B receptor internalization but did stimulate 5-HT2B receptor internalization ($t_{1/2} = 11.0 \pm 1.5$ min) in noncoexpressing cells (Fig. 2A; Table 3). Coexpression of 5-HT1B and 5-HT2B receptors did not significantly alter the effect of BW on the internalization of either receptor: the internalization kinetics of 5-HT2B receptors changed only slightly in the presence of 5-HT1B receptor ($t_{1/2} = 7.2 \pm 1.1$ min) (Fig. 2B; Table 3), whereas no internalization of 5-HT1B receptors was observed (Fig. 2C).

**Agonist-Dependent 5-HT1B Receptor Internalization.** To further investigate putative reciprocal interactions between 5-HT1B and 5-HT2B receptors, we studied the agonist-induced internalization kinetics of the 5-HT1B receptor. The selective agonist CP93129 (CP, 75 nM), induced 5-HT1B receptor internalization ($t_{1/2} = 14.1 \pm 0.7$) but did not affect 5-HT2B receptor distribution in noncoexpressing cells (Fig. 2D). We observed similar CP-induced internalization kinetics for both receptors ($t_{1/2} = 6.9 \pm 0.9$ min for 5-HT2B receptors; $t_{1/2} = 4.9 \pm 1.3$ min for 5-HT1B receptors) upon coexpression (Fig. 2E; Table 3). It is noteworthy that coexpression with 5-HT2B receptors caused a 3-fold increase in CP-induced internalization of 5-HT1B receptors ($t_{1/2} = 4.9 \pm 1.3$ min versus $t_{1/2} = 14.1 \pm 0.7$ min) (Fig. 2F; Table 3). These results reveal that, whereas coexpression with 5-HT2B receptor does not affect BW-induced 5-HT1B receptor internalization, the presence of 5-HT2B receptor does modulate CP-induced 5-HT1B receptor internalization.

**TABLE 2**

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<th>Receptors</th>
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<tr>
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<td>$K_D$ ($nM$)</td>
<td>$B_{max}$ ($nM$)</td>
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<tr>
<td>5-HT1B-YFP/5-HT2B</td>
<td>0.79 ± 0.15</td>
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<td>5-HT1B-YFP/5-HT2B-CFP</td>
<td>0.73 ± 0.12</td>
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Pharmacological properties of native or GFP-tagged coexpressed receptors

Saturation binding assays were performed using [125I]DOI (5-HT2B receptor) or [125I]GTI (5-HT1B receptor) on membrane fraction of cell homogenate (Membranes) or intact cells (Whole Cells) of the different stable clonal cell lines expressing the 5-HT1B receptor and the 5-HT2B receptor with or without tag. Binding data were analyzed using the iterative non-linear fitting software GraphPad Prism 2.0 to calculate dissociation constants ($K_D$) and maximum number of sites ($B_{max}$). Insertion of the various GFPs at the N-terminal part of the receptor did not affect receptor affinity. Intact cell $B_{max}$ represents only about 60% of that of binding on membranes. Co-expression of the receptor subtypes did not alter the receptor affinity for GTI or DOI (Table 1). Values are means ± S.E. of three independent determinations in triplicate.
Fig. 1. 5-HT stimulus on 5-HT$_{2B}$ and 5-HT$_{1B}$ receptor, dose dependence. Confocal studies in living cells were performed on various LMTK$^+/H11002$ -transfected cell lines plated on glass-bottomed dishes in Dulbecco’s modified Eagle’s medium without serum. Quantitative internalization kinetics of the GFP-tagged receptors was assessed by measuring the fluorescence intensity disappearance at the membrane compared with the intensity increase in the cytoplasm of approximately 10 ROI per cell and followed their relative intensity changes. Both signals could be fitted with a single exponential function over time, giving the same half-time. A, series of single confocal plane images taken from living cells expressing 5-HT$_{1B}$ receptor-GFP by time lapse video were used to evaluate the internalization kinetics after stimulation by 100 nM 5-HT (right). Distribution of 5-HT$_{1B}$ receptors expressed at 0 (0) and 50 (50) min of 5-HT stimulation. B, series of single confocal plane images was taken from living cells expressing 5-HT$_{2B}$ receptor-GFP by time lapse video. Internalization kinetics after stimulation by 100 nM 5-HT stimulation (right). Distribution of 5-HT$_{2B}$ receptors expressed at 0 and 50 min of stimulation. These images are representative of more than three cells observed in each of at least four independent experiments. Scale bars, 2$\mu$m. C, similar studies by confocal laser microscopy showed that 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors internalized with a single exponential kinetics after stimulation with 100 nM, 1$\mu$M, or 10$\mu$M 5-HT, and their half-lives were concentration-dependent. D, study by confocal laser microscopy showed that when 5-HT$_{2B}$ receptors were coexpressed with 5-HT$_{1B}$ receptors (5-HT$_{2B}^{1B}$), the 5-HT$_{2B}$ receptor internalized with a single exponential kinetics after stimulation with 100 nM 5-HT, but its half-life became five times faster. E, when 5-HT$_{2B}$ receptors were coexpressed with 5-HT$_{1B}$ receptors (5-HT$_{2B}^{1B}$ light gray bar), the 5-HT$_{1B}$ receptor internalized after stimulation with 100 nM 5-HT with a similar half-life to 5-HT$_{1B}$ receptor alone (black bar). F, independent radioligand binding experiments using $[^{125}\text{I}]{\text{DOI}}$ (5-HT$_{2B}$ receptor) or $[^{125}\text{I}]{\text{GTI}}$ (5-HT$_{1B}$ receptor) on intact living cells confirmed that stimulated with 100 nM 5-HT decreased the 5-HT$_{2B}$ receptor (5-HT$_{2B}^{1B}$ white bar) half-life but not that of 5-HT$_{1B}$ receptors (5-HT$_{1B}^{1B}$ light gray bar). Graphs display the mean± S.E.M. of at least three independent experiments.
Temperature Dependence of $5\text{-HT}_{1\text{B}}$ and $5\text{-HT}_{2\text{B}}$ Receptor Internalization. To verify that agonist-induced receptor internalization was due to an energy-dependent endocytic process, receptor internalization assays were performed at various temperatures. As expected, the kinetics of $5\text{-HT}$-induced receptor internalization were faster at higher temperatures for both receptors, irrespective of coexpression. In addition, the temperature dependence of agonist ($5\text{-HT}$ or subtype selective)-induced receptor internalization rate appeared linear for $5\text{-HT}_{1\text{B}}$ receptors and biphasic for $5\text{-HT}_{2\text{B}}$ receptor when expressed alone (Fig. 3, A and B).

Coexpression of both receptors did not seem to affect the effect of temperature on CP-induced $5\text{-HT}_{1\text{B}}$ receptor internalization (Fig. 3C). On the other hand, coexpression with $5\text{-HT}_{1\text{B}}$ receptors rendered the temperature dependence of BW-induced $5\text{-HT}_{2\text{B}}$ receptor internalization more linear (Fig. 3D). Furthermore, the temperature dependence of CP-induced $5\text{-HT}_{2\text{B}}$ receptor internalization rate was identical to that observed for $5\text{-HT}_{1\text{B}}$ receptors (Fig. 3E).

Microscopic Analysis Revealed No Receptor Colocalization. The apparent effect of receptor coexpression on agonist-induced $5\text{-HT}_{2\text{B}}$ and $5\text{-HT}_{1\text{B}}$ receptor internalization led us to hypothesized receptor heterodimerization. Therefore, we performed confocal microscopy on cells coexpressing CFP-$5\text{-HT}_{1\text{B}}$ and YFP-$5\text{-HT}_{2\text{B}}$ receptors. Cellular distribution analysis of the two receptors revealed approximately 20% colocalization in the plasma membrane before stimulation (Fig. 4). After 30 min of agonist stimulation, cytoplasmic

TABLE 3

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<tr>
<th>Ligand dependence of $5\text{-HT}<em>{1\text{B}}$ receptor and $5\text{-HT}</em>{2\text{B}}$ receptor internalization</th>
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<td>Quantitative internalization kinetics of the GFP-tagged receptors was assessed by measuring the fluorescence intensity disappearance at the membrane for more than 10 ROIs per cell and following the relative intensity changes of these ROI by time. In $5\text{-HT}<em>{2\text{B}}$ receptors or $5\text{-HT}</em>{1\text{B}}$ receptor transfected cells (single), this signal could be fitted with a single exponential function over time. Applying the same protocol on $5\text{-HT}<em>{1\text{B}}$ receptors in the presence of $5\text{-HT}</em>{1\text{B}}$ receptors transfected cells (double), a change in internalization rate could be observed. Values are means ± S.E. of more than three cells analyzed in at least four independent experiments.</td>
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<tr>
<td><strong>Agonist</strong></td>
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* P < 0.05 by Student’s t test $5\text{-HT}_{2\text{B}}$ versus $5\text{-HT}_{1\text{B}}$ receptors. 

# P < 0.05 by Student’s t test single versus double-transfected receptors.

Fig. 2. Ligand dependence of $5\text{-HT}_{1\text{B}}$ receptor and $5\text{-HT}_{2\text{B}}$ receptor internalization. Quantitative internalization kinetics of the GFP-tagged receptors was assessed by measuring the fluorescence intensity disappearance at the membrane of series of single confocal plane images taken from living transfected LMTK<sup>+</sup> cells by time lapse video. A–C, the fluorescence intensity disappearance has been fitted with a single exponential function over time and used to evaluate the internalization kinetics after stimulation by the preferential $5\text{-HT}_{2\text{B}}$ receptor agonist BW723C86 (BW) at the final concentration of 50 nM that produced no effect on the internalization of $5\text{-HT}_{1\text{B}}$ receptor alone. D–F, the fluorescence intensity disappearance has been fitted with a single exponential function over time and used to evaluate the internalization kinetics after stimulation by the selective $5\text{-HT}_{2\text{B}}$ receptor agonist CP93129 (CP) at the final concentration of 75 nM that produced no effect on the internalization of $5\text{-HT}_{2\text{B}}$ receptor alone. The x-axes display 0 to 30 min recording for fast internalization (A–C and E) and 0 to 60 min for slow internalization (D and F). Deduced half-life values are reported in Table 3 with statistics.
The colocalization of 5-HT_{2B} and 5-HT_{1B} receptors was still approximately 20%, irrespective of agonist. Using enhanced emission to measure FRET, we observed nearly no FRET signal at 5-HT_{1B}/5-HT_{2B} receptor colocalization points (Fig. 4). Thus, our image-based analysis of colocalization was not consistent with agonist-induced 5-HT_{1B}/5-HT_{2B} receptor complex formation.

**Serotonin 5-HT_{2B} and 5-HT_{1B} Receptor Internalization Occurs via Distinct Pathways.** In the absence of evidence supporting 5-HT_{1B}/5-HT_{2B} heterodimerization, we investigated the internalization pathways used by the receptors. We performed whole-cell binding studies on cells permeated with antibodies against proteins known to be involved in GPCR internalization. Cell surface receptor expression (B_{max}) was measured as a function of agonist exposure time to measure internalization half-life. It is noteworthy that the alveolysin and antibody treatments did not affect agonist and radioligand affinities (data not shown).

We observed that the internalization of 5-HT_{1B} receptors expressed alone was independent of GRK5,6 (data not shown) and clathrin but totally dependent on Cav1 and GRK2,3 when stimulated by 5-HT or CP (Fig. 5, A and B). The internalization of 5-HT_{2B} receptors expressed alone was independent of GRK5,6 (data not shown) and Cav1 but completely dependent on clathrin and β-arrestin-2 when stimulated by 5-HT or BW (Fig. 5, A–C). These results established that 5-HT_{1B} and 5-HT_{2B} receptors, when expressed alone, used distinct internalization pathways in an identical cell background.

We next applied the antibody knockdown strategy to cells coexpressing 5-HT_{1B} and 5-HT_{2B} receptors. When stimulated by 5-HT or CP, but not BW, the internalization of 5-HT_{2B} receptors became partially sensitive to Cav1 antibodies. Furthermore, the internalization of 5-HT_{2B} receptors was entirely sensitive to Arrestin-2 antibodies when stimulated with 5-HT in the absence or presence of 5-HT_{1B} receptors, and with CP in the presence of 5-HT_{1B} receptors (Fig. 5, A and B). However, 5-HT_{2B} receptor internalization was only partially inhibited by anti-β-Arrestin-2 when coexpressed with 5-HT_{1B} receptors and stimulated with BW (Fig. 5C). This result further suggested that 5-HT_{1B} receptors could affect the internalization pathway of 5-HT_{2B} receptors. Finally, the agonist-induced internalization of 5-HT_{2B} receptors was partially dependent on GRK2,3 when coexpressed with 5-HT_{1B} receptors and stimulated with CP (Fig. 5D).
with 5-HT<sub>1B</sub> receptors and stimulated with 5-HT, CP, or BW (Fig. 5). Thus, the effect of 5-HT<sub>1B</sub> receptor coexpression on 5-HT<sub>2B</sub> receptors caused a fraction of 5-HT<sub>2B</sub> receptors to internalize via a Cav1- and GRK2,3-dependent pathway.

With respect to 5-HT<sub>1B</sub> receptors, coexpression with 5-HT<sub>2B</sub> receptors caused 5-HT-induced 5-HT<sub>1B</sub> receptor internalization to become totally independent of Cav1 and GRK2,3. Furthermore, upon coexpression with 5-HT<sub>2B</sub> receptors, 5-HT-induced 5-HT<sub>1B</sub> receptor internalization was still independent of clathrin and Arrestin-2. In contrast, the Cav1/GRK2,3 dependence of CP-induced 5-HT<sub>1B</sub> receptor internalization was not affected by coexpression with 5-HT<sub>2B</sub> receptors. Thus, the effect of 5-HT<sub>2B</sub> receptor coexpression on 5-HT<sub>1B</sub> receptor internalization is to alter the 5-HT-induced internalization pathway from a fully Cav1-dependent pathway to one fully independent of both Cav1 and clathrin (Fig. 5).

Serotonin-Induced Stimulation of PKCε by 5-HT<sub>2B</sub> Receptors Regulates the Pathway of 5-HT<sub>1B</sub> Receptor Internalization. To investigate the non–Cav1-dependent/ non–clathrin-dependent internalization pathway used by 5-HT-stimulated 5-HT<sub>1B</sub> receptors coexpressed with 5-HT<sub>2B</sub> receptors, we tested the effect of various protein kinase inhibitors. The wide protein kinase inhibitor staurosporine (5
μM), which inhibits PKC, PKA, and protein kinase G, blocked 5-HT-induced 5-HT_{1B} receptor internalization when coexpressed with 5-HT_{2B} receptors (Fig. 6). H89 (5 μM), which inhibits PKA, protein kinase G, and PKC, but not other PKCs (Davies et al., 2000), had no effect on 5-HT_{1B} or 5-HT_{2B} receptor internalization. To further refine these results, we used PKC isotype-selective inhibitors to identify the PKC isozyme involved. We found that Gö 6850-Bisindolylmaleimide I (100 nM), a PKC inhibitor with high selectivity for PKC_{α, β, δ, ε} isozymes, completely prevented 5-HT-induced 5-HT_{2B} receptor internalization or that of 5-HT_{1B} receptors in the presence of 5-HT_{2B} receptors. This blocking effect was not observed with Gö 6976 (100 nM), which selectively inhibits the Ca^{2+}-dependent PKC_{β I, II}. Finally, the blocking effect of Gö 6850-Bisindolylmaleimide I was completely reproduced using PKC_{α} antibody knockdown (Fig. 6). These data indicate that the 5-HT stimulation of 5-HT_{2B} receptors triggers 5-HT_{1B} receptor internalization via a pathway that requires 5-HT_{2B} receptor dependent PKC_{α} activation.

**Discussion**

Given 1) the wide in vivo coexpression of 5-HT_{1B} and 5-HT_{2B} receptors (Kellermann et al., 1996; Ishida et al., 1998, 1999; Stefulj et al., 2000; Banes and Watts, 2003; Nicholson et al., 2003), 2) the established inhibitory effect of 5-HT_{2B} on 5-HT_{1B} receptor signaling (Tournois et al., 1998), 3) the clinical utility of 5-HT_{1B} Receptor agonists, and 4) the putative efficacy of 5-HT_{2B} receptor antagonists in treating migraines, knowledge about regulatory mechanisms between the two receptors is of high interest for understanding current and designing novel pharmaceuticals for therapeutic treatments. The main finding of this article is the asymmetric, agonist-dependent cross-regulation of 5-HT_{1B} and 5-HT_{2B} Receptor internalization. The evidence for this cross-regulation is that the 5-HT_{1B} receptor agonist CP causes 5-HT_{2B} receptor to internalize only if 5-HT_{1B} receptor is present, whereas the 5-HT_{2B} receptor agonist BW does not similarly affect 5-HT_{1B} receptors. However, CP-induced internalization of 5-HT_{1B} receptor is faster when 5-HT_{2B} receptors are present, demonstrating an effect of 5-HT_{2B} receptors on 5-HT_{1B} receptor internalization. Furthermore, coexpression with 5-HT_{2B} Receptors causes 5-HT_{1B} receptors to adopt a Cav1- and clathrin-independent but PKC-dependent 5-HT-induced internalization, whereas a portion of 5-HT_{2B} Receptors assumes a Cav1-dependent 5-HT-induced internalization pathway.

The present results demonstrate that individually, 5-HT_{1B}...
and 5-HT$_{2B}$ receptors expressed in nontransformed mouse fibroblast LMTK$^-$ cells use typically described agonist-dependent internalization pathways. The differences in the kinetics and temperature dependence of internalization strongly support the notion that these two receptors—when expressed alone—use different endocytic pathways, each agonist leading to specific output. The antibody knock-down experiments validate these findings and demonstrate that 5-HT$_{1B}$ receptors expressed alone internalize via a Cav1- and GRK2,3-dependent pathway, whereas 5-HT$_{2B}$ receptors expressed alone internalize via a clathrin-, β-arrestin-2-, and PKC$\varepsilon$-dependent pathway (Fig. 7A).

The coexpression of these two receptors influences their respective internalization kinetics according to the agonist used for stimulation, although in the absence of apparent colocalization (apparent lack of colocalization and of FRET). It is noteworthy that a slight acceleration of BW-induced internalization of 5-HT$_{2B}$ receptors can be observed when expressed with 5-HT$_{1B}$ receptors. However, a large acceleration of CP-induced 5-HT$_{1B}$ receptor internalization is triggered by the presence of 5-HT$_{2B}$ receptors. A marked modification of the thermodynamic profile of 5-HT$_{2B}$ receptor internalization is also observed in the presence of 5-HT$_{1B}$ receptors: the linear temperature dependence observed for internalization of 5-HT$_{1B}$ and 5-HT$_{1B}$/5-HT$_{2B}$ receptors, but not for 5-HT$_{2B}$ receptors expressed alone, strongly supports the notion that coexpression of 5-HT$_{1B}$ receptors with 5-HT$_{2B}$ receptors imposes a alternate internalization pathway on 5-HT$_{2B}$ receptors. Stimulation with 5-HT in the presence of CP activates 5-HT$_{1B}$ receptors that in turn activate GRK2,3, which leads to agonist-independent, Cav1-dependent internalization of 5-HT$_{2B}$ receptors. One likely possibility is that GRK2,3 phosphorylates 5-HT$_{2B}$ receptors in a way that enables them to use a Cav1-dependent internalization pathway (Fig. 7B).

Our experimental results implicate GRK2,3 in mediating the cross-regulation of 5-HT$_{2B}$ receptor internalization by 5-HT$_{1B}$ receptors. When 5-HT$_{2B}$ receptors are coexpressed with 5-HT$_{1B}$ receptors, CP activates 5-HT$_{1B}$ receptors that in turn activate GRK2,3, which leads to agonist-independent, Cav1-dependent internalization of 5-HT$_{2B}$ receptors. One possible explanation for this discrepancy is that the partial agonist stimulation could phosphorylate—either directly or indirectly—the 5-HT$_{1B}$ receptor, rendering it unable to internalize via a Cav1-dependent pathway. One inconsistency between our experimental data and the proposed model is that activation of 5-HT$_{2B}$ receptors by BW does not affect the 5-HT$_{1B}$ receptor internalization. Possible alternative, or additionally, both 5-HT$_{1B}$-induced 5-HT$_{1B}$ receptor GRK2,3 activation and 5-HT$_{2B}$-induced 5-HT$_{2B}$ receptor PKC$\varepsilon$.
activation are required for 5-HT_{1B} receptors to internalize via the Cav1- and clathrin-independent pathway (Fig. 7C). This cross-talk, which affects receptor internalization mechanics, is likely to explain the previously observed Gi uncoupling of 5-HT\textsubscript{1B} receptors by 5-HT\textsubscript{2B} receptors. Activation of the 5-HT\textsubscript{2B/2C} receptor has been shown to inhibit the 5-HT\textsubscript{1B} receptor function in two independent studies:

1. Using 5-HT\textsubscript{2C} and 5-HT\textsubscript{2A} receptors, stably transfected Chinese hamster ovary cells, it has been reported that activation of 5-HT\textsubscript{2C} receptors abolishes the endogenous 5-HT\textsubscript{1B} receptor-mediated inhibition of forskolin-stimulated cAMP accumulation. In contrast, activation of 5-HT\textsubscript{2A} receptors does not alter the 5-HT\textsubscript{1B} response and 5-HT\textsubscript{2C} receptor-mediated inhibition of 5-HT\textsubscript{1B} receptor function was blocked when 5-HT\textsubscript{2A} receptors were activated simultaneously (Berg et al., 1996).

2. Using the teratocarcinoma-derived cell line 1C11 that expresses 5-HT\textsubscript{1B} and 5-HT\textsubscript{2B} and then 5-HT\textsubscript{2A} receptors endogenously and sequentially, Tournois et al. (1998) showed that at day 2 of differentiation, when 5-HT\textsubscript{1B} and 5-HT\textsubscript{2B} receptor expression is induced, 5-HT\textsubscript{2B} receptors exert a dominant-negative regulation of the G\textsubscript{i2} coupled 5-HT\textsubscript{1B} Receptor; at day 4, when functional 5-HT\textsubscript{2A} receptors begin to be expressed, 5-HT\textsubscript{2A} receptor activation prevents the negative regulation exerted by 5-HT\textsubscript{2B} receptor on 5-HT\textsubscript{1B} receptor function. Therefore, it is plausible that 5-HT\textsubscript{2B} and 5-HT\textsubscript{2A} receptors should share a common intracellular internalization and signaling pathway by which to control 5-HT\textsubscript{1B} function, whereas 5-HT\textsubscript{2A} receptors use alternate pathway(s).

This newly described internalization route fits with other evidence supporting independent intracellular trafficking of 5-HT\textsubscript{1B} and 5-HT\textsubscript{2B} receptors (i.e., internalization of 5-HT\textsubscript{2B} receptors upon activation of 5-HT\textsubscript{1B} receptors despite the apparent lack of agonist-induced colocalization). Upon coexpression, stimulating one or the other receptor or both generates different cellular responses; this fact is supported by completely independent techniques (i.e., confocal microscopy image analysis and antibody knock-down coupled with whole-cell radioligand binding studies). This work provides the first evidence that one receptor may adopt different internalization pathways within the same cells upon the presence and stimulation of another receptor. Identified interactions regulate receptor internalization and could explain the observed coordination between 5-HT\textsubscript{1B} and 5-HT\textsubscript{2B} receptor internalization. Our work suggests that indirect events in trans are mediating the 5-HT\textsubscript{1B}/5-HT\textsubscript{2B} receptor cross-regulation that affects their cellular distribution during the endocytic process. Given the wide clinical use of 5-HT\textsubscript{1B} receptor agonists in the treatment of migraines, and the suspected prophylactic effect of 5-HT\textsubscript{2B} receptor antagonists, these newly identified functional interactions may be involved in therapeutic effects of these compounds. The phenomenon may also be relevant to the design of novel antimigraine therapies.

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