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Modified Receptor Internalization upon Co-expression of 5-HT_{1B} Receptor and 5-HT_{2B} Receptors

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Running title. Co-internalization of 5-HT_{1B} and 5-HT_{2B} receptors

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Abstract.

Serotonin 5-HT_{2B} receptors are often co-expressed with 5-HT_{1B} receptors, and crosstalk between the two receptors has been reported in various cell types. However, many mechanistic details underlying 5-HT_{1B} and 5-HT_{2B} receptors crosstalk have not been elucidated. We hypothesized that 5-HT_{2B} and 5-HT_{1B} receptors affect each other's signaling by modulating each other's trafficking. We thus examined the agonist stimulated internalization kinetics of fluorescent protein-tagged 5-HT_{2B} and 5-HT_{1B} receptors when expressed alone and upon co-expression in $LMTK^-$ murine fibroblasts. Time-lapse confocal microscopy and whole-cell radioligand binding analyses revealed that 5-HT_{2B} and 5-HT_{1B} receptors when expressed alone displayed distinct half-lives. Upon co-expression, serotonin-induced internalization of 5-HT_{2B} receptors was accelerated five-fold, and insensitive to a 5-HT_{2B} receptor antagonist. In this context, 5-HT_{2B} receptors did internalize in response to a 5-HT_{1B} receptor agonist. In contrast, co-expression did not render 5-HT_{1B} receptor internalization sensitive to a 5-HT_{2B} receptor agonist. The altered internalization kinetics of both receptors upon co-expression was likely not due to direct interaction as only low levels of co-localization were observed. Antibody knock-down experiments revealed that internalization of 5-HT_{1B} receptors (expressed alone) was entirely clathrin-independent and Caveolin1-dependent, while that of 5-HT_{2B} receptors (expressed alone) was Caveolin1-independent and clathrin-dependent. Upon co-expression, serotonin-induced 5-HT_{2B} receptor internalization became partially Caveolin1-dependent, and serotonin-induced 5-HT_{1B} receptor internalization became entirely Caveolin1-independent in a protein kinase Cepsilon-dependent fashion. In conclusion, these data demonstrate that co-expression of 5-HT_{1B} and 5-HT_{2B} receptors influences the internalization pathways and kinetics of both receptors.

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Introduction.

Serotonin (5-hydroxytryptamine, 5-HT) is a potent vasoactive molecule and also a major neurotransmitter in both the central and peripheral nervous systems (Hoyer et al., 2002). All 5-HT receptors, except 5-HT_3, belong to the rhodopsin-like G protein-coupled receptor (GPCR) superfamily. The 5-HT_{1B}, 5-HT_{2B}, and 5-HT_{2A} receptors were found in endothelial and smooth muscle cells from several human and mouse arteries at mRNA, protein and functional levels (Ullmer et al., 1995; Watts et al., 1996). Also, various human meningeal tissues have been found to co-express 5-HT_{1B} and 5-HT_{2B} receptor mRNA (Schmuck et al., 1996). Thus, given their co-expression and their role in regulating smooth muscle contractility (Banes and Watts, 2003), 5-HT_{1B} and 5-HT_{2B} receptors have been implicated in the pathogenesis of migraine headaches (Kalkman, 1994; Poissonnet et al., 2004; Schaerlinger et al., 2003), an idea supported by the efficacy of 5-HT_{1B} receptor agonist-based antimigraine therapy (Imitrex, 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 sub-types 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, crosstalk between receptor subtypes may represent an important additional regulatory mechanism at modulating sensitivity and/or signal transduction. Agonist-induced internalization of GPCRs uses a

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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 (Rapacciuolo 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 (Lefkowitz and Shenoy, 2005).

Among the described internalization mechanisms, 5-HT-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 microdomain proteins: 5-HT-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 arrestin-independent (Gray et al., 2003). A direct interaction between PSD-95 and the 5-HT_{2A} receptor at a type I PSD-95, Dlg, 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 GPCR kinase (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

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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 as monomers and heterodimers when co-expressed (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 co-expression 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 (Deraet et al., 2005; Porter et al., 2001; Schaerlinger et al., 2003), though the mechanism underlying this distinction has not been uncovered. In this work, we investigated potential 5-$HT_{1B/2B}$ receptor interactions by examining the co-localization 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 cells 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 utilized an antibody knockdown strategy to ascertain which pathways each receptor used for internalization. Our findings reveal that co-expression of 5-HT_{1B} and 5-HT_{2B} receptors affects both the kinetics of

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receptor internalization and the internalization pathway employed compared to either receptor expressed alone.

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Materials and Methods.

Reagents. RS-127445, 2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine was kindly provided by the Roche company; CP-93129, 1,4-Dihydro-3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo{3,2-b} pyridin-5-one dihydrochloride, BW-723C86, 1-{5-(2-thienylmethoxy)-1H-3-indolyl}propan-2-amine hydrochloride, H-89, N-{2-((p-Bromocinnamyl)amino)ethyl}-5-isoquinolinesulfonamide dihydrochloride, Gö 6850-Bisindolylmaleimide I, 2-{1-(3-Dimethylaminopropyl)-1H-indol-3-yl}-3-(1H-indol-3-yl)-maleimide, Gö 6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole, and all other chemicals were reagent grade, purchased from usual commercial sources. The radioactive compounds (6)-[^{125}I]1-(2,5-dimethoxy-4-iodophenyl)- 2-aminopropane hydrochloride ([^{125}I]DOI, 81.4 TBq/mmol) and [^{125}I]-serotonin-5-O-carboxymethyl-glycil-iodo-tyrosamine ([^{125}I]GTI, 81.4 TBq/mmol) were purchased from NEN Perkin Elmer. Already specificity-tested antibodies were used: rabbit antisera against rodent Cav1 (H-97, sc-7875), clathrin (H-300, sc-9069), GRK2,3 (H-222, sc-8329) and GRK5,6 (C-20, sc-565) and goat antiserum against rodent β-Arrestin-2 (D-18, sc-30938) and PKCε (C15, sc-214) (Santa Cruz Biotechnology; Santa Cruz, CA).

Mutagenesis and 5-HT$_{2B}$ receptor constructs-The fluorescent tagged fusion proteins of the mouse 5-HT$_{2B}$ and 5-HT$_{1B}$ receptors were generated by PCR-based sub-cloning. The receptor coding regions were sub-cloned 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$_{2B}$ and 5-HT$_{1B}$ receptor cDNAs were stably transfected into non-transformed murine fibroblast (LMTK$^-$) cells, which are devoid of endogenous 5-HT receptors as they do not exhibit a concentration-dependent rise in second messengers after 5-HT stimulation (Manivet et al., 2000). LMTK$^-$ cell lines were routinely cultured in

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Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum and 40 µg/ml gentamycin. The stably expressing mouse 5-HT_{1B} and 5-HT_{2B} 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 sub-cultured in serum free medium at least 24 h prior to experiments. Stable cell lines with different combination of receptor expression insured the absence of individual cell based effects.

Radioligand binding experiments-Radioligand binding experiments were performed using [^{125}I]-DOI or [^{125}I]-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/10^6 cells at 22°C under agitation as described (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 hr before the analysis on 35-mm glass bottom dishes (MatTek; NH, USA) and grown in a 5.4% CO_2 incubator. Twelve hours before the experiment, cells were washed in Dulbecco modified Eagle 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 Leica SP2AOBS confocal microscope (Manheim, 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 *xyzt* mode) were recorded sequentially every five minutes in two different emission intervals (462

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nm to 500 nm and 520 nm to 600 nm for CFP and YFP, respectively) with two different excitation wavelengths (458 nm 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 ten regions of interest (ROI) 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 a 488-nm excitation.

FRET measured by confocal microscopy-For the co-localization of 5-HT_{2B} and 5-HT_{1B} receptors in living cells, sensitized emission fluorescent resonance energy transfer (FRET) was used. Two additional channels, namely the FRET channel (excitation: 458 nm, emission: 520 nm to 600 nm) and a control channel (excitation: 514 nm, emission: 462 nm to 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 receptor. 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 up (not shown).

Co-localization calculation-We considered that two proteins were co-localized if the observed signals of the two corresponding labels were non-zero at the same pixel. The quantitative estimate of co-localization is given as the co-localization coefficients (Manders et al., 1992).

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To quantify the co-localized 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 non-linear regression model (GraphPad Prism 2.0). This allowed the calculation of dissociation constants (K_D) and the number of sites (B_{max}). All values represent the average of independent experiments ± SEM (n = number of experiments as indicated in the text). Comparisons between groups were performed using Student's unpaired *t* test or ANOVA and a Fischer test. Significance was set at $p<0.05$.

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Results

Radioligand saturation binding assays were performed on membranes from stable, clonal cell lines expressing either 5-HT_{1B} or 5-HT_{2B} receptors, bearing various N-terminal fluorescent protein tags as well as on cells co-expressing both receptors. The clones chosen for subsequent experiments were selected so as to approximate physiological receptor expression (B_{max} of approximately 100 fmol/mg protein) (Table 1). In control experiments with non-tagged receptors, we verified that none of the N-terminal fluorescent tags affected radioligand affinity (Table 1). Also, whole-cells radioligand binding experiments on clones expressing either fluorescent protein tagged or non-tagged receptors yielded a B_{max} approximately 60% of that measured on membrane preparations, suggesting that the fluorescent tag did not perturb membrane targeting (Table 1).

Kinetics of 5-HT-induced internalization of 5-HT_{2B} and 5-HT_{1B} 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 minutes. The time-dependent fluorescence intensity changes at the plasma membrane and in the cytoplasm both fit a one-phase exponential function (decrease for the plasma membrane, increase for the cytoplasm) and had similar half-lifes (5-HT_{2B} receptor cytoplasm $t_{1/2}$ = 23.0 ± 3.0 min, membrane $t_{1/2}$ = 22.2 ± 3.3 min; 5-HT_{1B} receptor cytoplasm $t_{1/2}$ = 9.8 ± 2.2 min, membrane $t_{1/2}$ = 10.7 ± 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 1A-B). To further confirm that we were measuring receptor endocytosis, we performed experiments with photoactivable (PA) GFP-tagged 5-HT_{1B} receptors and compared the rate of decrease in fluorescence intensity of the illuminated membrane region to that

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determined for plasma membrane YFP-tagged 5-HT_{1B} receptors. As before, for both GFP- and YFP-tagged 5-HT_{1B} receptors, the internalization kinetics fitted a one-phase exponential decay and were identical (5-HT_{1B}-PA-GFP $t_{1/2}$ = 9.8 ± 0.7 min; 5-HT_{1B}-YFP $t_{1/2}$ = 10.1 ± 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. While at 100 nM 5-HT-induced internalization of 5-HT_{1B} receptors was twice as fast as that of 5-HT_{2B} receptors, at higher concentrations of 5-HT internalization of both receptors occurred at similar rate. Furthermore, 5-HT-induced 5-HT_{2B} receptor internalization rate increased twice as fast as that of 5-HT_{1B} 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 co-expression altered 5-HT-induced 5-HT_{1B} and 5-HT_{2B} receptor internalization rates. Co-expression with the 5-HT_{1B} receptor resulted in a five-fold increase in the rate of 5-HT-induced 5-HT_{2B} receptor internalization ($2B^{1B}$) ($t_{1/2}$ = 4.0 ± 1.5 min vs. 23.0 ± 3.0) (Fig. 1D-E). On the other hand, co-expression with 5-HT_{2B} receptors had no effect on 5-HT-induced 5-HT_{1B} receptor internalization rate ($1B^{2B}$) ($t_{1/2}$ = 9.0 ± 1.0 min vs. 9.8 ± 2.2) (Fig. 1D-E). Kinetic whole-cell radioligand binding experiments corroborated our microscopy data revealing the asymmetric effect of receptor co-expression on 5-HT-induced 5-HT_{1B} and 5-HT_{2B} receptor internalization rate (Fig. 1E-F).

Agonist-dependent 5-HT_{2B} receptor internalization.

To determine whether the effect of 5-HT_{1B} receptors on 5-HT_{2B} receptor internalization kinetics involved activation of 5-HT_{2B} receptors, we stimulated cells co-expressing both receptors in the absence and presence of the highly selective 5-HT_{2B} receptor antagonist RS127445 (RS). In cells expressing 5-HT_{2B} receptor alone, 100 nM RS completely

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blocked 5-HT-induced 5-HT_{2B} receptor internalization. Strikingly, RS had no effect on 5-HT-induced 5-HT_{2B} receptor internalization in cells co-expressing 5-HT_{1B} receptors (Table 2). RS did not significantly affect 5-HT-induced 5-HT_{1B} receptor internalization irrespective of 5-HT_{2B} receptor co-expression.

To investigate whether modulation of internalization kinetics was agonist-dependent, we stimulated with the preferential 5-HT_{2B} receptor agonist BW723C86 (BW). Treatment with 50 nM BW induced no 5-HT_{1B} receptor internalization, but did stimulate 5-HT_{2B} receptor internalization ($t_{1/2}$ = 11.0 ± 1.5 min) in non-co-expressing cells (Fig. 2A-Table 2). Co-expression of 5-HT_{1B} and 5-HT_{2B} receptors did not significantly alter the effect of BW on the internalization of either receptor: the internalization kinetics of 5-HT_{2B} receptors changed only slightly in the presence of 5-HT_{1B} receptor ($t_{1/2}$ = 7.2 ± 1.1 min) (Fig. 2B-Table 2), while no internalization of 5-HT_{1B} receptors was observed (Fig. 2C)

Agonist-dependent 5-HT_{1B} receptor internalization.

To further investigate putative reciprocal interactions between 5-HT_{1B} and 5-HT_{2B} receptors, we studied the agonist-induced internalization kinetics of the 5-HT_{1B} receptor. The selective agonist CP93129 (CP, 75 nM), induced 5-HT_{1B} receptor internalization ($t_{1/2}$ = 14.1±0.7) but did not affect 5-HT_{2B} receptor distribution in non-co-expressing cells (Fig. 2D). We observed similar CP-induced internalization kinetics for both receptors ($t_{1/2}$ = 6.9 ± 0.9 min for 5-HT_{2B} receptors; $t_{1/2}$ = 4.9 ± 1.3 min for 5-HT_{1B} receptors) upon co-expression (Fig. 2E-Table 2). Interestingly, co-expression with 5-HT_{2B} receptors caused a three-fold increase in CP-induced internalization of 5-HT_{1B} receptors ($t_{1/2}$ = 4.9 ± 1.3 min vs. $t_{1/2}$ = 14.1 ± 0.7 min) (Fig. 2F-Table 2). These results reveal that, while co-expression with 5-HT_{2B} receptor does not affect BW-induced 5-HT_{1B} receptor internalization, the presence of 5-HT_{2B} receptor does modulate CP-induced 5-HT_{1B} receptor internalization.

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Temperature dependence of 5-HT$_{1B}$ and 5-HT$_{2B}$ 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-HT-induced receptor internalization were faster at higher temperatures for both receptors irrespective of co-expression. In addition, the temperature dependence of agonist (5-HT or subtype selective)-induced receptor internalization rate appeared linear for 5-HT$_{1B}$ receptors and biphasic for 5-HT$_{2B}$ receptor when expressed alone (Fig. 3A-B).

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

Microscopic analysis revealed no receptor co-localization.

The apparent effect of receptor co-expression on agonist-induced 5-HT$_{2B}$ and 5-HT$_{1B}$ receptor internalization led us to hypothesized receptor heterodimerization. Therefore, we performed confocal microscopy on cells co-expressing CFP-5-HT$_{1B}$ and YFP-5-HT$_{2B}$ receptors. Cellular distribution analysis of the two receptors revealed about 20% co-localization in the plasma membrane prior to stimulation (Fig 4). After 30 min of agonist stimulation, cytoplasmic co-localization of 5-HT$_{2B}$ and 5-HT$_{1B}$ receptors was still around 20% irrespective of agonist. Using enhanced emission to measure FRET, we observed nearly no FRET signal at 5-HT$_{1B}$/5-HT$_{2B}$ receptor co-localization points (Fig 4). Thus, our image-based

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analysis of co-localization 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. Notably, the alveolysin and antibody treatments did not affect agonist and radioligand affinities (not shown).

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

We next applied the antibody knockdown strategy to cells co-expressing 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. 5A-B). However, 5-HT$_{2B}$ receptor internalization was only partially inhibited by anti-Arrestin-2 when co-expressed 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-

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HT_{2B} receptors. Finally, the agonist-induced internalization of 5-HT_{2B} receptors was partially dependent on GRK2,3 when co-expressed with 5-HT_{1B} receptors and stimulated with 5-HT, CP, or BW (Fig. 5). Thus, the effect of 5-HT_{1B} receptor co-expression on 5-HT_{2B} receptors caused a fraction of 5-HT_{2B} receptors to internalize via a Cav1-, GRK2,3-dependent pathway.

With respect to 5-HT_{1B} receptors, co-expression with 5-HT_{2B} receptors caused 5-HT-induced 5-HT_{1B} receptor internalization to become totally independent of Cav1 and GRK2,3. Furthermore, upon co-expression with 5-HT_{2B} receptors, 5-HT-induced 5-HT_{1B} receptor internalization was still independent of clathrin and Arrestin-2. In contrast, the Cav1/GRK2,3 dependence of CP-induced 5-HT_{1B} receptor internalization was not affected by co-expression with 5-HT_{2B} receptors. Thus, the effect of 5-HT_{2B} receptor co-expression on 5-HT_{1B} 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_{2B} receptors regulates the pathway of 5-HT_{1B} receptor internalization.

To investigate the non-Cav1-, non-clathrin-dependent internalization pathway used by 5-HT-stimulated 5-HT_{1B} receptors co-expressed with 5-HT_{2B} receptors, we tested the effect of various protein kinase inhibitors. The wide protein kinase inhibitor staurosporine (5 μM), which inhibit PKC, PKA and PKG, blocked 5-HT-induced 5-HT_{1B} receptor internalization when co-expressed with 5-HT_{2B} receptors (Fig. 6). H89 (5 μM), which inhibits PKA, PKG, and PKCμ 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 PKCα-, βI-, βII-, γ-, δ-, and ε- isozymes, completely prevented 5-HT-induced 5-HT_{2B} receptor internalization

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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 α and βI. Finally, the blocking effect of Gö 6850-Bisindolylmaleimide was completely reproduced using PKCε 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 PKCε activation.

Discussion

Given (i) the wide *in vivo* co-expression of 5-HT_{1B} and 5-HT_{2B} receptors (Banes and Watts, 2003; Ishida et al., 1999; Ishida et al., 1998; Kellermann et al., 1996; Nicholson et al., 2003; Stefulj et al., 2000), (ii) the established inhibitory effect of 5-HT_{2B} on 5-HT_{1B} receptor signaling (Tournois et al., 1998), (iii) the clinical utility of 5-HT_{1B} receptor agonists, and (iv) 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 paper 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, while 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, co-expression with 5-HT_{2B} receptors causes 5-HT_{1B} receptors to adopt a Cav1-, clathrin-independent but PKCε-dependent 5-HT-induced internalization, while 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 non-transformed mouse fibroblast $LMTK^-$ cells use classically 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-, GRK2,3-dependent pathway, while 5-HT_{2B}

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receptors expressed alone internalize via a clathrin- Arrestin-2- and PKCε-dependent pathway (Fig7A).

The co-expression 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 co-localization and of FRET). Interestingly, 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 co-expression 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 RS shows the existence of multiple pathways for 5-HT_{2B} receptor internalization: while the 5-HT-induced internalization of 5-HT_{2B} receptors expressed alone is blocked by RS, co-expression with 5-HT_{1B} receptors renders 5-HT_{2B} receptor internalization sensitive to CP and insensitive to RS. The antibody knock-down experiments further validate that activation of 5-HT_{1B} receptors with CP in co-expressing cells results in a switch of 5-HT_{2B} receptor internalization from a totally clathrin-, Arrestin-2- and PKCε-dependent pathway to a Cav1, GRK2,3 partially dependent 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 co-expressed 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 likely

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possibility is that GRK2,3 phosphorylates 5-HT_{2B} receptors in such a way that enables them to use a Cav1-dependent internalization pathway (Fig. 7B).

Activation of 5-HT_{2B} receptors by 5-HT was shown to lead to PKC activation (Cox and Cohen, 1995; Launay et al., 2006). Our data also support the notion that PKC is responsible for the effect of 5-HT_{2B} receptors on 5-HT-induced 5-HT_{1B} receptor internalization (Fig. 7C). Using a combination of pharmacological inhibitors and antibody knockdown, we have identified PKCε as the isotype necessary for the 5-HT_{2B} receptor-dependent 5-HT_{1B} receptor internalization. PKCε 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 Cav1-dependent 5-HT_{1B} receptor internalization. One possible explanation for this discrepancy is that PKCε is poorly activated by the partial 5-HT_{2B} receptor agonist BW. Alternatively, or additionally, both 5-HT-induced 5-HT_{1B} receptor GRK2,3 activation and 5-HT-induced 5-HT_{2B} receptor PKCε activation are required for 5-HT_{1B} receptors to internalize via the Cav1, 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_{1B} receptors by 5-HT_{2B} receptors. Activation of the 5-$HT_{2B/2C}$ receptor has been shown to inhibit the 5-HT_{1B} receptor function in two independent studies: (i) Using 5-HT_{2C} and 5-HT_{2A} receptors stably transfected Chinese hamster ovary (CHO) cells, it has been reported that activation of 5-HT_{2C} receptors abolishes the endogenous 5-HT_{1B} receptor-mediated inhibition of forskolin-stimulated cAMP accumulation. In contrast, activation of 5-HT_{2A} receptors does not alter the 5-HT_{1B} response and 5-HT_{2C} receptor-mediated inhibition of 5-HT_{1B} receptor function was blocked when 5-HT_{2A} receptors were activated simultaneously (Berg et al., 1996). (ii) Using the

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

This newly described internalization route fits with other evidence supporting independent intracellular trafficking of 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors (i.e., internalization of 5-HT$_{2B}$ receptors upon activation of 5-HT$_{1B}$ receptors despite the apparent lack of agonist-induced co-localization). The fact that upon co-expression stimulating one or the other receptor or both generates different cellular responses is supported by completely independent techniques, i.e. confocal microcopy image analysis and antibody knock-down coupled with whole-cell radioligand binding studies. This work provides the first evidence that within the same cells, one receptor may adopt different internalization pathways upon the presence and stimulation of another receptor. Identified interactions regulate receptor internalization and could explain the observed co-ordination between 5-HT$_{1B}$ and 5-HT$_{2B}$ receptor internalization. Our work suggests that indirect events *in trans* are mediating the 5-HT$_{1B}$/5-HT$_{2B}$ receptor cross-regulation that affects their cellular distribution during the endocytic process. Given the wide clinical use of 5-HT$_{1B}$ receptor agonists in the treatment of migraines, and the suspected prophylactic effect of 5-HT$_{2B}$ 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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Legends for figures

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$^-$ transfected cell lines plated on glass bottom dishes in Dulbecco modified Eagle 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 about ten 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 panels) 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 were taken from living cells expressing 5-HT_{2B} receptor-GFP by time lapse video. Internalization kinetics after stimulation by100 nM 5-HT stimulation. (Right panels) Distribution of 5-HT_{2B} receptors expressed at 0 and 50 min of stimulation. These images are representative of more than 3 cells observed in each of at least 4 independent experiments. Bars 2 µM. (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 µM or 10 µM 5-HT and their half life were concentration-dependent. (D). Study by confocal laser microscopy showed that, when 5-HT_{2B} receptors were co-expressed with 5-HT_{1B} receptors (5-$HT2B^{1B}$), 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_{1B} receptors were co-expressed with 5-HT_{2B} receptors (5-$HT1B^{2B}$ light grey 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).

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(F). Independent radioligand binding experiments using [^{125}I]-DOI (5-HT$_{2B}$ receptor) or [^{125}I]-GTI (5-HT$_{1B}$ receptor) on intact living cells confirmed that, compared to 5-HT$_{2B}$ receptor expressed alone (dark grey bar) when the 5-HT$_{2B}$ receptor was co-expressed with 5-HT$_{1B}$ receptors, stimulation with 100 nM 5-HT decreased the 5-HT$_{2B}$ receptor (5-HT2B^{1B} white bar) half life but not that of 5-HT$_{1B}$ receptors (5-HT1B^{2B} light grey bar). Graphs display the mean±sem of at least 3 independent experiments.

FIG. 2. **Ligand dependency of 5-HT$_{1B}$ receptor and 5-HT$_{2B}$ 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$^-$ cells by time lapse video. (A-C). The fluorescence intensity disappearance have been fitted with a single exponential function over time and used to evaluate the internalization kinetics after stimulation by the preferential 5-HT$_{2B}$ receptor agonist BW723C86 (BW) at the final concentration of 50 nM that produced no effect on the internalization of 5-HT$_{1B}$ receptor alone. (D-F). The fluorescence intensity disappearance have been fitted with a single exponential function over time and used to evaluate the internalization kinetics after stimulation by the selective 5-HT$_{1B}$ receptor agonist CP93129 (CP) at the final concentration of 75 nM that produced no effect on the internalization of 5-HT$_{2B}$ receptor alone. The x-axes display 0 to 30 minutes recording for fast internalization (A,B,C,E) and 0 to 60 minutes for slow internalization (D,F). Deduced half life values are reported in table 2 with statistics.

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FIG. 3. **Temperature dependency of the of 5-HT$_{1B}$ receptor and $_{2B}$ receptors internalization.**

The receptors half life ($t_{1/2}$) were evaluated using transfected LMTK$^-$ cells expressing GFP-tagged receptors by measuring the fluorescence intensity disappearance at the membrane after fitting with a single exponential function over time. Upon 5-HT stimulation, the $t_{1/2}$ values in single receptor transfected cells was evaluated at different temperatures (22°C to 25°C and 37°C), for 5-HT$_{1B}$ receptors (A), for 5-HT$_{2B}$ receptors (B) or alone or co-expressed, after stimulation by CP for 5-HT$_{1B}$ receptors (C-E), or for 5-HT$_{2B}$ receptor (D-E) after BW stimulation. NS: no significant statistical difference; * $P<0.05$, more than 3 cells were analyzed in at least 4 independent experiments.

FIG. 4. **Single-cell fluorescence measurements for quantitative analysis.**

The cellular distribution of CFP-tagged 5-HT$_{2B}$ receptors (2B, red) co-expressed with YFP-5-HT$_{1B}$ receptors (1B green) was video-recorded and is displayed on a single confocal plane before (0 min) and after (30 min) stimulation with different agonists. The corresponding co-localizing points (1B^{2B}, yellow) and deduced FRET values are shown (right panels, white). (A). Distribution of 5-HT$_{2B}$ receptor expressed at 0 and 30 min of CP stimulation. (B). Distribution of 5-HT$_{2B}$ receptor expressed at 0 and 30 min of 5-HT stimulation. (C). Distribution of 5-HT$_{2B}$ receptor expressed at 0 and 30 min of BW stimulation. These images are representative of more than 3 cells observed in each of at least 4 independent experiments. Bars 2 µM.

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FIG. 5.**Whole-cell binding and antibody knockdown for quantitative analysis.** The amount of membrane receptor sites in transfected LMTK$^-$ cells was assessed by radioligand binding experiments using [^{125}I]-DOI (5-HT$_{2B}$ receptor) or [^{125}I]-GTI (5-HT$_{1B}$ receptor) on intact living cells 0, 5, and 10 minutes after agonist stimulation (K_D and Bmax). With unmodified K_D (not illustrated), the time-dependent changes in Bmax allowed to approximate the receptor half life in response to various agonists (left panels). Exposure of alveolysin-permeabilized cells to antibodies against Caveolin-1, Clathrin, Arrestin-2, or GRK2,3 modifies the receptor half life (expressed as fold over unexposed cells- right panels), upon agonist stimulation (A) 5-HT, (B) CP and (C) BW. Black bars, 5-HT$_{1B}$ receptors; light grey bars, 5-HT$_{1B}$ receptors co-expressed with 5-HT$_{2B}$ receptors (5-HT1B^{2B}); dark grey bars, 5-HT$_{2B}$ receptors; white bars, 5-HT$_{2B}$ receptors co-expressed with 5-HT$_{1B}$ receptors (5-HT2B^{1B}). Graphs display the mean±sem of at least 3 independent experiments.

FIG. 6.**Whole-cell binding and pharmacological treatment for quantitative analysis.** The amount of membrane receptor sites in transfected LMTK$^-$ cells was assessed by radioligand binding experiments using [^{125}I]-DOI (5-HT$_{2B}$ receptor) or [^{125}I]-GTI (5-HT$_{1B}$ receptor) on intact living cells 0, 5, and 10 minutes after agonist stimulation (K_D and Bmax). With unmodified K_D (not illustrated), the time-dependent changes in Bmax allowed to approximate the receptor half life in response to various protein kinase blockers, (A) 5-HT, (B) 5-HT+staurosporine (5 μM), (C) 5-HT+H89 (5 μM), (D) 5-HT+Gö 6976 (100 nM), and (E) 5-HT+Gö 6850-Bisindolylmaleimide I (100 nM). (F) Exposure of alveolysin-permeabilized cells to antibodies against PKCε modifies the receptor half life, upon 5-HT stimulation. Black bars, 5-HT$_{1B}$ receptors; light grey bars, 5-HT$_{1B}$ receptors co-expressed with 5-HT$_{2B}$ receptors (5-HT1B^{2B}); dark grey bars, 5-HT$_{2B}$ receptors; white bars, 5-HT$_{2B}$ receptors

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co-expressed with 5-HT$_{1B}$ receptors (5-HT2B^{1B}). Graphs display the mean±sem of 3 independent experiments.

FIG. 7. **Model of interactions between 5-HT$_{2B}$ and 5-HT$_{1B}$ receptors in their internalization pathways.**

A) When expressed as a single receptor in cells devoid of endogenous 5-HT receptors expression and upon 5-HT stimulation, 5-HT$_{1B}$ receptor internalizes via a Cav1 and GRK2,3 dependent pathway, while 5-HT$_{2B}$ receptors internalizes via clathrin and Arrestin-2 dependent pathway. B) When co-expressed, the two receptors respond differently to selective agonists. A selective 5-HT$_{2B}$ receptor agonist, BW, does not trigger any internalization of co-expressed 5-HT$_{1B}$ receptor and acts as 5-HT on the 5-HT$_{2B}$ receptor internalization (not illustrated). By contrast, stimulation of 5-HT$_{1B}$ receptor by CP, a selective 5-HT$_{1B}$ receptor agonist, makes the 5-HT$_{1B}$ receptor internalizing via Cav1 and GRK2,3 as 5-HT. While inefficient on cells expressing 5-HT$_{2B}$ receptor alone, CP triggers also the internalization of co-expressed 5-HT$_{2B}$ receptor via Cav1, GRK2,3, and Arrestin-2 imposing for these two receptors the same kinetic for internalization. GRK2,3 becomes a partner of this trans-internalization of 5-HT$_{2B}$ receptors. C) The co-stimulation of both receptors by 5-HT makes 5-HT$_{2B}$ receptors to internalize via its classical clathrin but also via 5-HT$_{1B}$ triggered Cav1-dependent pathways (large arrow), which imposes in return 5-HT$_{1B}$ receptors to adopt new internalization pathways independent of both clathrin and Cav1 but that becomes dependent on 5-HT$_{2B}$ receptor-induced PKCε (small arrow).

Table 1 Pharmacological properties of native or GFP-tagged receptors

	Membrane		Whole cells	
	K_D (nM)	Bmax (fmol/mg prot)	K_D (nM)	Bmax (fmol/mg prot)
5-HT_{1B}-YFP clone 9	0.82±0.14	119±5*	0.84±0.15	73±5
5-HT_{1B} clone 5	0.56±0.11	72±4	/	/
5-HT_{1B}-PA-GFP clone 3	0.83±0.13	122±5*		
5-HT_{2B}-PA-GFP clone 9	26.8±6.3	138±8*	26.3±6.5	76±3
5-HT_{2B} clone 17	16.5±4.5	65±5	/	/
5-HT_{2B}-YFP clone 22	28.8±5.8	132±8*		

Receptors	5-HT_{1B} R		5-HT_{2B}R		5-HT_{1B}R		5-HT_{2B}R	
	K_D (nM)	Bmax	K_D (nM)	Bmax	K_D (nM)	Bmax	K_D (nM)	Bmax
5-HT_{1B}-YFP/5-HT_{2B}PAGFP	0.79±0.15	69±6	19.1±4.5	80±9	0.73±0.10	64±5	26.0±3.5	80±8
5-HT_{1B}-YFP/5-HT_{2B}-CFP	0.73±0.12	87±9	27.1±5.8	81±8				

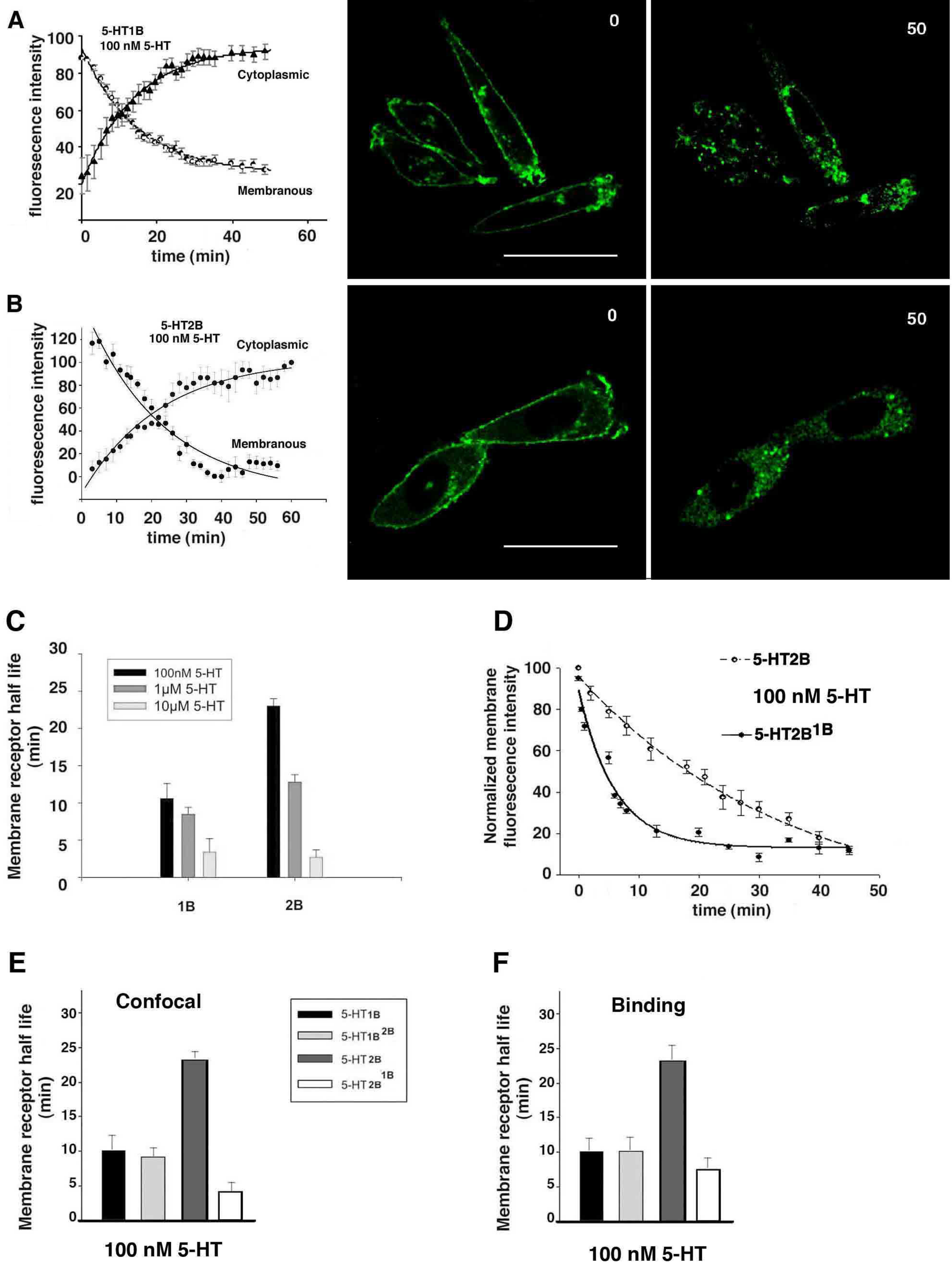
Saturation binding assays were performed using [^{125}I]-DOI (5-HT_{2B} receptor) or [^{125}I]-GTI (5-HT_{1B} receptor) on membrane fraction of cell homogenate (Membranes) or intact cells (Whole cells) of the different stable clonal cell lines expressing either the 5-HT_{1B} receptor, the 5-HT_{2B} receptor alone or co-expressing both receptor subtypes, with our 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 (Bmax). Insertion of the various GFPs at the N-terminal part of the receptor did not affects the receptor affinity. Intact cells Bmax 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. * $P<0.05$ vs. non-tagged receptors by Student's t test. Values are means±standard errors of 3 independent determinations in triplicate.

Table 2 Ligand dependence of 5-HT_{1B} receptor and 5-HT_{2B} receptor internalization.

Agonist	Conc (nM)	1B T1/2 min	2B T1/2 min
"SINGLE"			
5-HT	100	10.6±2.8*	23.0±1.0#
5-HT/RS	100/100	10.8±0.9*	∞#
BW	50	∞*	11.0±1.5#
CP93	75	14.1±0.7#*	∞#
"DOUBLE"			
5-HT	100	9.0±1.0	4.0±1.5
5-HT/RS	100/100	7.7±1.6	4.1±2.0
BW	50	∞*	7.2±2.2
CP93	75	4.9±2.3	6.9±0.9

Quantitative internalization kinetics of the GFP-tagged receptors was assessed by measuring the fluorescence intensity disappearance at the membrane for more than ten ROI per cell and followed the relative intensity changes of these ROI by time. In 5-HT_{2B} receptors or 5-HT_{1B} receptors transfected cells (single), this signal could be fitted with a single exponential function over time. Applying the same protocol on 5-HT_{2B} receptors in the presence of 5-HT_{1B} receptors transfected cells (double), a change in internalization rate could be observed. * $P<0.05$ by Student's t test 5-HT_{1B} vs. 5-HT_{2B} receptors, # $P<0.05$ by Student's t test single vs. double transfected receptors. Values are means±standard errors of more than 3 cells analyzed in at least 4 independent experiments.

Figure 1



A
5-HT1B
100 nM 5-HT
Cytoplasmic
Membranous
fluoresecence intensity
time (min)
0
50
B
5-HT2B
100 nM 5-HT
Cytoplasmic
Membranous
fluoresecence intensity
time (min)
0
50
C
Membrane receptor half life (min)
100nM 5-HT
1μM 5-HT
10μM 5-HT
1B
2B
D
5-HT2B
100 nM 5-HT
5-HT2B1B
Normalized membrane fluoresecence intensity
time (min)
E
Confocal
5-HT1B
5-HT1B2B
5-HT2B
5-HT2B1B
Membrane receptor half life (min)
100 nM 5-HT
F
Binding
Membrane receptor half life (min)
100 nM 5-HT

Figure 2

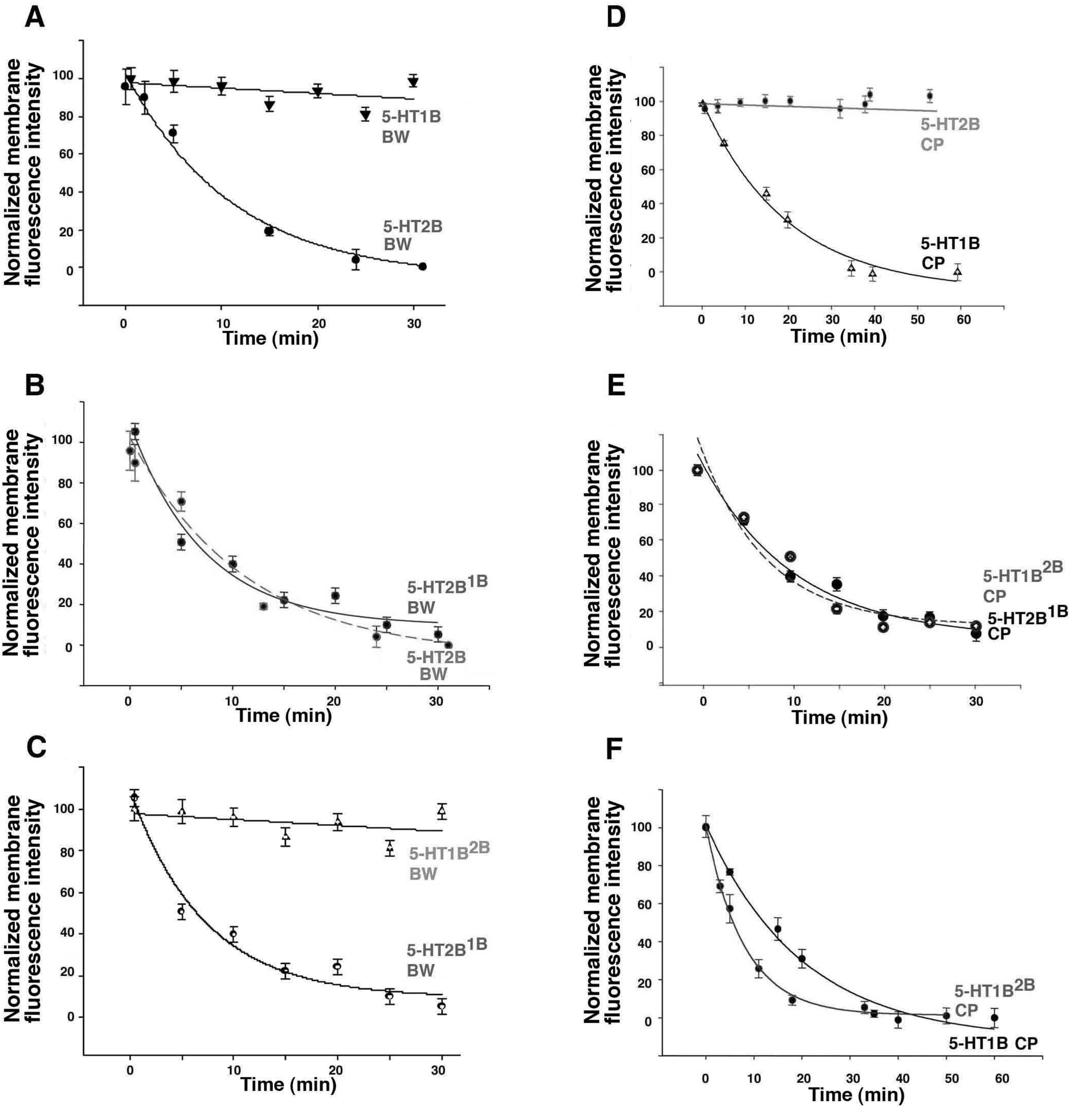

A
Normalized membrane fluorescence intensity
Time (min)
5-HT1B BW
5-HT2B BW
B
5-HT2B^1B BW
5-HT2B BW
C
5-HT1B^2B BW
5-HT2B^1B BW
D
5-HT2B CP
5-HT1B CP
E
5-HT1B^2B CP
5-HT2B^1B CP
F
5-HT1B^2B CP
5-HT1B CP

Figure 3

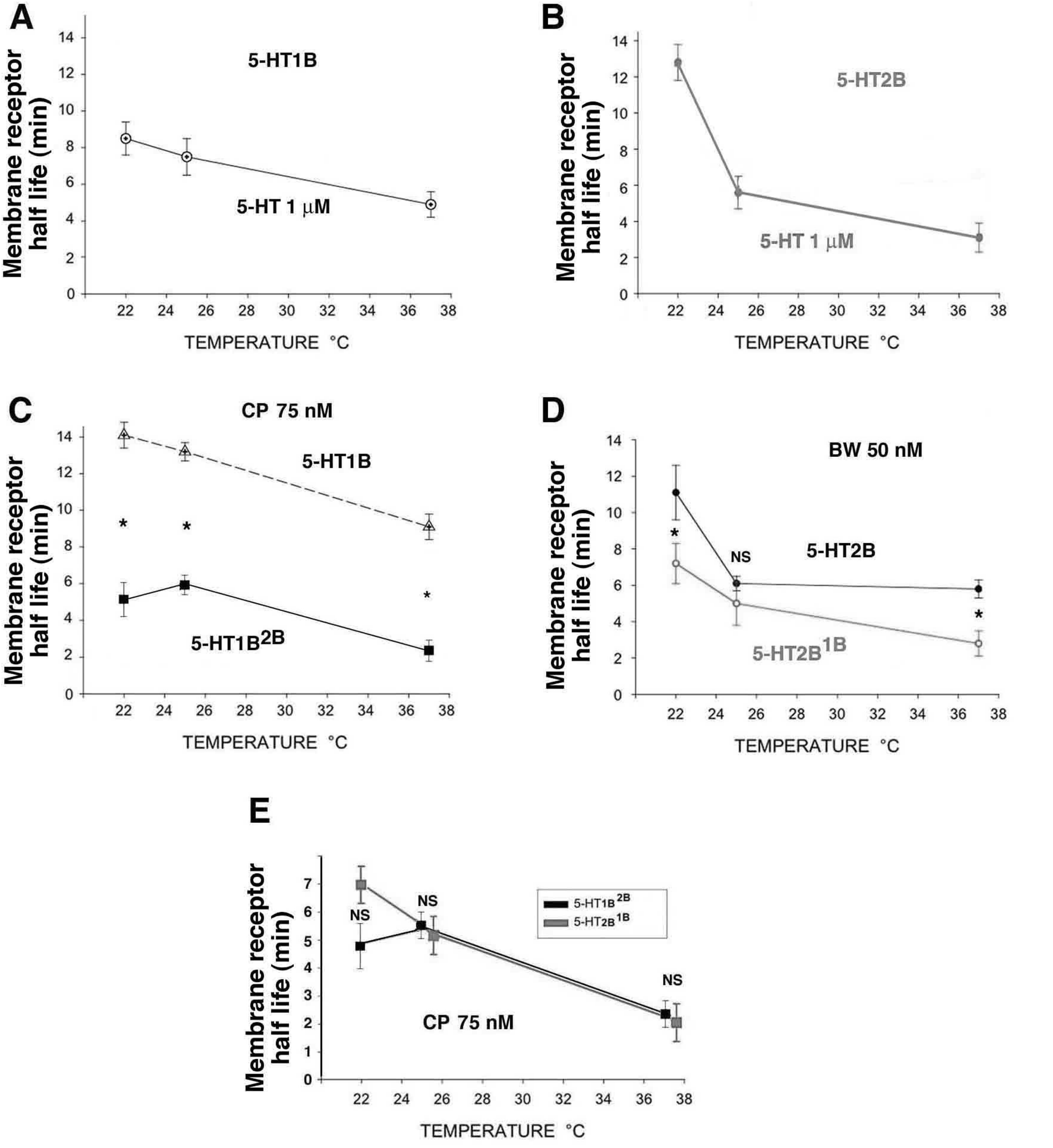
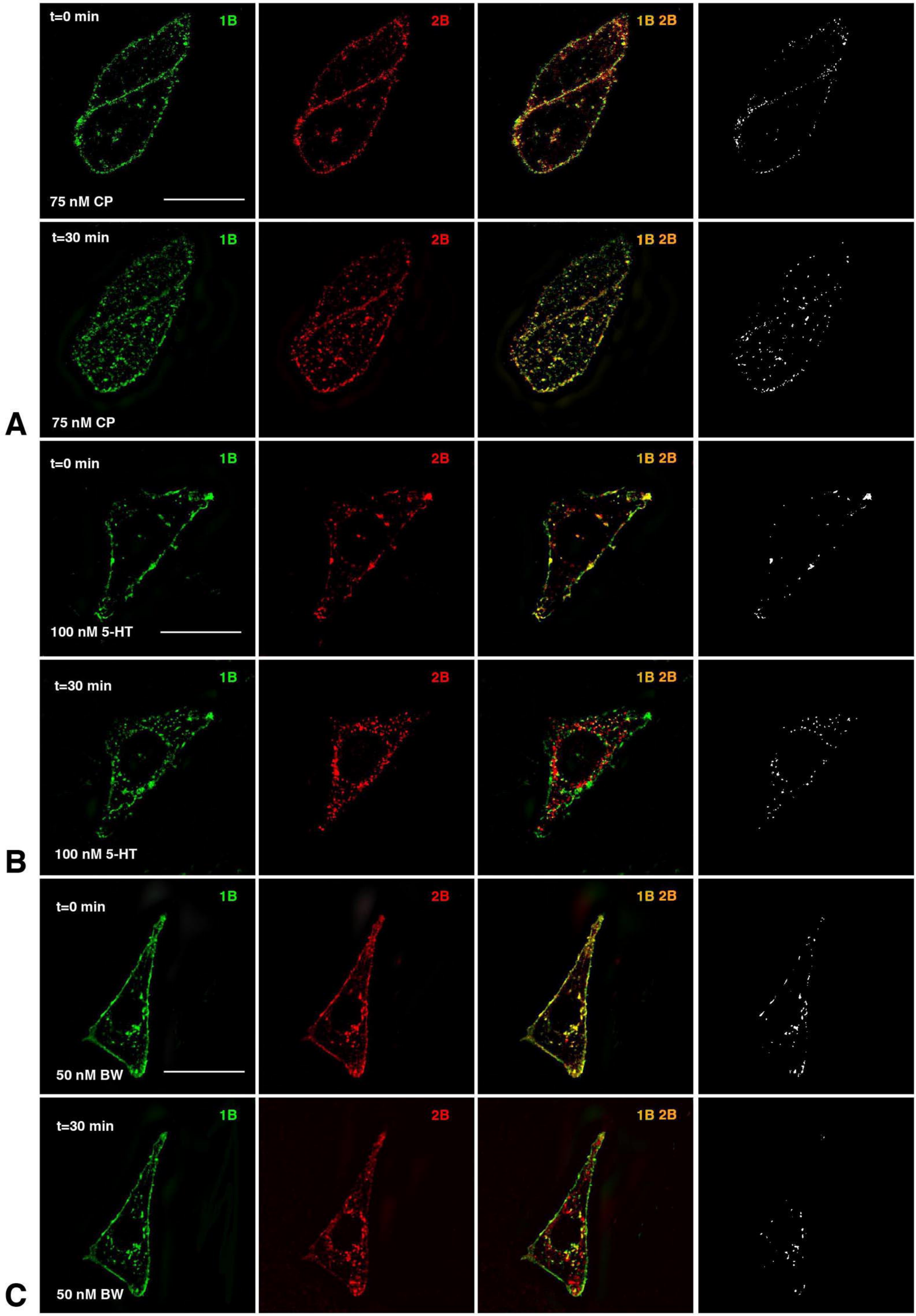

A
5-HT1B
5-HT 1 μM
Membrane receptor half life (min)
TEMPERATURE °C
B
5-HT2B
5-HT 1 μM
Membrane receptor half life (min)
TEMPERATURE °C
C
CP 75 nM
5-HT1B
5-HT1B2B
Membrane receptor half life (min)
TEMPERATURE °C
D
BW 50 nM
NS
5-HT2B
5-HT2B1B
Membrane receptor half life (min)
TEMPERATURE °C
E
NS
NS
NS
5-HT1B 2B
5-HT2B 1B
CP 75 nM
Membrane receptor half life (min)
TEMPERATURE °C

Figure 4



t=0 min
1B
2B
1B 2B
75 nM CP
t=30 min
1B
2B
1B 2B
75 nM CP
A
t=0 min
1B
2B
1B 2B
100 nM 5-HT
t=30 min
1B
2B
1B 2B
100 nM 5-HT
B
t=0 min
1B
2B
1B 2B
50 nM BW
t=30 min
1B
2B
1B 2B
50 nM BW
C

Figure 5

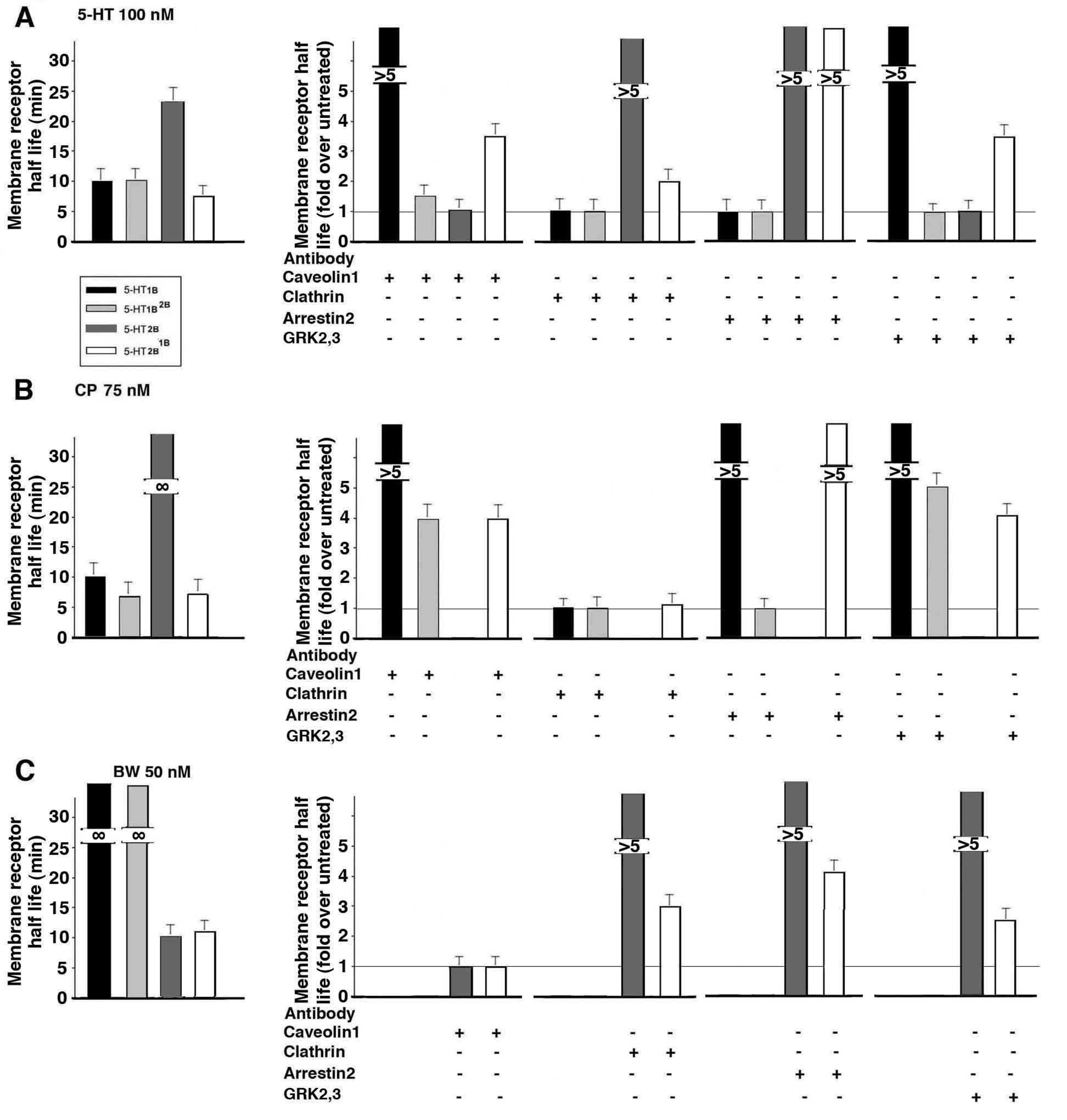
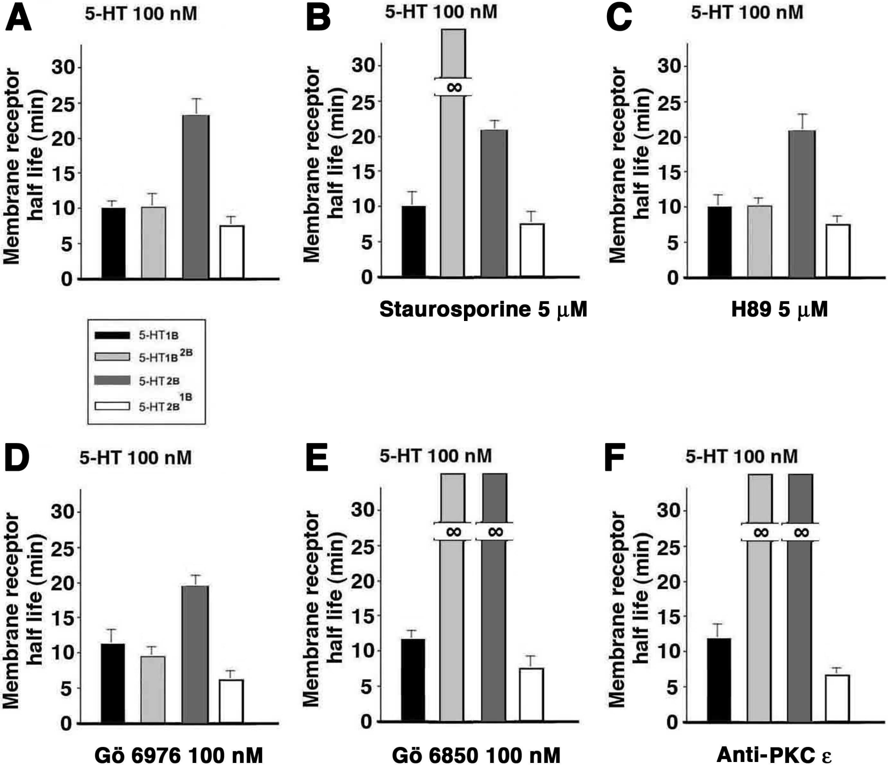
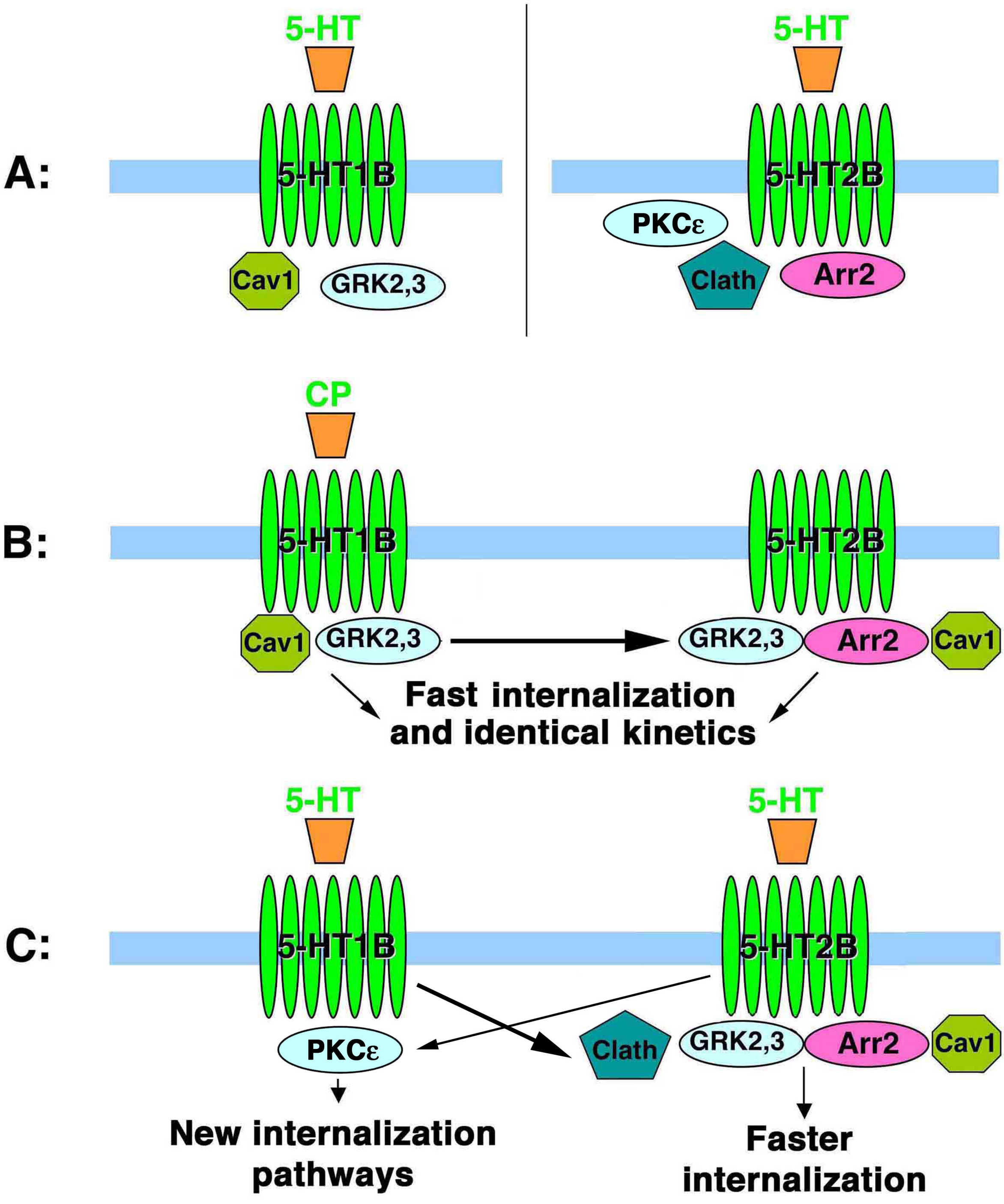

A
5-HT 100 nM
Membrane receptor half life (min)
Membrane receptor half life (fold over untreated)
>5
5-HT1B
5-HT1B 2B
5-HT2B
5-HT2B 1B
Antibody
Caveolin1
Clathrin
Arrestin2
GRK2,3
B
CP 75 nM
∞
C
BW 50 nM

Figure 6



A
5-HT 100 nM
Membrane receptor half life (min)
0
5
10
15
20
25
30
5-HT1B
5-HT1B 2B
5-HT2B
5-HT2B 1B
B
5-HT 100 nM
∞
Staurosporine 5 μM
C
5-HT 100 nM
H89 5 μM
D
5-HT 100 nM
Gö 6976 100 nM
E
5-HT 100 nM
∞
∞
Gö 6850 100 nM
F
5-HT 100 nM
∞
∞
Anti-PKC ε

Figure 7


A:
5-HT
5-HT1B
Cav1
GRK2,3
5-HT
5-HT2B
PKCε
Clath
Arr2
B:
CP
5-HT1B
Cav1
GRK2,3
5-HT2B
GRK2,3
Arr2
Cav1
Fast internalization
and identical kinetics
C:
5-HT
5-HT1B
PKCε
New internalization
pathways
5-HT
5-HT2B
Clath
GRK2,3
Arr2
Cav1
Faster
internalization