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The Dynamin-dependent, Arrestin-independent Internalization of 5-Hydroxytryptamine 2A (5-HT_{2A}) Serotonin Receptors Reveals Differential Sorting of Arrestins and 5-HT_{2A} Receptors during Endocytosis*[S]

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5-Hydroxytryptamine 2A (5-HT_{2A}) receptors, a major site of action of clozapine and other atypical antipsychotic medications, are, paradoxically, internalized *in vitro* and *in vivo* by antagonists and agonists. The mechanisms responsible for this paradoxical regulation of 5-HT_{2A} receptors are unknown. In this study, the arrestin and dynamin dependences of agonist- and antagonist-mediated internalization were investigated in live cells using green fluorescent protein (GFP)-tagged 5-HT_{2A} receptors (SR2-GFP). Preliminary experiments indicated that GFP tagging of 5-HT_{2A} receptors had no effect on either the binding affinities of several ligands or agonist efficacy. Likewise, both the native receptor and SR2-GFP were internalized via endosomes *in vitro*. Experiments with a dynamin dominant-negative mutant (dynamin K44A) demonstrated that both agonist- and antagonist-induced internalization were dynamin-dependent. By contrast, both the agonist- and antagonist-induced internalization of SR2-GFP were insensitive to three different arrestin (Arr) dominant-negative mutants (Arr-2 V53D, Arr-2-(319–418), and Arr-3-(284–409)). Interestingly, 5-HT_{2A} receptor activation by agonists, but not antagonists, induced greater Arr-3 than Arr-2 translocation to the plasma membrane. Importantly, the agonist-induced internalization of 5-HT_{2A} receptors was accompanied by differential sorting of Arr-2, Arr-3, and 5-HT_{2A} receptors into distinct plasma membrane and intracellular compartments. The agonist-induced redistribution of Arr-2 and Arr-3 into intracellular vesicles and plasma membrane compartments distinct from those involved in 5-HT_{2A} receptor internalization implies novel roles for Arr-2 and Arr-3 independent of 5-HT_{2A} receptor internalization and desensitization.

5-Hydroxytryptamine 2A (5-HT_{2A})[1] receptors are essential for the actions of 5-HT in a wide variety of physiological processes, including vascular and nonvascular smooth muscle contraction and platelet aggregation (1). 5-HT_{2A} receptors also mediate the actions of hallucinogens such as lysergic acid diethylamide, 4-iodo-2,5-dimethoxyamphetamine, and *N*,*N*-dimethyltryptamine by activating 5-HT_{2A} receptors located on cortical pyramidal neurons (2, 3). Additionally, atypical antipsychotic drugs such as clozapine, risperidone, and olanzapine may mediate some of their unique actions via antagonism of 5-HT_{2A} receptors (4).

For many years, it has been clear that a large number of G-protein-coupled receptors (GPCRs), including opiate receptors (5–7) and β-adrenergic receptors (8, 9), are found in coated vesicles and various intracellular vesicles associated with endocytic pathways. Studies with the β-adrenergic receptor in particular have delineated a general pathway for agonist-mediated internalization by which agonist-induced activation of receptors leads to receptor phosphorylation and then the binding of arrestin to phosphorylated receptors (10, 11). Arrestin binding appears to facilitate translocation of phosphorylated GPCRs to clathrin-coated pits and the eventual internalization of GPCRs via the endosome pathway. Interactions of the C terminus of arrestin with clathrin (12) and the AP-2 adaptor protein (13) appear to be essential for arrestin-mediated internalization of many GPCRs.

5-HT_{2A} receptors are subject to unique modes of regulation compared with other GPCRs. Thus, as previously reported, both agonists and antagonists induce down-regulation and internalization of 5-HT_{2A} receptors *in vitro* and *in vivo* (14–16). Since antagonist binding does not lead to receptor activation, it is difficult to imagine how antagonists could induce either internalization or arrestin binding, assuming that the process delineated for β-adrenergic receptors is universal for GPCRs.

Accordingly, in this set of studies, we examined the arrestin and dynamin sensitivities for the agonist- and antagonist-mediated internalization of 5-HT_{2A} receptors *in vitro*. As we report, both the agonist- and antagonist-induced internalization of 5-HT_{2A} receptors are dynamin-dependent and arrestin-independent. Furthermore, we report that even though the agonist-

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[S] The on-line version of this article (available at *http://www.jbc.org*) contains Image Sequences 1–4 in Quick-time Movie Format.

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[1] The abbreviations used are: 5-HT_{2A}, 5-hydroxytryptamine 2A; 5-HT, 5-hydroxytryptamine; GPCR, G-protein-coupled receptor; Arr, arrestin; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BODIPY-FL, 4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-pentanoic acid, succinimidyl ester.

induced internalization is arrestin-independent, activation of 5-HT$_{2A}$ receptors leads to a redistribution of βArr-1 (Arr-2), β-Arr-2 (Arr-3), and truncated Arr-2 (Arr-2(319–418)) into plasma membrane domains and intracellular vesicles distinct from those containing 5-HT$_{2A}$ receptors. Our results imply a novel and distinct sorting pathway for Arr-2 and Arr-3 that occurs independently of 5-HT$_{2A}$ receptor internalization.

EXPERIMENTAL PROCEDURES

Constructs and Antibodies—A green fluorescent protein (GFP)-tagged rat 5-HT$_{2A}$ fusion protein (SR2-GFP) was constructed by amplifying the entire coding region of the 5-HT$_{2A}$ receptor including 15 base pairs of the 5′-untranslated region in-frame using *Pfu* polymerase and subcloning it into the vector pEGFP-N2 (CLONTECH). Clones containing inserts in the appropriate orientation were verified by automated sequencing (Cleveland Genomics, Inc.) of the entire insert. A rat arrestin-2 dominant-negative mutant (Arr-2 V53D) was provided by Dr. Marc Caron (Duke University); the carboxyl-terminal arrestin-2 (Arr-2(319–418); CT-Arr-2) and arrestin-3 (Arr-3(284–409); CT-Arr-3) dominant-negative mutants were prepared as previously detailed, as were Arr-2 and Arr-3 full-length eukaryotic expression vectors (12, 17, 18). Mono- and polyclonal anti-GFP antibodies were from CLONTECH; polyclonal 5-HT$_{2A}$ receptor amino terminus-specific and arrestin-3-specific antibodies have been previously described (14, 17). A rabbit polyclonal CT-Arr-2-specific antibody was generated using a glutathione *S*-transferase fusion protein containing residues 357–418 of bovine arrestin-2 as antigen. The rat 5-HT$_{2A}$ receptor was FLAG epitope-tagged on the amino-terminal extracellular domain by subcloning into pCMV-TAG$_{2B}$ (Stratagene) with the introduction of a consensus Factor Xa cleavage site (IEGR) between the FLAG epitope and the amino terminus of 5-HT$_{2A}$ to yield FLAG-2A. In brief, polymerase chain reaction amplification was performed using the oligonucleotide primers 5′-aaaggatccatcgagggccgcggaggtatggaaattctttgtgaag-3′ and 5′-tttggatcctcacacacagctaacctttttc-3′, introducing a *Bam*HI cleavage site for in-frame insertion into pCMV-TAG$_{2B}$. The entire insert was subjected to automated sequencing to confirm that the FLAG tag was in-frame and that no polymerase chain reaction-induced mutations occurred during the amplification.

Transfection and Expression of 5-HT$_{2A}$ Receptors and Arrestins in HEK-293 Cells—HEK-293 cells were plated onto 6-well, 35-mm plates at ~0.5×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. At 24 h after plating, cells were transfected with 2 μg of DNA using Fugene 6 (Roche Molecular Biochemicals) exactly as described by the manufacturer. For cotransfection experiments, the total amount of DNA transfected was kept constant by the addition of empty vector (pcDNA3). At 24 h after transfection, cells were split into 24-well plates containing polylysine-coated coverslips and grown for an additional 24 h in DMEM supplemented with 10% dialyzed serum. The next day, the medium was removed; and after washing with serum-free medium, the cells were incubated overnight with serum-free DMEM to remove 5-HT present in the serum.

Immunocytochemistry—Following various treatments, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, lightly permeabilized on ice (0.2% Triton X-100 in PBS) for 20 min, and incubated with blocking buffer (1% bovine serum albumin and 1% gelatin in PBS) for 1 h. Cells were then incubated overnight with the following antibodies diluted in blocking buffer: a 5-HT$_{2A}$ receptor amino terminus-specific antibody (Ab51; 1:3000 dilution) (14), mono- and polyclonal anti-GFP antibodies (1:5000 dilution), anti-CT-Arr-2 antibody (1:3000 dilution), and a monoclonal anti-arrestin-2 antibody (1:3000 dilution) (19). After warming to room temperature and washing with PBS, cells were incubated with a 1:200 dilution of either BODIPY-FL-labeled goat anti-mouse antibody or Texas Red-labeled goat anti-rabbit antibody (diluted in blocking buffer) for 1 h. Cells were then washed with PBS and mounted for fluorescent confocal microscopic evaluation as previously detailed (14). For confocal microscopy, all images were taken at an overall magnification via the microscope of ×1000; and in selected images, electronic magnification greater than ×1000 was obtained.

Live Cell Confocal Microscopy—For these studies, HEK-293 cells were plated onto sterile 35-mm cell wells that contained coverslips affixed to the bottom in DMEM with 10% fetal calf serum. Cells were then transfected as described above, incubated with DMEM containing 10% dialyzed fetal calf serum for 24 h, and switched to serum-free DMEM for an additional 24-h incubation. On the day of the experiment, cells were switched to indicator-free HEPES-buffered serum-free DMEM and placed on the heated stage of a Zeiss confocal microscope. In addition to a heated stage, a plastic tent enclosed the stage and objective so that a stream of heated air maintained the microscope objective and air surrounding the stage at 37 °C for the duration of the experiment. Images were automatically collected at 30-s intervals, and subsequent image sequences were used to compose movies using Quicktime Pro.

TABLE I
Pharmacological characterization of SR2-GFP expressed in HEK-293 cells

Data represent means of K_i determinations *versus* [^{3}H]ketanserin. For [^{3}H]ketanserin, data represent K_d determination. K_{act} and V_{max} values for 5-HT stimulation of phosphatidylinositol hydrolysis for the native (K_{act} = 88.9 ± 36 nM; V_{max} = 3048 ± 188 cpm) and GFP-tagged (K_{act} = 23 ± 11 nM; V_{max} = 3110 ± 175 cpm) receptors were similar.

Ligand	K_i	
	Native 5-HT$_{2A}$	SR2-GFP
Ketanserin	0.7 nM	0.4 nM
Spiperone	0.98 nM	1.12 nM
5-HT	1.07 μM	0.87 μM

Internalization Assays—Internalization was quantified as previously described using a computer-based image analysis system (14, 15). Typically, 30–100 cells were quantified per experiment in a blinded fashion, and all experiments were replicated at least three times. Data were analyzed via Student's *t* test for comparison of independent mean values, with $p < 0.05$ considered significant.

Surface Biotinylation Assays—Surface biotinylation was done as described previously (15) with modifications. In brief, HEK-293 cells were cotransfected with FLAG-2A and either Arr-2 or CT-Arr-2 as described above in 100-mm dishes. The next day, cells were split onto 6-well polylysine-coated plates with DMEM + 10% dialyzed fetal calf serum; and 24 h later, the medium replaced with serum-free DMEM. The next day, cells were incubated with 5-HT (10 μM) or vehicle (PBS) for 15 min, placed on ice, rinsed with 2 ml of ice-cold biotinylation buffer (10 mM boric acid, 154 mM NaCl, 7.2 mM KCl, and 1.8 mM $CaCl_2$, pH 8.4), and then surface-biotinylated in a total volume of 1.0 ml with 0.8 mM biotin disulfide *N*-hydroxysuccinimide ester for 15 min on ice. The reaction was quenched with 2 ml of quenching buffer (0.192 M glycine, 25 mM Tris, 1.8 mM $CaCl_2$, and 154 mM NaCl, pH 8.3), and the cells were then lysed in 1 ml of lysis buffer (20 mM HEPES, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate, and 1× complete protease inhibitor mixture (−EDTA), pH 7.40) for 15 min on ice. After clarification of lysates by centrifugation (14,000 × *g* for 20 min at 4 °C), lysates were incubated in a total volume of 1.0 ml with 50 μl of streptavidin-agarose for 2 h at 4 °C with constant mixing. Biotinylated proteins were then purified by centrifugation, followed by three washes with lysis buffer at 4 °C. After the final wash, biotinylated FLAG-2A was liberated from the agarose by the addition of 100 μl of SDS sample buffer and heating at 65 °C for 1 min. Samples containing equivalent quantities of protein were then run on 10% SDS-polyacrylamide gels and transferred to nitrocellulose as previously described (15).

For Western blot analysis, blots were blocked with Tris-buffered saline with Tween 20 (150 mM NaCl, 50 mM Tris-Cl, and 0.1% Tween 20, pH 7.40; TBST) containing 5% nonfat dry milk (blocking buffer) for 1 h at room temperature. Blots were then incubated with a 1:2000 dilution of a monoclonal anti-FLAG antibody (Sigma) in blocking buffer for 2 h. Following extensive washing with Tris-buffered saline, blots were incubated with secondary antibody (peroxidase-conjugated goat anti-mouse, 1:2000; Accurate Chemical and Science Corp.) in blocking buffer for 1 h, washed extensively with TBST, and washed twice with Tris-buffered saline. Blots were then incubated for 1 min with chemiluminescence substrate and visualized and quantified using a chemiluminescence imaging system (Kodak Digital Science).

RESULTS

Characterization of GFP-tagged 5-HT$_{2A}$ Receptors—In initial studies, we characterized the pharmacology and functional activity of GFP-tagged 5-HT$_{2A}$ receptors (SR2-GFP) and compared SR2-GFP with wild-type receptors expressed in HEK-293 cells. Table I shows that SR2-GFP and wild-type 5-HT$_{2A}$ receptors have similar expression levels and agonist and antagonist pharmacologies. These results demonstrate that GFP tagging does not lead to detectable changes in 5-HT$_{2A}$ receptor pharmacology or functional activity. Our prior studies of ago-

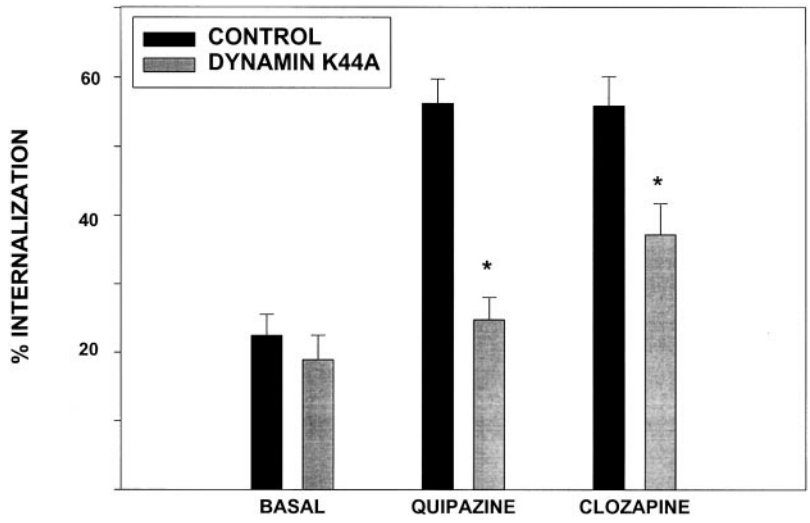

FIG. 1. **Agonist- and antagonist-induced internalization of 5-HT$_{2A}$ receptors are dynamin-dependent.** Cells transfected with SR2-GFP + pcDNA3 (empty vector control) or SR2-GFP + dynamin K44A were exposed to vehicle, 5-HT (10 μM for 15 min), or clozapine (10 μM for 30 min). Cells were then fixed and prepared for immunofluorescent confocal microscopy, and internalization was quantified as described under "Experimental Procedures." Data represent means ± S.E. of internalized receptor from a large number of cells (100–200 cells) from a representative experiment that was replicated three times. *, $p < 0.01$ *versus* control (Student's *t* test).

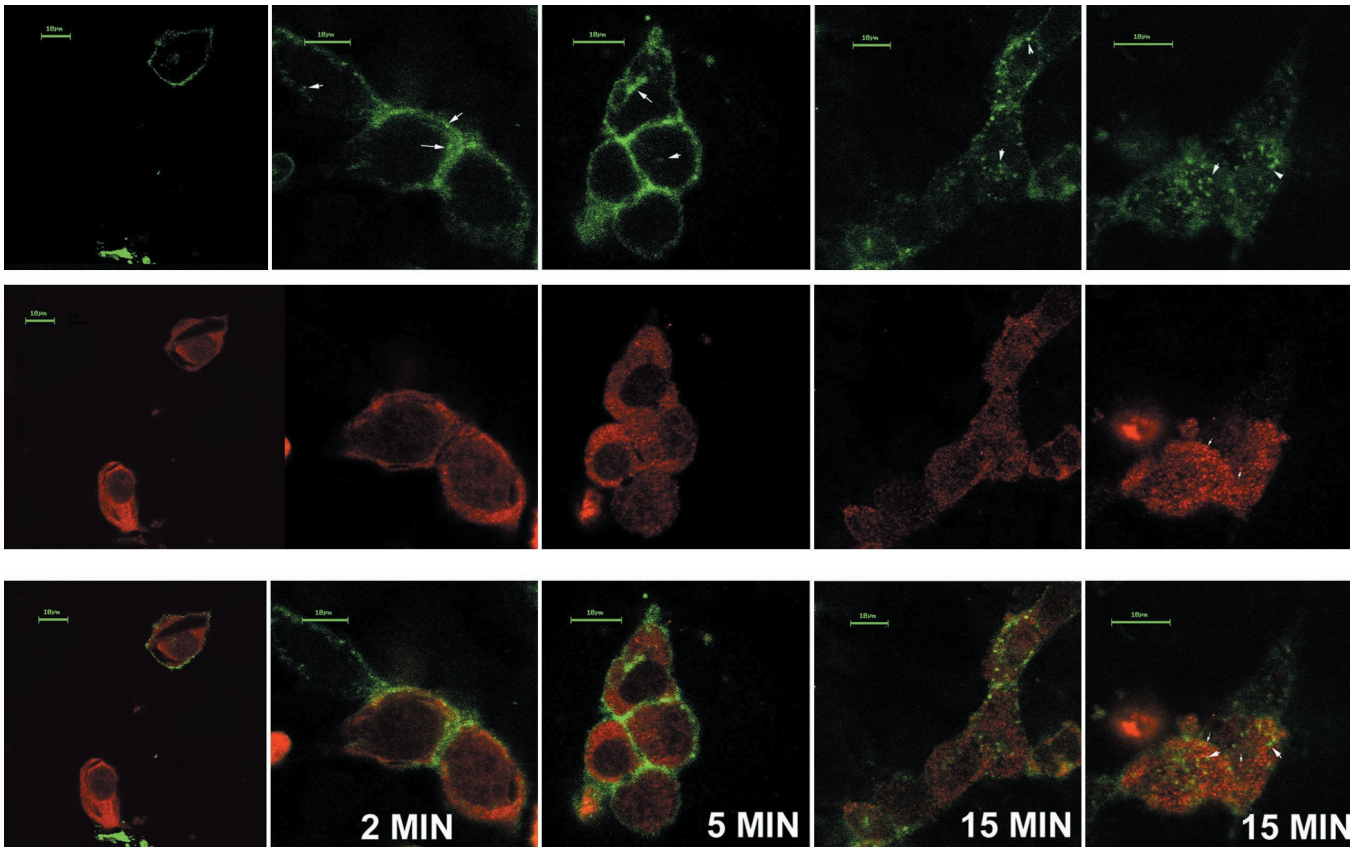

FIG. 2. **Agonist-induced 5-HT$_{2A}$ receptor internalization results in a differential redistribution of arrestin-2.** Cells were transfected with SR2-GFP (*green*) and Arr-2 (*red*), exposed to 5-HT (10 μM) for various time periods, and then prepared for immunofluorescent confocal microscopy as described under "Experimental Procedures." Data represent representative images taken from an experiment that was replicated three times with identical results. *Arrows* indicate differential localization of Arr-2 and SR2-GFP. With the exception of the series of panels on the right, all images were taken from the middle of representative cells. The last series of panels shows a higher power view taken at the level of the plasma membrane from a representative cell after 15 min of 5HT exposure.

nist-induced internalization of 5-HT$_{2A}$ receptors suggested an endosome-mediated process (14). To verify that GFP tagging did not alter the trafficking of 5-HT$_{2A}$ receptors, we performed dual-label, real-time experiments with SR2-GFP and Texas Red-labeled transferrin. Preliminary studies (data not shown) indicated that SR2-GFP was internalized coincident with transferrin, in agreement with our prior studies on the native receptor (14).

Agonist- and Antagonist-mediated Internalization Are Dynamin-dependent—We next cotransfected SR2-GFP with a dynamin dominant-negative mutant (dynamin K44A) to investigate the dynamin dependences of agonist- and antagonist-mediated internalization. For these studies, image sequences were converted to Quick-time Movie Format (see Supplemental Material). These image sequences clearly demonstrate that 5-HT induced a rapid internalization of SR2-GFP in live cells (Image Sequence 1 in Supplemental Material) and that the process of internalization was abolished by cotransfection with dynamin K44A (Image Sequence 2 in Supplemental Material). These image sequences also show that 5-HT induced a change

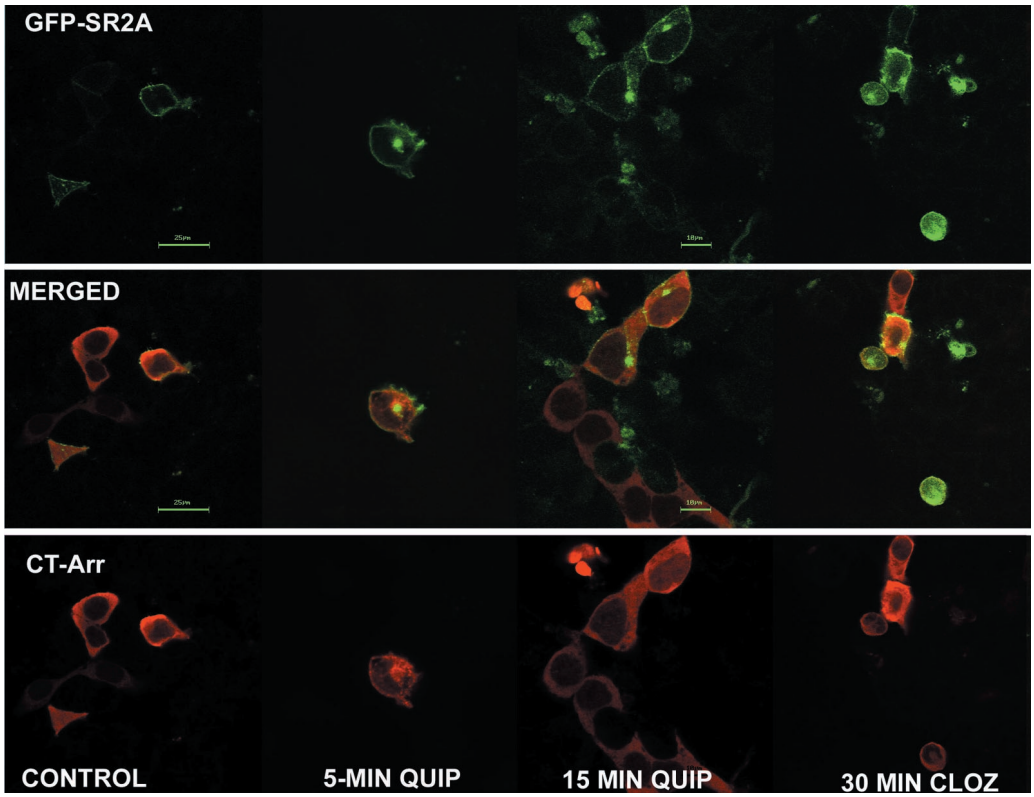

FIG. 3. **Dominant-negative arrestin does not alter agonist- or antagonist-induced internalization of 5-HT$_{2A}$ receptors.** For these experiments, cells were transfected with SR2-GFP + Arr-2-(319–418), stimulated with quipazine (*QUIP*; 100 μM) or clozapine (*CLOZ*; 10 μM) for various time periods, and then prepared for confocal microscopy as described under "Experimental Procedures." A polyclonal CT-Arr-2-specific antibody was used with a Texas Red-labeled secondary antibody to visualize Arr-2-(319–418) (*red*), whereas SR2-GFP (*green*) was visualized using GFP fluorescence. Images are shown from the middle of representative cells from a typical experiment that was replicated with identical results four times.

in shape (Image Sequence 1 in Supplemental Material), which was unaffected by cotransfection with dynamin K44A (Image Sequence 2 in Supplemental Material). Further image sequences of live cells (Image Sequence 3 in Supplemental Material) demonstrate that clozapine induced internalization of 5-HT$_{2A}$ receptors without inducing a change in shape. By contrast, cells exposed to vehicle (Image Sequence 4 in Supplemental Material) showed neither shape change nor receptor internalization. These results indicate that shape change and agonist-mediated receptor internalization are independent processes. Analysis of a large number of cells from several experiments is shown in Fig. 1. As shown, 5-HT and clozapine induced similar degrees of internalization and were both equally sensitive to dynamin K44A. These results indicate that both agonist- and antagonist-induced internalization of 5-HT$_{2A}$ receptors are dynamin-dependent.

Agonist- and Antagonist-induced Internalization Are Arrestin-independent—We next examined the effects of agonist and antagonist exposure on arrestin subcellular distribution using Arr-2 and SR2-GFP. As shown in Fig. 2, agonist exposure induced minimal Arr-2 translocation. Similar results were obtained with cells expressing GFP-Arr-2 and native 5-HT$_{2A}$ receptors (data not shown). After longer time periods of agonist exposure, a redistribution of Arr-2 became evident. As shown in Fig. 2, by 5 min of agonist exposure, a punctate redistribution of Arr-2 in the cytoplasm became evident, which was pronounced by 15 min of agonist exposure. At this time period as well, punctate accumulations of Arr-2 at the plasma membrane were also evident, although it was clear that these punctate accumulations were distinct from those containing SR2-GFP. No redistribution of Arr-2 was seen in cells that were exposed

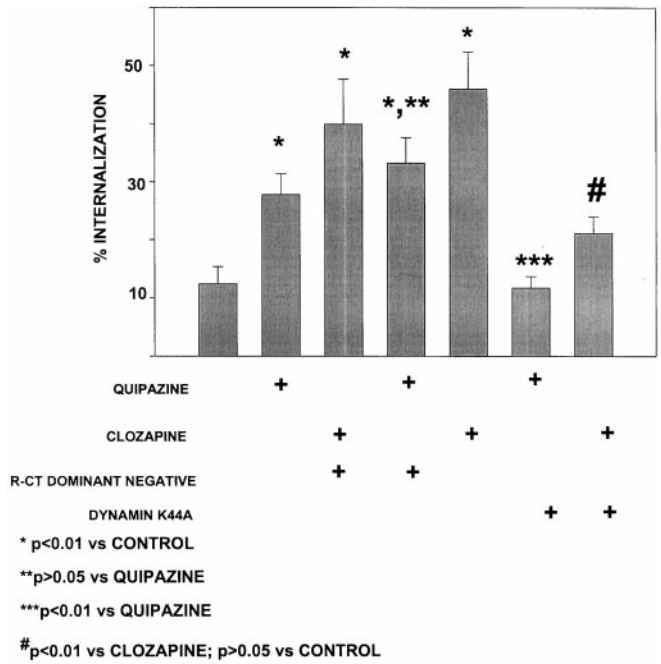

FIG. 4. **Dynamin-dependent, arrestin-independent internalization of 5-HT$_{2A}$ receptors.** Shown are the means ± S.E. from a representative experiment in which 100–200 cells were imaged and internalization of SR2-GFP was analyzed as described under "Experimental Procedures." Cells were transfected with SR2-GFPA ± pcDNA3 (empty vector control), dynamin K44A (positive control), or Arr-2-(319–418).

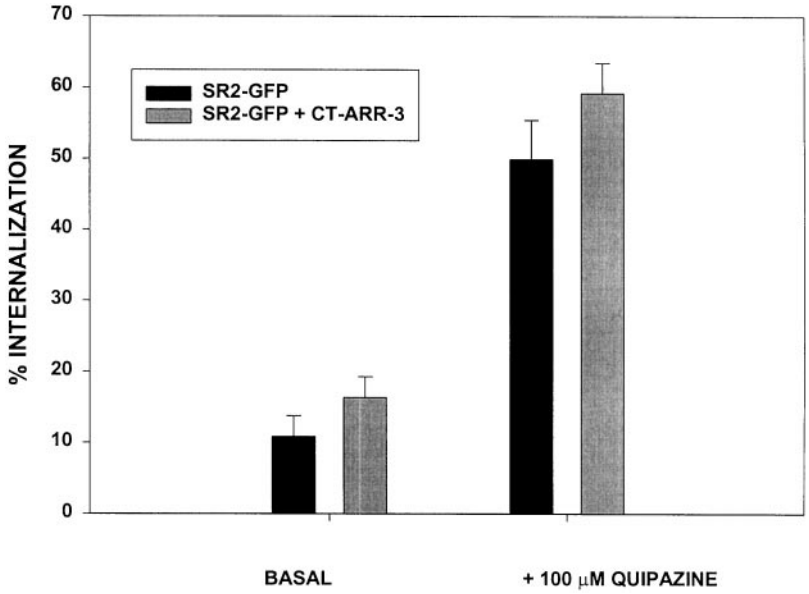

FIG. 5. **Agonist-induced internalization of 5-HT$_{2A}$ receptors is insensitive to an arrestin-3 dominant-negative mutant.** HEK-293 cells were transfected with pRcCMV-SR2 + GFP-Arr-3 or pRcCMV-SR2 + Arr-3-(284–409). Shown are the means ± S.E. from experiments in which 50–100 cells were imaged and internalization of the 5-HT$_{2A}$ receptors was quantified as described under "Experimental Procedures."

to 5-HT but not cotransfected with 5-HT$_{2A}$ receptors (data not shown).

We next examined the arrestin dependences of agonist- and antagonist-induced internalization using three dominant-negative arrestins: 1) the carboxyl-terminal clathrin-binding domain of arrestin-2 (Arr-2-(319–418); CT-Arr-2) (20, 21); 2) the Arr-2 V53D mutant, which has been used by many others (22); and 3) an arrestin-3 dominant-negative mutant (Arr-3-(284–409); CT-Arr-3) (17). As shown in Fig. 3, the agonist quipazine (100 μM) and the antagonist clozapine (10 μM) both induced internalization of SR2-GFP, which was unaffected by coexpressing CT-Arr-2.

We also quantified results from a large number of cells that were transfected with SR2-GFP + pcDNA3 (empty vector; negative control), SR2-GFP + dynamin K44A (positive control), or SR2-GFP + CT-Arr-2. As shown in Fig. 4, cotransfection with CT-Arr-2 had no significant effect on either agonist- or antagonist-induced internalization of SR2-GFP. Similar negative results were found with the dominant-negative mutant Arr-2 V53D (data not shown). As a positive control, the dynamin K44A mutant inhibited both agonist- and antagonist-induced internalization. Fig. 5 shows that CT-Arr-3, an Arr-3 dominant-negative mutant, also had no effect on agonist-induced internalization of 5-HT$_{2A}$ receptors.

A closer examination of the confocal images revealed a distinctive redistribution of Arr-2, Arr-3, and 5-HT$_{2A}$ receptors. Thus, after 5 min of agonist exposure, a redistribution of CT-Arr-2 to the plasma membrane and distinct presumably endocytotic vesicles was apparent. As shown at the 5-min time point in Fig. 6, minimal colocalization of CT-Arr-2 and SR2-GFP was seen in endocytotic vesicles, even though there *was* apparent translocation of CT-Arr-2 to the plasma membrane. Interestingly, the CT-Arr-2 immunofluorescent puncta were clearly distinct from those in which SR2-GFP was localized (Fig. 6). Separate dual-label studies using a polyclonal CT-Arr-2-specific antibody and a monoclonal anti-AP-2 antibody indicated that the vesicles in which CT-Arr-2 is localized are AP-2 containing organelles (data not shown), in verification of prior studies (18).

Next, we examined the effect of quipazine on Arr-3 translocation. As shown in Fig. 7, quipazine (100 μM) induced a modest and rapid translocation of Arr-3 to the plasma membrane of cells transfected with GFP-Arr-3 and native 5-HT$_{2A}$ receptors. At later time periods, a redistribution of GFP-Arr-3 into intracellular vesicles distinct from those containing 5-HT$_{2A}$ receptors was evident (Fig. 7). No colocalization of GFP-Arr-3 and 5-HT$_{2A}$ receptors to intracellular vesicles was evident at any of the time points studied. Additionally, clozapine exposure led to neither GFP-Arr-3 translocation nor colocalization of GFP-Arr-3 with 5-HT$_{2A}$ receptors (Fig. 7). These results indicate that the agonist-induced internalization of 5-HT$_{2A}$ receptors leads to a redistribution of GFP-Arr-3 to subcellular domains that are distinct from those occupied by 5-HT$_{2A}$ receptors. These results also indicate that clozapine induces internalization of 5-HT$_{2A}$ receptors without inducing Arr-3 translocation. Control experiments were performed with HEK-293 cells cotransfected with GFP-Arr-3 and β_2-adrenergic receptors. In agreement with prior studies (23), isoproterenol (10 μM) exposure induced a rapid translocation of GFP-Arr-3 to the plasma membrane (data not shown). Additionally, in cells transfected only with GFP-Arr-3, 5-HT exposure did not change GFP-Arr-3 distribution (data not shown).

Additionally, we sought to determine the effect of CT-Arr-2 on 5-HT$_{2A}$ receptor internalization using a biochemical assay of internalization. For these studies, we cotransfected HEK-293 cells with FLAG-2A and either Arr-2 or CT-Arr-2. Internalization of FLAG-2A was quantified by measuring the loss of surface-biotinylated FLAG-2A by Western blot analysis. As shown in Fig. 8, FLAG-2A was internalized to a similar extent in cells cotransfected with either Arr-2 (61 ± 7%) or CT-Arr-2 (59 ± 6%). Additionally, the extent of internalization induced by 5-HT (61%) was quite similar to that measured by our image analysis technique (45–61%).

To determine whether the differential sorting of 5-HT$_{2A}$ receptors, Arr-2, and CT-Arr-2 was due to some artifact related to GFP tagging of 5-HT$_{2A}$ receptors, we did additional experiments using a FLAG-tagged 5-HT$_{2A}$ receptor (FLAG-2A). For these experiments, we cotransfected FLAG-2A with either Arr-2 or CT-Arr-2 and visualized FLAG-2A with a monoclonal anti-FLAG antibody and Arr-2/CT-Arr-2 with a polyclonal CT-Arr-2-specific antibody. As shown in Fig. 9 (*A–C*), after 5 min of 5-HT exposure, differential sorting of FLAG-2A and Arr-2 was seen. A similar pattern of differential sorting was seen for FLAG-2A and CT-Arr-2 (Fig. 9, *D–F*) following 5 min of 5-HT exposure. These studies indicate that FLAG-2A and SR2-GFP are sorted in an identical manner.

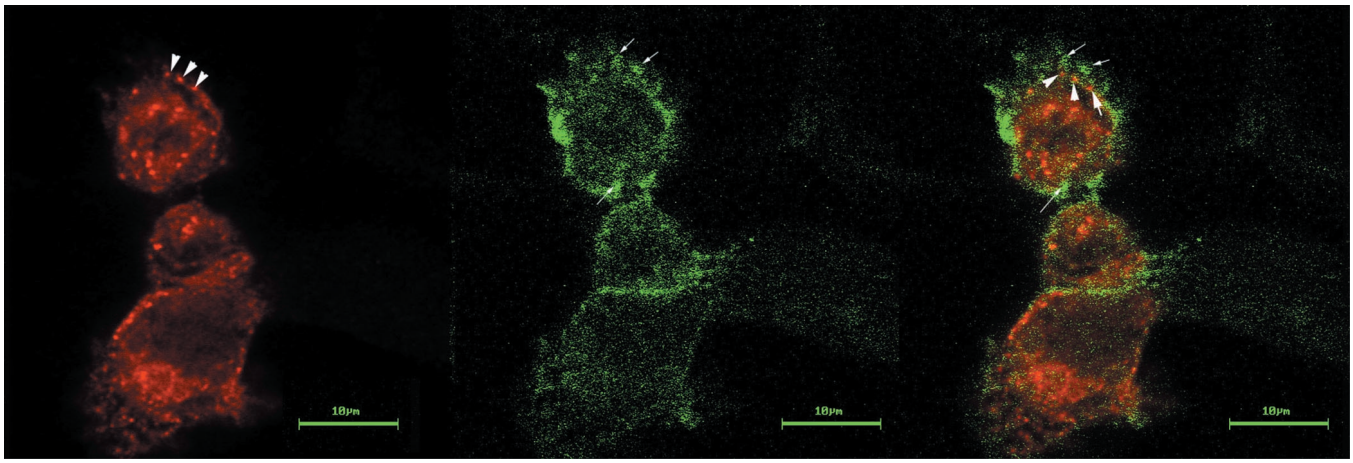

FIG. 6. **Arrestin-2 carboxyl terminus is differentially sorted from internalized 5-HT$_{2A}$ receptors.** Shown is a close-up view of a high power (magnification × 1000) image of a representative HEK-293 cell cotransfected with SR2-GFP (*green*) and Arr-2(319–418) (*red*), stimulated with 5-HT (10 μM) for 5 min, and prepared for confocal microscopy as described under "Experimental Procedures." *Arrowheads* demonstrate Arr-2(319–418) immunoreactive puncta; *arrows* represent SR2-GFP immunoreactive vesicles. The experiment was replicated three times with identical results.

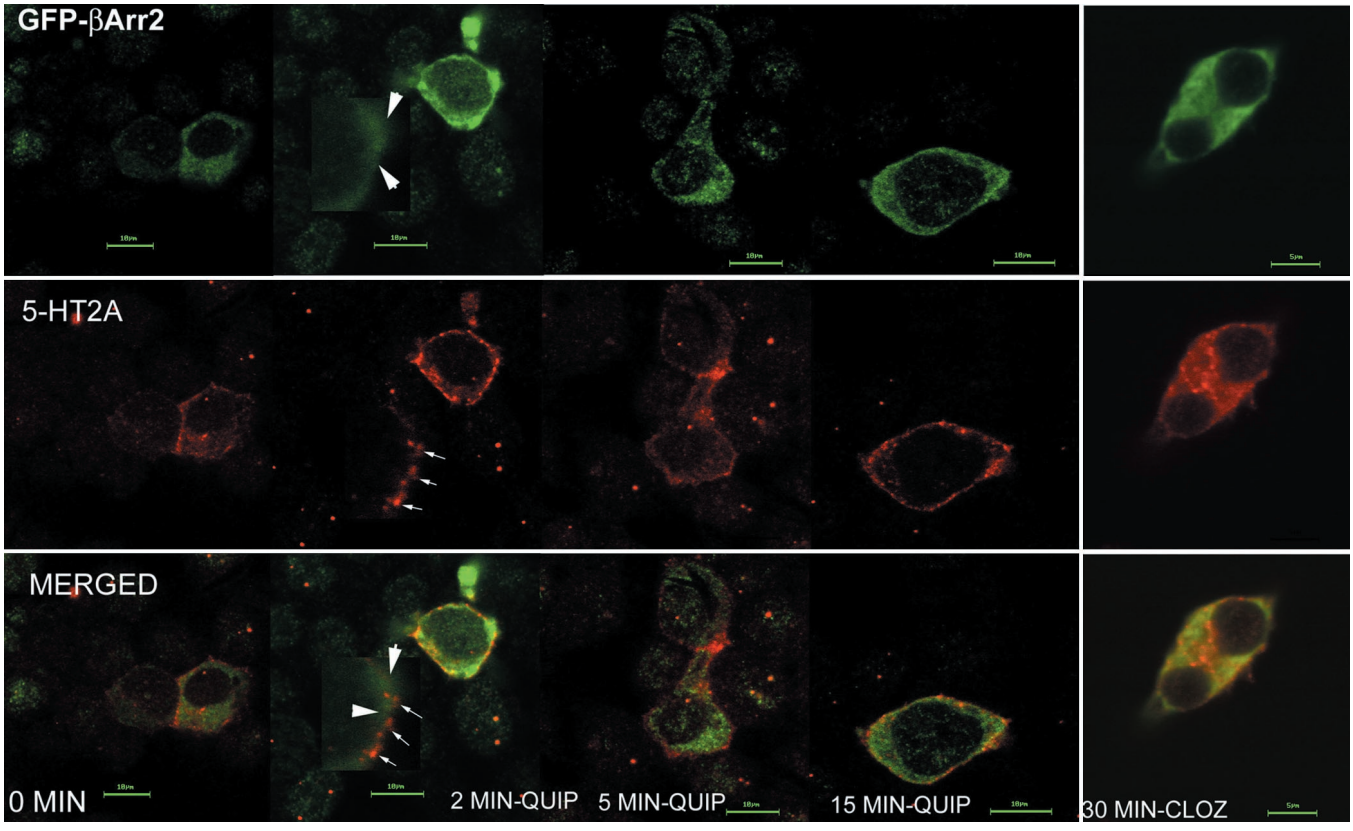

FIG. 7. **Agonist-induced 5-HT$_{2A}$ receptor internalization leads to a differential sorting of arrestin-3 and 5-HT$_{2A}$ receptors.** For these experiments, HEK-293 cells were cotransfected with pRcCMV-SR2 to visualize 5-HT$_{2A}$ receptors (*red*) and with GFP-Arr-3 (*GFP-βArr2*) (*green*) to visualize Arr-3, stimulated with quipazine (*QUIP*; 100 μM) or clozapine (*CLOZ*; 10 μM) for various time periods, and then prepared for confocal microscopy as described under "Experimental Procedures." The *inset* of the 2-min time period shows magnified images of the plasma membrane from the same cells. *Arrows* indicate 5-HT$_{2A}$ receptor immunoreactivity, whereas *arrowheads* represent GFP-Arr-3 fluorescence. The experiment was replicated three times with identical results.

DISCUSSION

The major findings of this study are that 1) agonist- and antagonist-induced internalization of 5-HT$_{2A}$ receptors in HEK-293 cells are dynamin-dependent and arrestin-independent and 2) the agonist-induced internalization of 5-HT$_{2A}$ receptors leads to a differential sorting of 5-HT$_{2A}$ receptors and β-arrestin-1 (Arr-2) and β-arrestin-2 (Arr-3) into distinct plasma membrane and intracellular compartments. These results demonstrate that novel sorting pathways exist for arrestins and 5-HT$_{2A}$ receptors. Additionally, since 5-HT$_{2A}$ receptor activation leads to a redistribution of Arr-2 and Arr-3, our findings imply that internalization-independent functions exist for Arr-2 and Arr-3.

In addition to these novel observations, the results are important because they clarify a long-standing conundrum related to the study of 5-HT$_{2A}$ receptors. Since 1980 (24), it has been noted by a large number of investigators that various 5-HT$_{2A}$ antagonists can induce 5-HT$_{2A}$ receptor down-regula-

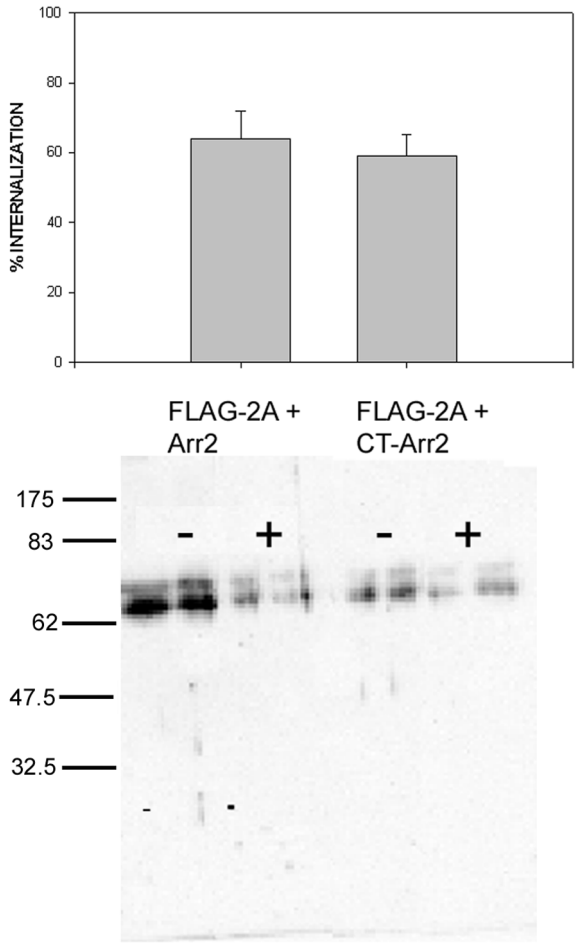

FIG. 8. **CT-Arr-2 does not inhibit 5-HT$_{2A}$ receptor internalization as assessed by surface biotinylation.** HEK-293 cells were cotransfected with FLAG-2A and either Arr-2 or CT-Arr-2 and prepared for surface biotinylation as described under "Experimental Procedures." 15 min after vehicle (−) or 5-HT (10 μM; +) exposure. The *upper panel* shows mean percent internalization (loss of surface receptors) for n = three separate determinations, whereas the *lower panel* shows a representative Western blot. The experiment was replicated twice.

tion *in vivo* and *in vitro* (see Refs. 1 and 25 for reviews). Our prior studies (26), as well as those of others (16), have clearly demonstrated that the antagonist-induced down-regulation of 5-HT$_{2A}$ receptors occurs without significant changes in 5-HT$_{2A}$ receptor gene transcription. These results imply that the antagonist-induced down-regulation of 5-HT$_{2A}$ receptors occurs via post-transcriptional mechanisms. Our recent *in vivo* and *in vitro* studies, in which we demonstrated that antagonists induce 5-HT$_{2A}$ receptor internalization *in vivo* and *in vitro*, imply that antagonist-mediated internalization is a prominent pathway for 5-HT$_{2A}$ receptor down-regulation (15).

Studies delineating the cellular mechanisms responsible for GPCR internalization, however, have implied that antagonists cannot induce internalization and/or down-regulation because the internalization process is dependent upon receptor activation, followed by subsequent receptor phosphorylation and arrestin binding. 5-HT$_{2A}$ antagonists do not activate 5-HT$_{2A}$ receptors using any measure of receptor activity (phosphatidylinositol hydrolysis or arachidonic acid release) and do not induce shape change (present findings). In fact, clozapine, the antagonist used in these studies, has been demonstrated to be a potent antagonist with *negative intrinsic activity* at 5-HT$_{2A}$ receptors (27). Thus, it is difficult to reconcile a large number of prior findings that 5-HT$_{2A}$ antagonists can induce receptor internalization and down-regulation with most of the current models of GPCR regulation.

Our present results, which indicate that both the clozapine- and quipazine-induced internalization are sensitive to a dynamin dominant-negative mutant (dynamin K44A) and insensitive to three different arrestin dominant-negative mutants (Arr-2 V53D, Arr-2-(319–418), and Arr-3-(284–409)), show that 5-HT$_{2A}$ agonists and antagonists induce internalization via an *arrestin-independent* pathway. Since the pathway is dynamin-sensitive and since 5-HT$_{2A}$ receptors internalize via clathrin-coated vesicles (28) and transferrin-containing endosomes (Ref. 14 and present results), it is clearly an endosome-mediated process. It is unlikely that internalization occurs via caveolae since the present studies demonstrated that agonist-induced internalization occurs coincident with transferrin receptor internalization, which is a well accepted marker of endosome-mediated internalization. Additionally, our prior studies demonstrated that internalized receptors do not colocalize with caveolin *in vitro* (14, 28).

Our results also show that, although arrestins are not involved in 5-HT$_{2A}$ receptor internalization, activation of 5-HT$_{2A}$ receptors by agonists induces a translocation/redistribution of Arr-2 and Arr-3. Intriguingly, although Arr-2 and truncated Arr-2 (Arr-2-(319–418)) were redistributed to intracellular vesicles, minimal colocalization with 5-HT$_{2A}$ receptors was detected. Prior studies have implicated the C terminus of Arr-2 as containing both clathrin-binding (20) and AP-2 adaptor protein-binding (13) motifs. Our results imply that the C terminus of Arr-2 may be recruited to distinct plasma membrane and endocytic subcellular domains independently of GPCR internalization. This conclusion is supported by preliminary findings from triple-label studies in which we found that Arr-2 and CT-Arr-2 are redistributed, in part, to AP-2 containing vesicles that are distinct from those containing 5-HT$_{2A}$ receptors (data not shown).

Quite recent studies (29) suggest that at least two distinct classes of GPCRs exist, depending upon their affinities for Arr-2 and Arr-3. The 5-HT$_{2A}$ receptor appears to fall into Group B since 5-HT$_{2A}$ receptors induce a very modest redistribution of Arr-2 and Arr-3. Prior studies by us (19) have demonstrated that Arr-2 and Arr-3 can bind to the purified third intracellular loop of the 5-HT$_{2A}$ receptor *in vitro* and that 5-HT$_{2A}$ receptors are colocalized with Arr-2 and Arr-3 in some, but not all, cortical neurons. The present studies imply that unknown neuron-specific factors regulate the interactions of Arr-2 and Arr-3 with 5-HT$_{2A}$ receptors in cortical neurons because minimal colocalization of Arr-2/Arr-3 and 5-HT$_{2A}$ receptors was evident in HEK-293 cells.

If Arr-2 and Arr-3 are induced to redistribute by 5-HT$_{2A}$ receptor stimulation, but fail to regulate internalization, what is the role for redistribution of Arr-2 to punctate domains on the plasma membrane and distinct intracellular vesicles? The present studies do not answer these important questions, although work in progress suggests that the C terminus of Arr-2 may regulate mitogen-activated protein kinase (p42/44) activation via a pathway distinct from that used to internalize 5-HT$_{2A}$ receptors.[2]

Several control experiments were performed to verify that the results we obtained were not due to artifacts related to GFP tagging of 5-HT$_{2A}$ receptors or to the presence of endogenous 5-HT receptors in HEK-293 cells. Thus, we found that native 5-HT$_{2A}$ receptors and FLAG-tagged and GFP-tagged 5-HT$_{2A}$ receptors were differentially sorted from Arr-2 and Arr-3 during endocytosis. Additionally, we verified that CT-Arr-2 did not

[2] J. Woods and B. L. Roth, manuscript in preparation.

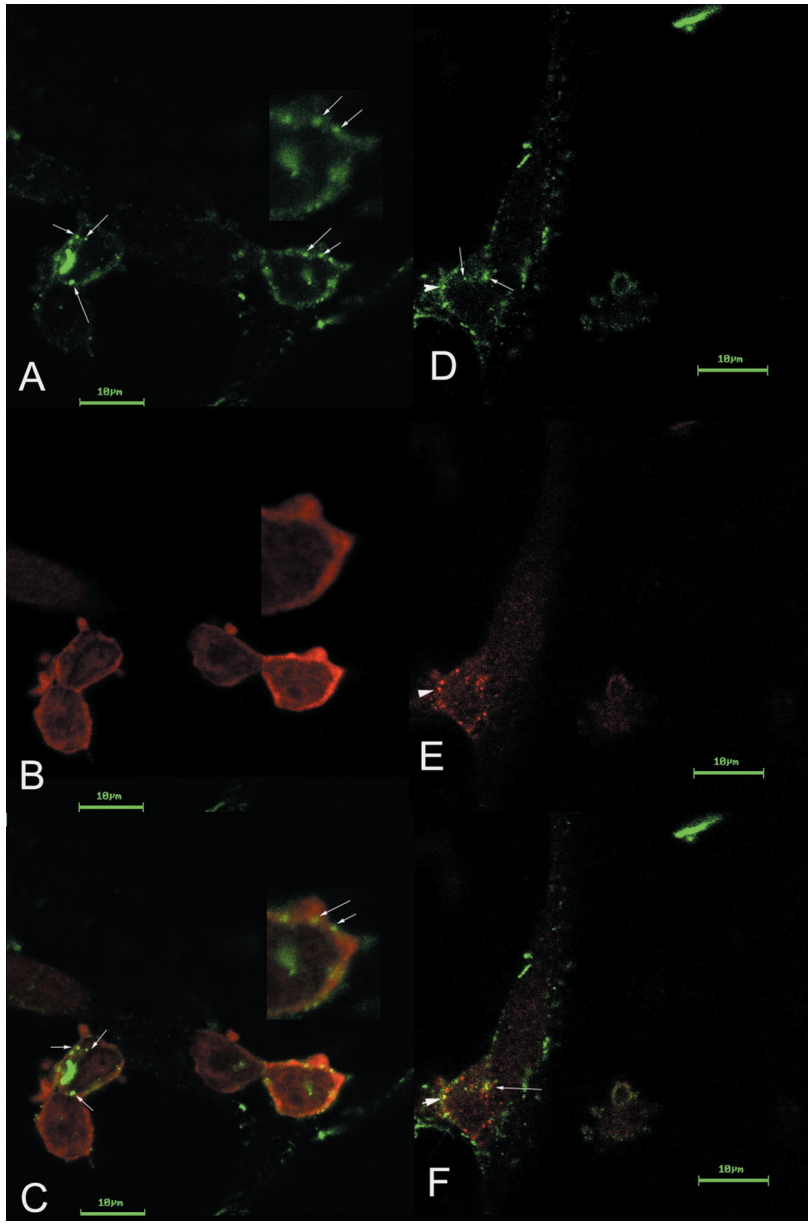

FIG. 9. **Differential sorting of FLAG-2A, Arr-2, and CT-Arr-2 following 5-HT exposure in HEK-293 cells.** For these studies, HEK-293 cells were co-transfected with FLAG-2A and either Arr-2 (*A–C*) or CT-Arr-2 (*D–F*), stimulated for 5 min with 10 μM 5-HT, and then prepared for immunofluorescent confocal microscopy as described under "Experimental Procedures." FLAG-2A was visualized using a mouse monoclonal anti-FLAG antibody and a BODIPY-FL-tagged anti-mouse secondary antibody, whereas Arr-2 and CT-Arr-2 were visualized using rabbit polyclonal anti-CT-Arr-2 antibodies and Texas Red-tagged anti-rabbit secondary antibodies. The *inset* shows a magnified image of the plasma membrane of a typical cell. *Arrows* indicate FLAG-2A immunofluorescence, whereas the *arrowhead* indicates the one vesicle in which CT-Arr-2 and FLAG-2A were colocalized. The experiment was replicated with identical results three times.

inhibit 5-HT$_{2A}$ receptor internalization using a biochemical assay of internalization (surface biotinylation). In fact, the extent of internalization induced by 5-HT was the same whether internalization was measured by confocal microscopy or by surface biotinylation (~60% in both cases). Finally, in untransfected cells, no redistribution of Arr-2 or Arr-3 was seen after 5-HT exposure.

Taken together, these results demonstrate that 5-HT$_{2A}$ receptors may induce plasma membrane and intracellular sorting of Arr-2 and Arr-3 distinct from that of internalized 5-HT$_{2A}$ receptors. Importantly, internalization *per se* is not a sufficient signal for Arr-2 or Arr-3 redistribution since clozapine, which induces internalization but does not activate 5-HT$_{2A}$ receptors, does not alter the subcellular distribution of Arr-2 or Arr-3. Additionally, our results demonstrate that the dynamin-dependent, arrestin-independent modes of GPCR internalization occur coincident with redistribution of Arr-2 and Arr-3 to the plasma membrane. Taken together, these results are sufficient to conclude that novel internalization-independent modes of arrestin redistribution occur following 5-HT$_{2A}$ receptor activation.

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