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The Interaction of a Constitutively Active Arrestin with the Arrestin-Insensitive 5-HT_{2A} Receptor Induces Agonist-Independent Internalization

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ABSTRACT

5-HT_{2A} serotonin receptors are unusual among G-protein coupled receptors in that they can be internalized and desensitized, in some cell types, in an arrestin-independent manner. The molecular basis of the arrestin-insensitivity of 5-HT_{2A} receptors is unknown but is probably caused, in part, by the apparent lack of agonist-induced 5-HT_{2A} receptor phosphorylation. Because the arrestin-insensitivity of 5-HT_{2A} receptors is cell-type selective, we used a "constitutively active" arrestin mutant that can interact with agonist-activated but nonphosphorylated receptors. We show here that this "constitutively active" arrestin mutant (Arr2-R169E) can force 5-HT_{2A} receptors to be regulated by arrestins. Cotransfection of 5-HT_{2A} receptors with Arr2-R169E induced agonist-independent 5-HT_{2A} receptor internalization, and a constitutive translocation of the Arr2-R169E mutant to the plasma membrane, whereas

wild-type Arrestin-2 had no effect. Additionally, Arr2-R169E, unlike wild-type arrestin-2, induced a significant decrease in efficacy of agonist-induced phosphoinositide hydrolysis with an unexpected increase in agonist potency. Radioligand binding assays demonstrated that the fraction of receptors in the high-affinity agonist binding-state increased with expression of Arr2-R169E, indicating that Arr2-R169E stabilizes the agonist-high affinity state of the 5-HT_{2A} receptor (R*). Intriguingly, the agonist-independent interaction of Arr2-R169E with 5-HT_{2A} receptors was inhibited by inverse agonist treatment and is thus probably caused by the high level of 5-HT_{2A} receptor constitutive activity. This is the first demonstration that a constitutively active arrestin mutant can both induce agonist-independent internalization and stabilize the agonist-high affinity state of an arrestin-insensitive G protein coupled receptor.

Serotonin_{2A} [5-hydroxytryptamine_{2A} (5-HT_{2A})] receptors are essential for mediating a large number of physiologic processes in the periphery and in the central nervous system, including platelet aggregation, smooth muscle contraction, and the modulation of mood and perception. Many drugs of diverse therapeutic classes mediate their actions, at least in part, by interactions with 5-HT_{2A} receptors. Most but not all hallucinogens (Roth et al., 2002), including lysergic acid diethylamide and *N,N'*-dimethyltryptamine, function as agonists at 5-HT_{2A} receptors, whereas all clinically approved atypical antipsychotic drugs are potent 5-HT_{2A} receptor antagonists. 5-HT_{2A} receptors are localized to the apical dendrites of pyramidal neurons in the frontal cortex and are

found predominantly intracellularly in neurons (Willins et al., 1997; Cornea-Hebert et al., 1999).

Many prior studies have suggested a general mechanism of G protein-coupled receptor (GPCR) desensitization involving the phosphorylation of the intracellular domains of GPCRs (Ferguson, 2001). Receptor phosphorylation also promotes the binding of arrestins to the intracellular loops and carboxy-terminal tails of agonist-activated GPCRs, further preventing G proteins from coupling (Ferguson, 2001). Arrestin binding to phosphorylated receptors is accomplished by the disruption of a "polar core" within the arrestin molecule by the highly charged receptor-attached phosphate moiety, resulting in the transition of arrestin to its active high-affinity receptor binding state (Gurevich and Benovic, 1995; Gurevich and Benovic, 1997; Vishnivetskiy et al., 1999). Indeed, mutations that destabilize the "polar core" of both visual and nonvisual arrestins result in enhanced binding of arrestin with nonphosphorylated agonist-activated receptors (Gure-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; DOI, 2,5-dimethoxy-4-iodophenylisopropylamine; BODIPY-FL, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid, succinimidyl ester; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PI, phosphoinositide; DynK44A, Dynamin K44A; GRK, G protein-coupled receptor kinase; Arr2-wt, Arrestin-2; Arr2-R169E, Arrestin-2 (R169E).

vich and Benovic, 1995; Gray-Keller et al., 1997; Kovoor et al., 1999). In addition to uncoupling receptors from G proteins, the interaction of many GPCRs with arrestins promotes the targeting of desensitized receptors to clathrin-coated pits for their subsequent internalization (Ferguson, 2001). After endocytosis and sorting to the endosomal compartment, GPCRs may be rapidly dephosphorylated and recycled back to the plasma membrane (a process termed resensitization) or targeted to lysosomes for degradation (a process involved in down-regulation) (Gray and Roth, 2002).

Although arrestins play essential roles in the desensitization and internalization of many GPCRs, it is clear that these mechanisms are by no means universal. Thus, we have recently demonstrated that 5-HT_{2A} receptor internalization follows the clathrin-mediated endocytic pathway and is dynamin-dependent (Berry et al., 1996; Bhatnagar et al., 2001). However, we also found that 5-HT_{2A} receptor endocytosis is independent of the action of arrestins and, interestingly, results in a redistribution of arrestins into intracellular vesicles and plasma membrane compartments distinct from those containing internalized 5-HT_{2A} receptors (Bhatnagar et al., 2001). Additionally, 5-HT_{2A} receptor desensitization and resensitization is arrestin-independent and is unaffected by overexpression of the G protein-coupled receptor kinases GRK2 and GRK5 (Gray et al., 2001). The basis of the arrestin-independent regulation of 5-HT_{2A} receptors in HEK293 cells is unknown but is probably caused by an apparent lack of agonist-induced 5-HT_{2A} receptor phosphorylation (Gray and Roth, 2001; Vouret-Craviari et al., 1995; B. Roth, unpublished observations). In neurons, however, 5-HT_{2A} receptors, which are predominantly intracellular (Willins et al., 1997; Cornea-Hebert et al., 1999), colocalize with endogenous arrestin-2 and arrestin-3 (Gelber et al., 1999). Thus, this arrestin-insensitivity of 5-HT_{2A} receptors is, apparently, cell-type dependent and may signify novel modes of regulation of 5-HT_{2A} receptors by arrestins. These prior findings predicted to us that a 'constitutively active' (e.g., Arr2-R169E) arrestin mutant might serve to rescue the arrestin-insensitivity of 5-HT_{2A} receptors in culture and provide us with important insights into the potential role(s) arrestins may have in modulating 5-HT_{2A} receptor signaling in other cell types (e.g., neurons).

In the present studies, therefore, we examined the effect of the receptor phosphorylation-independent arrestin-2 mutant (Arr2-R169E) on 5-HT_{2A} receptor signaling and regulation in transfected HEK-293 cells. Coexpression of 5-HT_{2A} receptors with Arr2-R169E results in substantial basal receptor internalization indicating a forced reversal of the arrestin-independent trafficking of the 5-HT_{2A} receptor. Overexpression of Arr2-R169E also results in 5-HT_{2A} receptors that are "locked" into an agonist high-affinity state that shows diminished signaling, as demonstrated by an increase in agonist potency with decreased efficacy. Taken together, these results demonstrate that a constitutively active arrestin mutant forces the agonist-independent internalization of arrestin-insensitive 5-HT_{2A} receptors, suggesting that the arrestin-insensitivity of 5-HT_{2A} receptors is probably caused not by the inability of arrestins to interact with the receptor but by other factors. The ability of Arr2-R169E to induce the constitutive internalization of 5-HT_{2A} receptors in HEK-293 cells suggests that arrestins may also play a role in the

maintenance of the intracellular localization of 5-HT_{2A} receptors in neurons.

Materials and Methods

Materials and Constructs. HEK-293 cells were purchased from the American Type Culture Collection (Manassas, VA). [³H]Inositol (21.0 Ci/mmol), [³H]ketanserin (63.3 Ci/mmol), and [¹²⁵I]2,5-dimethoxy-4-iodophenylisopropylamine (DOI) were obtained from PerkinElmer (Boston, MA). Quipazine, 5-hydroxytryptamine (5-HT), DOI, phenoxybenzamine, clozapine, spiperone, ritanserin, ketanserin, and dithiobis(succinimidylpropionate) were purchased from Sigma (St. Louis, MO). An amino-terminal FLAG epitope-tagged rat 5-HT_{2A} receptor was constructed as described previously (Bhatnagar et al., 2001). Wild-type and R169E mutant arrestin-2 constructs were described previously (Kovoor et al., 1999). The cDNA of the dominant-negative mutant of dynamin I (DynK44A) was the generous gift of Dr. M. G. Caron (Duke University Medical Center, Durham, NC). The anti-arrestin2-ct antibody was the generous gift of Dr. Jeffrey Benovic (Thomas Jefferson University Medical School, Philadelphia, PA). The polyclonal 5-HT_{2A} receptor antibody (Ab51) specific to the amino terminus was described previously (Berry et al., 1996). The monoclonal anti-arrestin-2 antibody was purchased from BD Transduction Laboratories (Lexington, KY). The monoclonal anti-transferrin receptor antibody was the generous gift of Dr. Martin Snider (Case Western Reserve University, Cleveland, OH). The polyclonal anti-FLAG antibody and anti-FLAG-M2 agarose beads were purchased from Sigma (St. Louis, MO). Goat anti-rabbit-horseradish peroxidase and goat anti-rabbit-Texas Red were purchased from Vector Laboratories (Burlingame, CA) and goat anti-rabbit-BODIPY-FL was from Molecular Probes (Eugene, OR).

Cell Culture and Transfection. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. HEK-293 cells were transfected in 10-cm dishes at 60 to 80% confluence with 2 µg of receptor DNA and 4 µg of cotransfected DNA using Fugene6 (Roche, Indianapolis, IN) exactly as described by the manufacturer. For transfection of receptor alone, the total amount of DNA transfected was kept constant with the addition of empty vector (pcDNA3).

Determination of Phosphoinositide Hydrolysis. At 24 h after transfection, cells were split into poly(L-lysine)-coated 24-well plates and grown for an additional 24 h in DMEM supplemented with 5% dialyzed fetal calf serum. Cell cultures were grown at 37°C in 5% CO₂. Twenty-four hours later, the cells were washed with inositol-free DMEM and incubated for an additional 18 h with inositol-free DMEM containing 1 µCi/ml [³H]inositol and 5% dialyzed fetal calf serum. Receptor stimulation was performed in 1× Hank's balanced salt solution supplemented with 11 mM glucose and 10 mM LiCl, incubated for 30 min at 37°C. The reaction was terminated by aspiration and the addition of 1 ml of 10 mM formic acid. Isolation and measurement of total phosphoinositides was performed exactly as described previously (Gray et al., 2001). Desensitization and resensitization experiments were performed as described previously (Gray et al., 2001).

Binding Assays. Competition binding assays were performed with [³H]ketanserin or [¹²⁵I]DOI in total volumes of 0.25 ml at room temperature for 1 h with 5 to 20 µg of cells harvested with hypotonic buffer (50 mM Tris-HCl, pH 7.4) as described previously (Choudhary et al., 1992) in 50 mM Tris-Cl buffer, pH 7.4. Saturation binding assays were performed with saturating concentrations of [³H]ketanserin as described previously (Gray et al., 2001). Nonspecific binding was defined as radioactivity bound in the presence of 10 µM clozapine or spiperone and represented less than 10% of total binding. Membranes were harvested with a Brandel cell harvester followed by three ice-cold washes onto polyethylenimine-pretreated (0.3%) Whatman GF/C filters. Radioactivity bound to filters was quantified by liquid scintillation or gamma counting. Specific binding was nor-

malized to total membrane protein concentrations determined using an assay kit from Bio-Rad (Hercules, CA) with bovine serum albumin as the standard.

Immunocytochemistry and Confocal Microscopy. Twenty-four hours after transfection, cells were plated onto poly(L-lysine)-coated coverslips in DMEM supplemented with 5% dialyzed fetal calf serum, then switched to serum-free DMEM 18 h before treatment. Cells were treated with vehicle, 10 μ M 5-HT, or 1 μ M ritanserin for 10 min, placed on ice, then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, lightly permeabilized on ice (0.3% Triton X-100 in PBS) for 20 min, and incubated with blocking buffer (5% milk in PBS) for 1 h. Cells were then incubated overnight with either a 5-HT_{2A} receptor amino terminus-specific antibody (Ab51; 1:3000 dilution) (Berry et al., 1996), a monoclonal anti-Arrestin antibody (1:2000 dilution), or a monoclonal anti-transferrin receptor antibody (1:200 dilution), each diluted in blocking buffer. Cells were then washed twice with PBS and incubated with a 1:200 dilution of either Texas Red-labeled goat anti-rabbit antibody or BODIPY-FL-labeled horse anti-mouse antibody for 1 h in blocking buffer. Cells were then washed with PBS and mounted for fluorescent confocal microscopic evaluation as previously detailed (Bhatnagar et al., 2001). For confocal microscopy, all images were taken at an overall optical magnification of 1000 \times ; in selected images, electronic magnification greater than 1000 \times was obtained. For quantification, images were taken of several fields with multiple cells from three independent experiments and quantified in a blinded fashion with Meta-View 4.5 imaging software (Universal Imaging Corporation, Downingtown, PA). Briefly, percentage intracellular immunofluorescence was determined by the equation: [(mean intracellular pixel intensity - mean background pixel intensity) \times region area]/[(mean total cellular pixel intensity - mean background pixel intensity) \times region area] \times 100.

Arrestin Cross-Linking and Western Blotting. Quantification of arrestin binding was performed essentially as previously detailed (Freedman et al., 1997; Min et al., 2002) for β_2 -adrenergic receptors. Briefly, 24 h after transfection, cells were split into poly(L-lysine)-coated six-well plates in DMEM supplemented with 5% dialyzed fetal calf serum. Cells were switched to serum-free DMEM after 24 h and grown for an additional 18 h. Before experiment, cells were washed three times with 0.15 M NaCl, 20 mM HEPES, pH 7.4, at 37°C. Cells were treated with vehicle or 10 μ M 5-HT for 10 min in a total volume of 900 μ l at 37°C. After incubation, each well received 100 μ l of 25 mM dithiobis(succinimidylpropionate) in dimethyl sulfoxide. The cross-linking reaction was allowed to proceed for 30 min at room temperature with rocking (Freedman et al., 1997; Min et al., 2002). Cells were placed on ice and washed three times with ice-cold 0.15 M NaCl, 20 mM HEPES pH 7.4 then incubated with lysis buffer [50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 \times Complete, EDTA-free protease inhibitor cocktail (Roche)] at 4°C with gentle shaking. Cells were scraped into microcentrifuge tubes and spun at 14000g for 20 min at 4°C. Lysates were normalized for protein levels determined using the Bio-Rad assay kit (Hercules, CA) with bovine serum albumin as the standard. 800 μ l of normalized supernatants were transferred to fresh tubes with 40 μ l anti-FLAG-M2 agarose beads (Sigma; St. Louis, MO) and rotated at 4°C for 2 h. Beads were washed 3 times with wash buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl). The cross-linked complexes were eluted with 15 μ g of 3 \times -FLAG peptide (Sigma) in 100 μ l of wash buffer at 4°C for 30 min with gentle shaking. Beads were spun briefly and supernatant was added to 100 μ l of 2 \times SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 100 mM dithiothreitol) to reduce the cross-linked complex. Samples were heated to 95°C for 10 min then resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Blots were blocked in blocking buffer (Tris-buffered saline with 0.01% Tween 20 and 5% nonfat dried milk) for 1 h at room temperature. The blots were then incubated for 2 h at room temperature with anti-arrestin2-ct (1:10,000) or polyclonal anti-FLAG (1:2,000) in

blocking buffer. Blots were washed three times for 10 min each at room temperature with Tris-buffered saline containing 0.05% Tween 20 and incubated with goat anti-rabbit-horseradish peroxidase (1:2000) for 1 h at room temperature in blocking buffer. Blots were washed and visualized with the use of Lumi-light Western blotting substrate (Roche). Chemiluminescence was detected and quantified with a Kodak digital imaging system adjusted to prevent image saturation. Immunoprecipitated arrestins were normalized to amount in lysate.

Data Analysis. Phosphoinositide hydrolysis (PI) hydrolysis assays were performed in triplicate and repeated at least three times and were analyzed by nonlinear regression using Prism 3.0 software (GraphPad, San Diego, CA). Statistical significance of the data were determined by two-tailed paired *t* test and was defined as *p* < 0.05 and was analyzed by GraphPad Prism. Binding data were analyzed using GraphPad Prism and the weighted, nonlinear least-squares curve fitting program LIGAND (Munson and Rodbard, 1980).

Results

A Constitutively Active Arrestin Induces Basal Internalization of 5-HT_{2A} Receptors. We have recently shown that 5-HT_{2A} receptors are internalized, desensitized, and resensitized in an arrestin-independent manner in HEK-293 cells (Bhatnagar et al., 2001; Gray et al., 2001). We hypothesize that the basis of this arrestin-independent regulation is the inability of 5-HT_{2A} receptors to efficiently activate arrestins because of the apparent lack of agonist-induced phosphorylation (Vouret-Craviari et al., 1995; Gray and Roth, 2001). A direct prediction of this hypothesis is that a constitutively active arrestin mutant (Arr2-R169E) should rescue the arrestin-independent phenotype of 5-HT_{2A} receptors. As shown in Fig. 1A, 5-HT_{2A} receptors are predominantly found on the cell surface when cotransfected with wild-type arrestin-2 (Arr2-wt) and are rapidly internalized after stimulation with 5-HT (Fig. 1B). To our knowledge, this is the first demonstration that a constitutively active arrestin induces agonist-independent internalization of a GPCR.

Agonist stimulation resulted in approximately 40 to 50% internalization with cells cotransfected with 5-HT_{2A} receptors and wt-Arr2 (Fig. 2) in agreement with our previous studies (Berry et al., 1996; Bhatnagar et al., 2001). Additionally, stimulation of 5-HT_{2A} receptors elicits no translocation of Arr2-wt (Fig. 1B) (Bhatnagar et al., 2001), unlike the response seen with most other GPCRs. Cotransfection of Arr2-R169E with 5-HT_{2A} receptors, however, results in a significant increase in the level of constitutive internalization (Figs. 1C and 2) to levels even higher than that observed for agonist-stimulated cells transfected with Arr2-wt and 5-HT_{2A} receptors. Significantly, treatment of Arr2-R169E transfected cells with 5-HT did not result in any further increase in the amount of intracellular 5-HT_{2A} receptors (Figs. 1D and 2). To determine whether the increase in intracellular receptors induced by Arr2-R169E represents constitutive internalization impaired plasma membrane translocation, we cotransfected 5-HT_{2A} receptors with Arr2-R169E and stained for 5-HT_{2A} receptors and transferrin receptors (a marker for clathrin-coated vesicles and early endosomes). As shown in Fig. 3A, the intracellular 5-HT_{2A} receptors (red) are mainly colocalized with transferrin receptors (green). As shown in Fig. 3B, 5-HT_{2A} receptor internalization induced by Arr2-R169E was blocked by a cotransfected dominant-negative dynamin mutant (DynK44A) that

we have previously demonstrated to block 5-HT_{2A} receptor internalization in HEK-293 cells (Bhatnagar et al., 2001; Gray et al., 2001). Additionally, some 5-HT_{2A} receptor immunofluorescence showed a “beaded” appearance along the cell surface when cotransfected with DynK44A consistent with our previous reports (Bhatnagar et al., 2001), and this colocalized with Arr2-R169E immunofluorescence (Fig. 3B, arrows). Taken together, these results demonstrate that the increase in intracellular 5-HT_{2A} receptors upon cotransfection of Arr2-R169E is probably caused by the constitutive, agonist-independent internalization of 5-HT_{2A} receptors via clathrin-coated pits to the early endosomal compartment. Another important finding was that, in the presence of 5-HT_{2A} receptors, Arr2-R169E was constitutively translocated to the cell surface, although in cells not transfected with receptor, Arr2-R169E maintained a cytoplasmic distribution (Figs. 4A and 1, C and D), suggesting a specific association between 5-HT_{2A} receptors and Arr2-R169E. Taken together, these results demonstrate that 5-HT_{2A} receptors,

which internalize in an arrestin-independent fashion, are forcibly internalized by a constitutively active arrestin.

The agonist independence of Arr2-R169E-induced 5-HT_{2A} receptor internalization is probably caused by the high level of 5-HT_{2A} receptor constitutive activity (Shapiro et al., 2002). Therefore, we treated the transfected cells for 15 min with ritanserin, a potent 5-HT_{2A} receptor inverse agonist (Shapiro et al., 2002), before confocal microscopy. As shown in Fig. 4B, inverse agonist treatment caused a translocation in Arr2-R169E from the cell surface back to a cytosolic distribution. These results suggest that the interaction of 5-HT_{2A} receptors with the constitutively active arrestin requires the receptors to be in an active conformational state.

The Direct Association of Wild-Type and Mutant Arrestin-2 with 5-HT_{2A} Receptors Occurs in an Agonist-Independent Fashion. To determine whether Arr2-wt and Arr2-R169E directly interacted with 5-HT_{2A} receptors, we have used a cross-linking approach that has been previously demonstrated to facilitate the immunoprecipitation of nonvi-

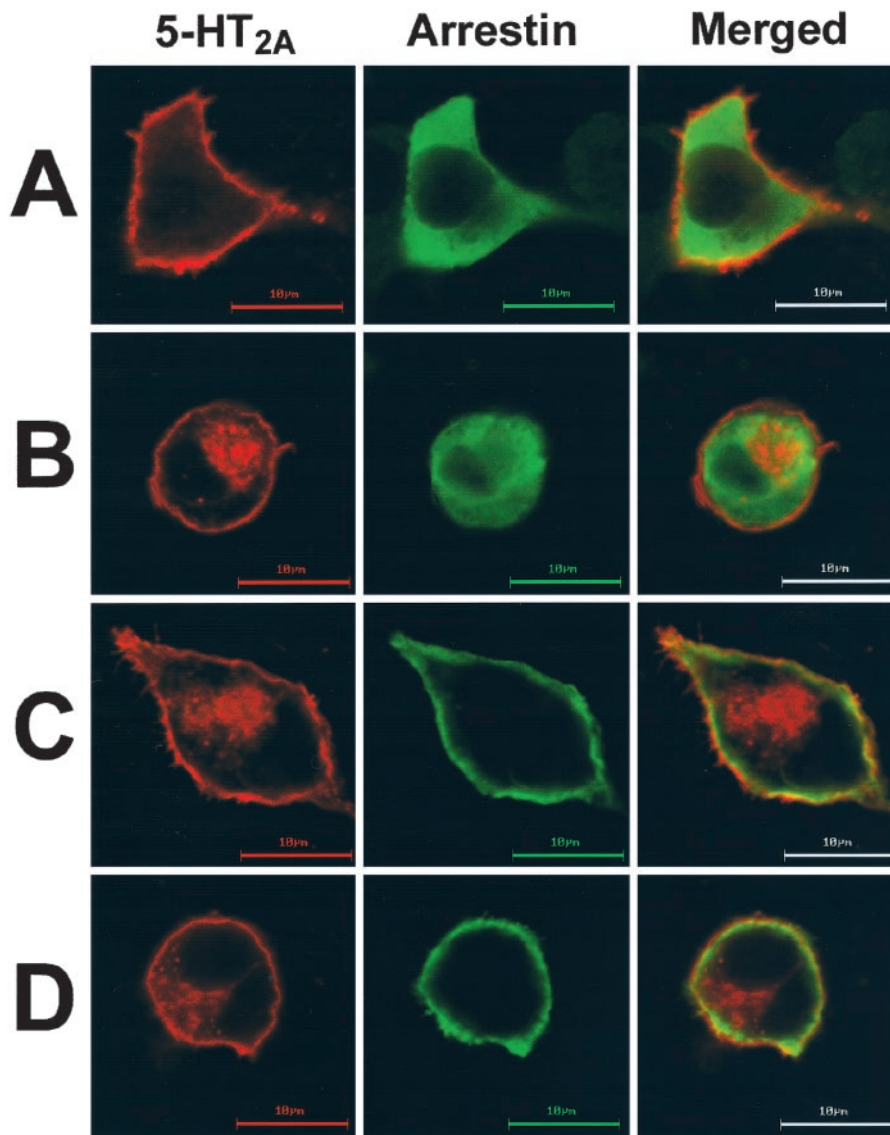


Fig. 1. Effect of Arr2-wt and Arr2-R169E on 5-HT_{2A} receptor internalization in HEK-293 cells. 5-HT_{2A} receptors (red) were cotransfected with Arr2-wt (A, B, green) or Arr2-R169E (C, D, green) and treated with vehicle (A, C) or 10 μM 5-HT for 10 min (B, D) and prepared as described under *Materials and Methods*. The experiment was replicated three times with identical results.

sual arrestins with GPCRs (Freedman et al., 1997; Min et al., 2002). Thus, to reliably detect receptor-arrestin complexes, we have used a cell-permeable, homobifunctional, cleavable cross-linking reagent before immunoprecipitation. Interestingly, low levels of Arr2-wt were found cross-linked to 5-HT_{2A}

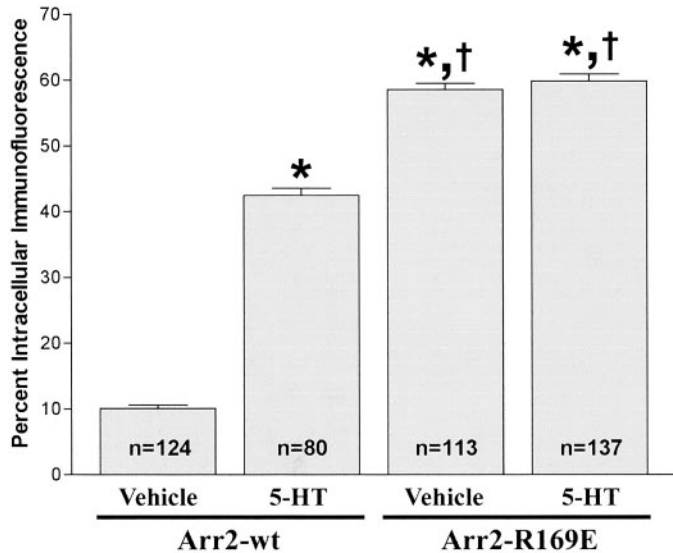


Fig. 2. Quantification of 5-HT_{2A} receptor internalization. Approximately 100 cells from three independent experiments were quantified in a blinded fashion. Percentage of intracellular immunofluorescence was determined by: [(mean intracellular pixel intensity – mean background pixel intensity) × region area]/[(mean total cellular pixel intensity – mean background pixel intensity) × region area] × 100. Bars represent mean ± S.E.M. for number of cells listed on bar. *, $p < 0.05$, statistically different from receptor plus Arr2-wt, no 5-HT. †, $p < 0.05$, statistically different from receptor plus Arr2-wt, 5-HT stimulated.

receptors that did not increase after agonist stimulation with 5-HT (Fig. 5, A and B). The lack of a significant increase in Arr2 binding to 5-HT_{2A} receptors after agonist stimulation provides biochemical confirmation of our confocal studies. By contrast, ~20-fold more Arr2-R169E was immunoprecipitated in the cross-linked complexes than Arr2-wt (Fig. 5, A and B), although again, no significant increase was noted after agonist stimulation. Taken together, these results demonstrate that the 5-HT_{2A} receptor associates poorly with Arr2-wt (whether agonist is present or not) but interacts with Arr2-R169E in a novel agonist-independent fashion. It is likely that the relatively poor association of Arr2-wt is responsible for the apparent arrestin-insensitivity previously demonstrated for 5-HT_{2A} receptors (Bhatnagar et al., 2001; Gray et al., 2001).

Arr2-R169E Decreases Signaling of 5-HT_{2A} Receptors. To determine whether the interactions of Arr2-wt and the Arr2-R169E mutant have functional consequences, PI dose-response curves were generated with three chemically distinct 5-HT_{2A} receptor agonists: the endogenous full agonist 5-HT and two partial agonists, DOI and quipazine. As shown in Fig. 6 and Table 1, Arr2-wt and Arr2-R169E each results in statistically significant reductions in the efficacy of PI hydrolysis compared with receptor alone. Cotransfection with Arr2-wt resulted in an approximately 40% reduction in efficacy, whereas Arr2-R169E reduced the efficacy by 75 to 85% (Fig. 6; Table 1). These results suggest that 5-HT_{2A} receptors may be functionally desensitized, conceivably because of the uncoupling of the receptor from its heterotrimeric G protein, Gq. However, effects of receptor expression levels needed to be examined because transient cotransfection of proteins often affects their relative expression levels,

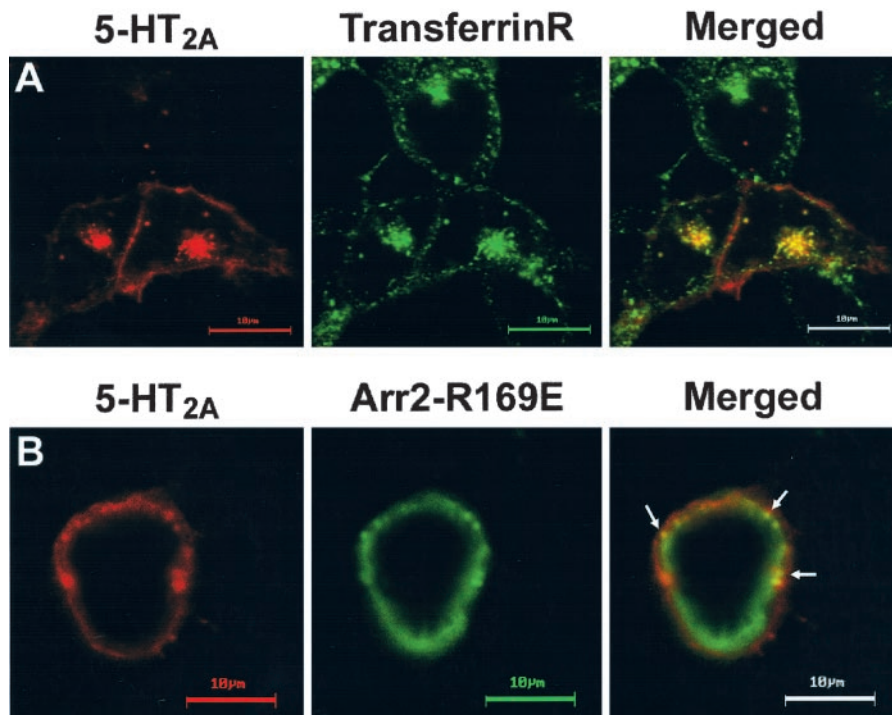


Fig. 3. A, colocalization of constitutively internalized 5-HT_{2A} receptors with transferrin receptors in HEK-293 cells. 5-HT_{2A} receptors were cotransfected with Arr2-R169E and stained for 5-HT_{2A} receptors (red) and transferrin receptors (green) as described under *Materials and Methods*. B, blockade of constitutive 5-HT_{2A} receptor internalization by DynK44. 5-HT_{2A} receptors were cotransfected with Arr2-R169E and DynK44A and stained for 5-HT_{2A} receptors (red) and Arr2-R169E (green) as described under *Materials and Methods*. Arrows, colocalization of 5-HT_{2A} receptors and Arr2-R169E in a “beaded” appearance on the cell surface.

as does the presence of receptor reserve. Thus, we performed transfections such that no receptor reserve was present, as described previously (Gray et al., 2001). As shown in Fig. 7, A and B, the maximal stimulation levels and expression levels of cells cotransfected with Arr2-wt or Arr2-R169E were then normalized to the maximal stimulation and expression of cells expressing 5-HT_{2A} receptors alone. Expression levels were determined by saturation binding experiments with [³H]ketanserin as described under *Materials and Methods*. Cells were harvested in hypotonic buffer (50 mM Tris-HCl, pH 7.4), which allows for the measurement of the entire cellular complement of 5-HT_{2A} receptors (data not shown). Cotransfection of 5-HT_{2A} receptors with Arr2-wt or Arr2-R169E resulted in statistically significant reductions of both maximal stimulation and expression. However, when stimulation levels were normalized to expression levels, Arr2-wt no longer had an effect on 5-HT_{2A} receptor signaling, whereas Arr2-R169E still resulted in about a 60% reduction in signaling (Fig. 7C). These results imply that Arr2-R169E functionally inactivates 5-HT_{2A} receptors, whereas the reduced signaling seen with Arr2-wt is predominantly caused by the reduced expression of 5-HT_{2A} receptors. Further examination of the PI hydrolysis dose-response curves revealed that Arr2-R169E but not Arr2-wt resulted in a statistically sig-

nificant increase in the potency (decreased EC₅₀) of all three agonists. This was unexpected but suggests that even though binding of Arr2-R169E uncouples the 5-HT_{2A} receptor from G protein, it stabilizes a high-affinity agonist state (R*) of the 5-HT_{2A} receptor.

Arr2-R169E Enriches the Population of 5-HT_{2A} Receptors in an Agonist-High-Affinity (R*) Conformation. To further investigate the increase in agonist potency for stimulating PI hydrolysis by 5-HT_{2A} receptors cotransfected with Arr2-R169E, we performed agonist and antagonist radioligand competition assays to examine the percentage of receptors in various states. Assays setting the 5-HT_{2A} receptor-selective antagonist [³H]ketanserin against the agonists 5-HT, DOI, and quipazine best fit a typical one-site model of binding (Fig. 8, A-C). Interestingly, there was a significant reduction in the total antagonist binding seen when cells were cotransfected with both Arr2-wt and Arr2-R169E (Fig. 8, A-C; Table 2), in concordance with the saturation binding data in Fig. 7. In contrast, competition of agonist binding, measured with [¹²⁵I]DOI, with each of the agonists best fit a two-site model of binding; interestingly, Arr2-R169E resulted in a significant increase in the total binding to a high-affinity state of the receptor (Fig. 8, D-F). As shown in Table 2, cotransfection of 5-HT_{2A} receptors with

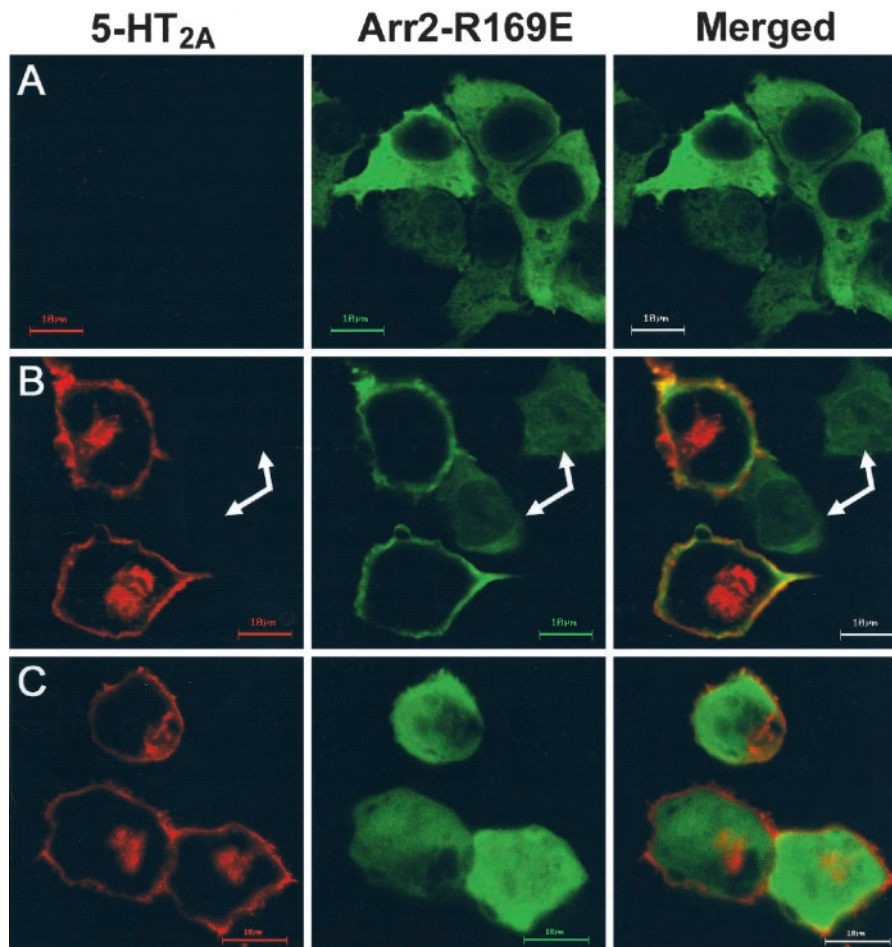


Fig. 4. Ritanserin, a 5-HT_{2A} receptor inverse agonist, reverses the 5-HT_{2A} receptor-Arr2-R169E interaction. Arr2-R169E (green) was cotransfected with empty vector (A) or 5-HT_{2A} receptors (B, red) into HEK-293 cells and prepared as described under *Materials and Methods*. Arrows point to cells that are apparently transfected with Arr2-R169E but with no 5-HT_{2A} receptor expressed. B, ritanserin, a 5-HT_{2A} receptor inverse agonist, blocks the 5-HT_{2A}-Arr2-R169E interaction. 5-HT_{2A} receptors (red) were cotransfected with Arr2-R169E (green) and treated with 1 μM ritanserin for 15 min then stained as described under *Materials and Methods*.

Arr2-R169E resulted in a significant increase in the fraction of receptors in the high-affinity conformation. Taken together, these results suggest that the interaction of Arr2-R169E with 5-HT_{2A} receptors stabilizes an agonist high-affinity state that is inefficiently coupled to G proteins (R*). This is an important result because it provides the first demonstration that the binding properties of an arrestin-insensitive GPCR can be modulated by a constitutively active arrestin mutant.

Arr2-R169E Does Not Alter the Kinetics of 5-HT_{2A} Receptor Desensitization and Resensitization. To examine potential functional effects of Arr2-R169E on 5-HT_{2A} receptors in HEK-293 cells, we looked at 5-HT_{2A} receptor desensitization and resensitization. As shown in Fig. 9A, cotransfection of 5-HT_{2A} receptors with Arr2-wt or Arr2-R169E had no effect on the normalized time course of agonist-induced desensitization. It is important to note that

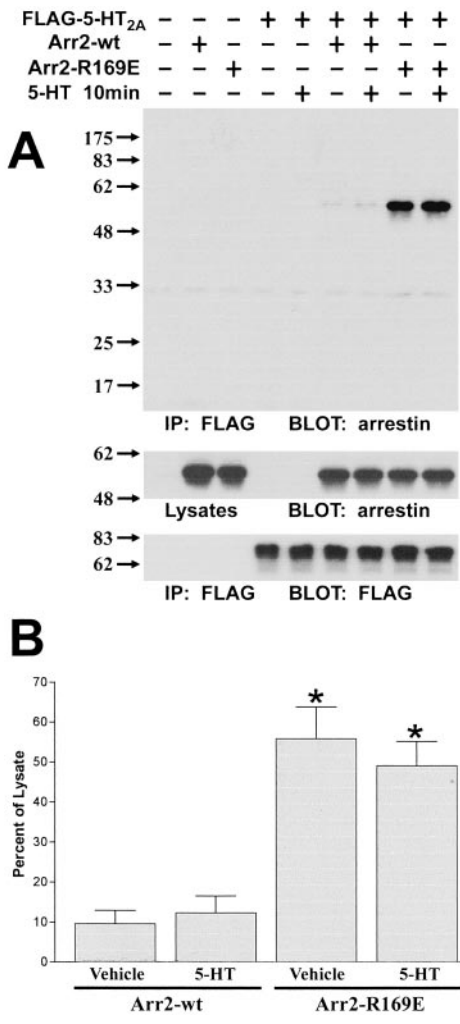


Fig. 5. Cross-linking and immunoprecipitation of Arr2-wt and Arr2-R169E with FLAG-tagged 5-HT_{2A} receptors. After transfection and treatment, cells were incubated with a cell-permeable cross-linking reagent and the cross-linked complexes were immunoprecipitated with anti-FLAG beads as described under *Materials and Methods*. A, top blot is a representative immunoprecipitation experiment probed for arrestin-2. The middle blot consists of cell lysates probed for arrestin-2, and the bottom blot concerns the immunoprecipitated samples probed with an anti-FLAG antibody. B, quantification of Arr2-wt and Arr2-R169E in FLAG immunoprecipitates as a percentage of arrestin in lysates. *, *p* < 0.05, statistically different from Arr2-wt, no 5-HT, by Student's *t* test.

although the receptor population is apparently 'pre-desensitized' by Arr2-R169E, when the remaining amount of signaling is normalized to 100% and desensitization is assessed, the rate and extent of further desensitization is maintained. This suggests that although the population of receptors that

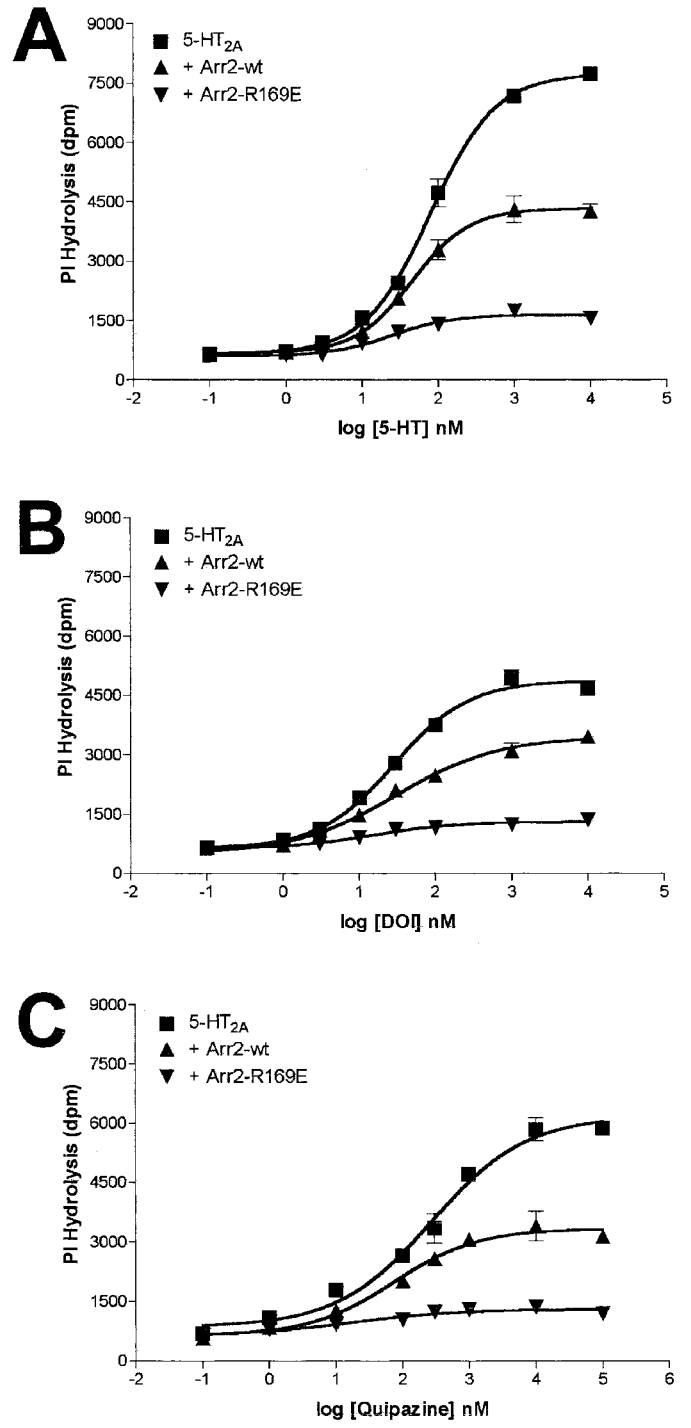


Fig. 6. Effect of Arr2-wt and Arr2-R169E on agonist-stimulated 5-HT_{2A} receptor-mediated PI hydrolysis in HEK-293 cells. A, dose response to 5-HT when 5-HT_{2A} receptors were cotransfected with empty vector, Arr2-wt, or Arr2-R169E. B, dose response to DOI when 5-HT_{2A} receptors were cotransfected with empty vector, Arr2-wt, or Arr2-R169E. C, dose response to quipazine when 5-HT_{2A} receptors were cotransfected with empty vector, Arr2-wt, or Arr2-R169E. The data represent the mean ± S.E.M. values from one representative experiment performed in triplicate.

is coupled to Gq is reduced by Arr2-R169E, those that are still available to signal are regulated in an arrestin-independent manner (Gray et al., 2001). In addition, Fig. 9B shows that cotransfection with Arr2-wt or Arr2-R169E has no effect on the time course or extent of 5-HT_{2A} receptor resensitization. For most GPCRs, it would be expected that the increased internalization induced by Arr2-R169E would increase the extent of recycling and resensitization, but we have recently demonstrated that internalization is not involved in 5-HT_{2A} receptor desensitization or resensitization (Gray et al., 2001). In fact, blocking internalization with a dominant-negative dynamin mutant (DynK44A) increases the rate of 5-HT_{2A} receptor resensitization in HEK-293 cells (Bhatnagar et al., 2001; Gray et al., 2001). Thus, as a positive control, we have again demonstrated that DynK44A increases the rate of 5-HT_{2A} receptor resensitization (Fig. 9B). Taken together, these results suggest that although Arr2-R169E induces a novel high-affinity/inactive state of the 5-HT_{2A} receptor, which is constitutively internalized, receptor desensitization and resensitization remain arrestin-independent.

Discussion

The major finding of this study is that a constitutively active arrestin mutant (Arr2-R169E) interacts in an agonist-independent manner with the 5-HT_{2A} receptor, thereby inducing agonist-independent internalization of the 5-HT_{2A} receptor, which we have previously demonstrated to be insensitive to regulation by arrestins in HEK-293 cells (Bhatnagar et al., 2001; Gray et al., 2001). To our knowledge, this is the first report that arrestins can induce substantial agonist-independent internalization of a GPCR. Arr2-R169E thereby reverses the arrestin-insensitive phenotype of the 5-HT_{2A} receptor in HEK-293 cells. To our knowledge, this is also the first report that an inverse agonist can reverse a spontaneously formed receptor-arrestin interaction. Additionally, we showed that the binding of Arr2-R169E, which interacts in a receptor phosphorylation-independent manner, stabilizes a high-affinity agonist binding state (R*) of the receptor, although it negatively affects the ability of the 5-HT_{2A} receptor to signal. Our results imply that the arrestin-insensitivity of 5-HT_{2A} receptors is caused not by the inability of arrestins to interact with the 5-HT_{2A} receptor but

by other, as-yet-undefined factors. Finally, because our prior studies showed that neuronal and intracellular 5-HT_{2A} receptors colocalize with arrestin in vivo (Gelber et al., 1999), our findings suggest that arrestins could be involved in maintaining the intracellular localization of 5-HT_{2A} receptors in neurons.

A general mechanism of GPCR desensitization involves the phosphorylation of the intracellular domains of GPCRs and the subsequent binding of arrestins, both of which serve to uncouple GPCRs from G proteins (Ferguson, 2001). Arrestin binding also promotes the targeting of desensitized receptors to clathrin-coated pits for their internalization (Ferguson, 2001). This well-documented targeting of GPCRs to the clathrin-coated pit/endosome pathway (Roth et al., 1981; Bennett et al., 1985; Chuang et al., 1986; von Zastrow and Kobilka, 1992) is critical because it allows for the resensitization and recycling of receptors (Yu et al., 1993; Pippig et al., 1995) and may be involved in down-regulation (Gray and Roth, 2002).

Although arrestins play crucial roles in the desensitization and internalization of many GPCRs, it is clear that these mechanisms are by no means universal (Pals-Rylandsdam et al., 1997; Lee et al., 1998; Walker et al., 1999; Heding et al., 2000; Bhatnagar et al., 2001; Gilbert et al., 2001; Gray et al., 2001; Lamb et al., 2001). Thus, we have recently demonstrated that 5-HT_{2A} receptor internalization, desensitization, and resensitization are arrestin-independent, (Bhatnagar et al., 2001; Gray et al., 2001). In addition to 5-HT_{2A} receptors, several other GPCRs manifest arrestin-independent internalization. For example, the m1, m3, and m4 muscarinic acetylcholine receptors have been shown to internalize independent of arrestins (Lee et al., 1998), whereas internalization of the m2 muscarinic acetylcholine receptor is arrestin-dependent (Pals-Rylandsdam et al., 1997; Lee et al., 1998). In addition, the secretin receptor (Walker et al., 1999), the B₂ bradykinin receptor (Lamb et al., 2001), and the gonadotropin-releasing hormone receptor (Heding et al., 2000) internalize independent of arrestins.

Despite their arrestin-insensitivity in some in vitro systems, we have previously demonstrated that, in neurons, 5-HT_{2A} receptors, which are predominantly found intracellularly (Willins et al., 1997; Cornea-Hebert et al., 1999), colocalize with endogenous arrestin-2 and arrestin-3 (Gelber et al., 1999). Thus, it is unclear why 5-HT_{2A} receptors are insensitive to arrestins in vitro but probably interact with

TABLE 1

Relative efficacies (with respect to receptor alone treated with 5-HT) and potencies for PI hydrolysis of 5-HT, DOI, and quipazine at 5-HT_{2A} receptors cotransfected with Arr2-wt and Arr2-R169E in HEK-293 cells

Values in parentheses are with respect to receptor alone treated with corresponding agonist

	<i>n</i>	Relative Efficacy	EC ₅₀	log EC ₅₀ ± S.E.M.
			<i>nM</i>	
5-HT				
5-HT _{2A}	3	1.000 ± 0.000	74.6	1.873 ± 0.073
+ Arr2-wt	3	0.598 ± 0.029	55.9	1.748 ± 0.066
+ Arr2-R169E	3	0.249 ± 0.029	16.6	1.220 ± 0.156*
DOI				
5-HT _{2A}	3	0.642 ± 0.022(1.000 ± 0.022)	27.8	1.444 ± 0.040
+ Arr2-wt	3	0.392 ± 0.028(0.610 ± 0.028)	29.2	1.465 ± 0.044
+ Arr2-R169E	3	0.160 ± 0.006(0.249 ± 0.006)	15.6	1.194 ± 0.038*
Quipazine				
5-HT _{2A}	3	0.795 ± 0.004(1.000 ± 0.004)	324.8	2.512 ± 0.149
+ Arr2-wt	3	0.452 ± 0.011(0.569 ± 0.011)	175.1	2.243 ± 0.224
+ Arr2-R169E	3	0.187 ± 0.014(0.235 ± 0.014)	35.8	1.554 ± 0.178*

* Statistically different from receptor alone $P < 0.05$ Student's *t* test

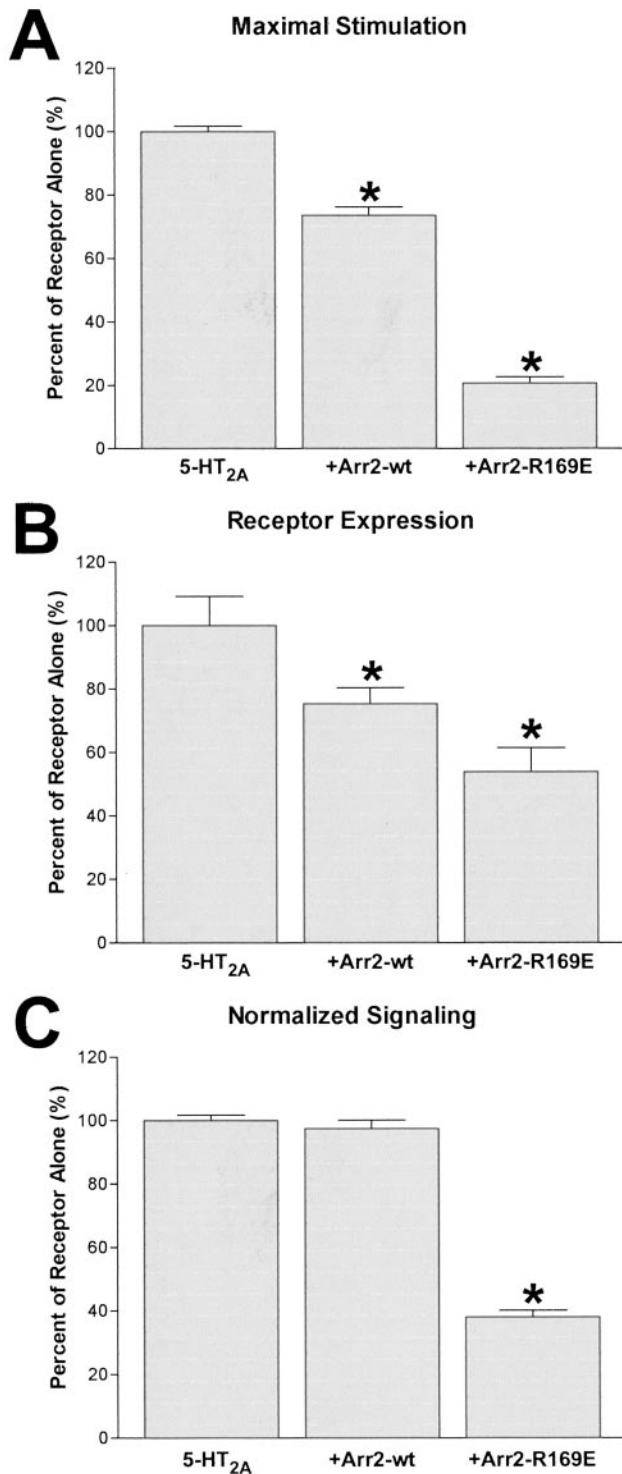


Fig. 7. Effect of Arr2-wt and Arr2-R169E on 5-HT_{2A} signaling normalized to receptor expression levels in the absence of receptor reserve. **A**, maximal level of 5-HT stimulated PI hydrolysis in HEK-293 cells transiently cotransfected with 5-HT_{2A} receptor and empty vector, Arr2-wt, or Arr2-R169E and normalized to maximal stimulation of receptor alone. **B**, 5-HT_{2A} receptor expression levels determined by saturation binding assay with [³H]ketanserin as described under *Materials and Methods* when cotransfected with empty vector, Arr2-wt, or Arr2-R169E and normalized to expression level of receptor alone. **C**, 5-HT_{2A} receptor signaling normalized to relative expression levels (percentage signal/percentage expression × 100). Data represent the mean ± S.E.M. values from five independent experiments performed in triplicate. Signaling and expression determinations for each independent experiment were from the same transfection. *, $p < 0.05$, significantly different from receptor alone by Student's *t* test.

arrestins in neurons in vivo. Most likely, neurons contain additional proteins involved in 5-HT_{2A} receptor regulation that are necessary for their interactions with arrestins. We have hypothesized that 5-HT_{2A} receptors are regulated, in HEK-293 cells, in an arrestin-insensitive manner because of an apparent lack of agonist-stimulated phosphorylation (Vouret-Craviari et al., 1995; Gray and Roth, 2001; B. Roth, unpublished observations). In this regard, it is interesting that μ -opioid receptors desensitize at a slower rate than δ -opioid receptors, because the agonist-stimulated phosphorylation of μ -opioid receptors results in a less efficient activation of arrestin binding (Lowe et al., 2002). Indeed, 5-HT_{2A} receptors desensitize at a slow rate compared with many GPCRs, such as the β_2 -adrenergic receptor (Gray et al., 2001). To investigate the hypothesis that 5-HT_{2A} receptors are not regulated by arrestins because of inefficient arrestin activation, we have used a constitutively active arrestin-2 mutant (Arr2-R169E) that is receptor phosphorylation-state independent (Kovoor et al., 1999). If Arr2-R169E was capable of reversing the arrestin insensitivity of 5-HT_{2A} receptors in HEK-293 cells, insights into the role of arrestins in 5-HT_{2A} receptor regulation in neurons might be gleaned.

Arrestin binding to phosphorylated receptors is accomplished by disruption of the "polar core" within the arrestin molecule by highly charged receptor-attached phosphates, resulting in the transition of arrestin to its active high-affinity receptor binding state (Gurevich and Benovic, 1995, 1997; Vishnivetskiy et al., 1999). Mutations destabilizing this "polar core" enhance the binding of arrestin with non-phosphorylated agonist-activated receptors (Gurevich and Benovic, 1995; Gray-Keller et al., 1997; Kovoor et al., 1999). Studies using Arr2-R169E demonstrated that it binds to and desensitizes β_2 -adrenergic receptors regardless of phosphorylation-state and desensitizes the δ -opioid receptor (Kovoor et al., 1999). Interestingly, Arr2-R169E restores agonist-induced desensitization of a truncated δ -opioid receptor lacking the critical phosphorylation sites (Kovoor et al., 1999). Transfection of Arr2-R169E also promoted the agonist-dependent endocytosis of internalization-defective thyrotropin-releasing hormone receptor mutants lacking critical phosphorylation sites (Hanyaloglu et al., 2001). Thus, it was likely that the use of Arr2-R169E might provide important insights into the molecular basis of the arrestin-insensitivity of 5-HT_{2A} receptors.

In the present studies, we have demonstrated that expression of Arr2-R169E results in the constitutive internalization of 5-HT_{2A} receptors through a high degree of basal interaction. Interestingly, there was a low level of interaction of wild-type arrestin-2 with FLAG-tagged 5-HT_{2A} receptors in cross-linked immunoprecipitation experiments, although this level did not increase as would be expected with the addition of agonist. These results provide cellular confirmation of prior in vitro studies in which we demonstrated that purified visual and nonvisual arrestins interact with the nonphosphorylated third intracellular loop of the 5-HT_{2A} receptor in vitro (Gelber et al., 1999). Thus, the internalization of arrestin-insensitive 5-HT_{2A} receptors could be invoked by a constitutively active arrestin suggesting that 5-HT_{2A} receptors are capable of being regulated by arrestins under special circumstances. Thus, the intracellular distribution of 5-HT_{2A} receptors in neurons may be maintained

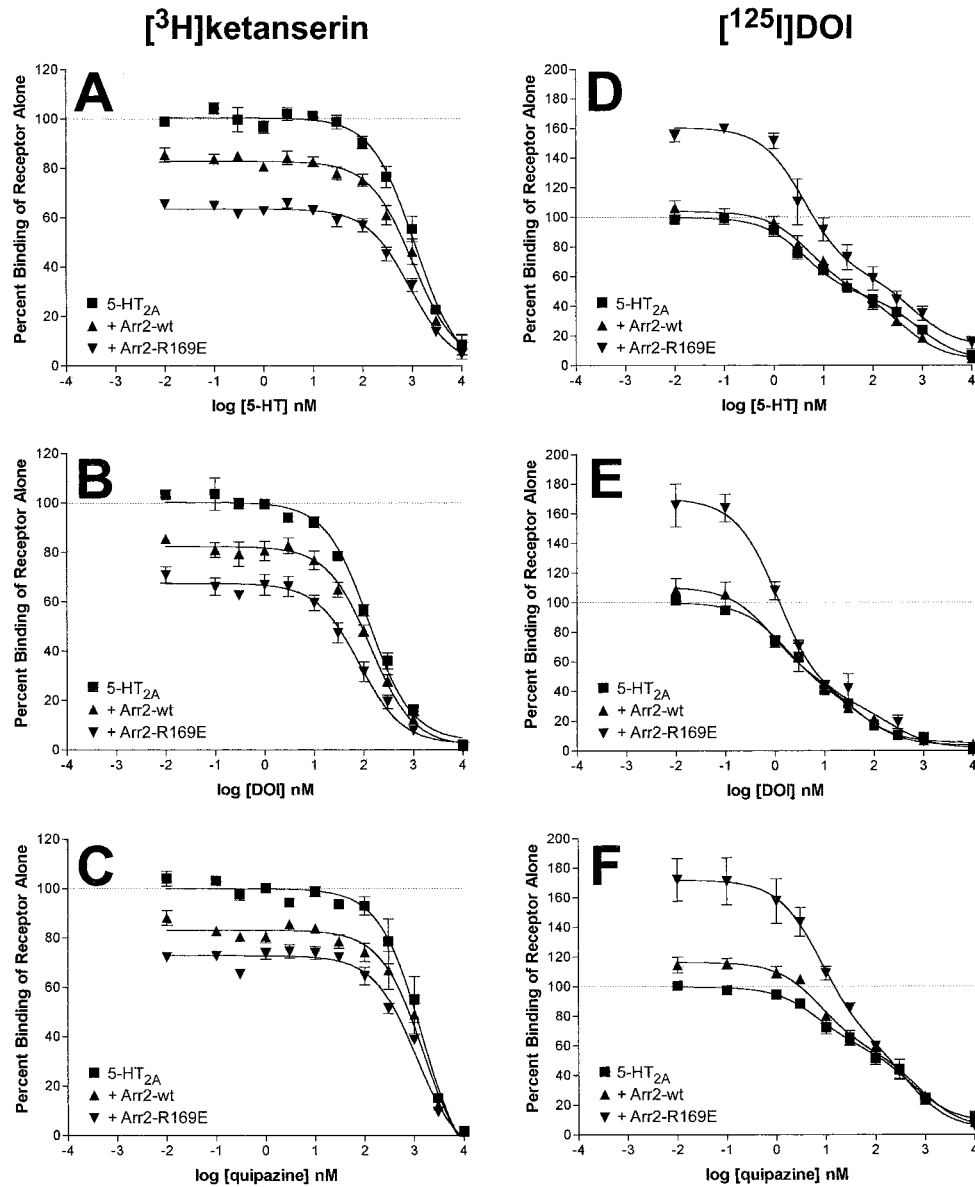


Fig. 8. Agonist and antagonist competition binding isotherms for 5-HT_{2A} receptor cotransfected with empty vector, Arr2-wt, or Arr2-R169E. HEK-293 cells transiently cotransfected with 5-HT_{2A} receptors and either empty vector, Arr2-wt, or Arr2-R169E were pelleted as described under *Materials and Methods*. A to C, competition curves of [³H]ketanserin, a 5-HT_{2A} receptor-selective antagonist, with the agonists 5-HT (A), DOI (B), and quipazine (C). D–F, competition curves of the radiolabeled agonist [¹²⁵I]DOI with 5-HT (D), DOI (E), and quipazine (F). All curves represent data from three independent experiments performed in duplicate and are normalized to the maximal binding of 5-HT_{2A} receptor alone.

TABLE 2

Binding affinities of 5-HT, DOI, and quipazine versus agonist and antagonist radioligands at the 5-HT_{2A} receptor cotransfected with Arr2-wt or Arr2-R169E in HEK-293 cells
 Values in parentheses are log K_i ± S.E.M.

	K _i			
	[³ H]Ketanserin	[¹²⁵ I]DOI		Receptors in High-Affinity State %
		High-Affinity Site	Low-Affinity Site	
	nM	nM	nM	
5-HT				
5-HT _{2A}	858.2(2.934 ± 0.059)	5.348(0.728 ± 0.110)	787.7(2.896 ± 0.176)	44.7 ± 3.7
+ Arr2-wt	760.4(2.881 ± 0.083)	6.273(0.797 ± 0.129)	708.3(2.850 ± 0.247)	46.4 ± 9.7
+ Arr2-R169E	668.4(2.825 ± 0.064)	5.979(0.777 ± 0.109)	2020 (3.305 ± 0.201)*	75.5 ± 6.3*
DOI				
5-HT _{2A}	80.1(1.903 ± 0.121)	1.330(0.124 ± 0.096)	29.4(1.479 ± 0.154)	40.7 ± 9.3
+ Arr2-wt	92.5(1.966 ± 0.153)	1.224(0.088 ± 0.069)	29.8(1.475 ± 0.105)	44.1 ± 9.2
+ Arr2-R169E	49.6(1.695 ± 0.097)	0.956(-0.019 ± 0.108)	151.3(2.180 ± 0.221)*	80.8 ± 6.1*
Quipazine				
5-HT _{2A}	1052 (3.022 ± 0.099)	10.2 (1.009 ± 0.149)	473.8(2.680 ± 0.160)	46.4 ± 9.9
+ Arr2-wt	1143 (3.058 ± 0.127)	8.53 (0.931 ± 0.195)	712.7(2.853 ± 0.242)	47.6 ± 7.2
+ Arr2-R169E	740.1(2.869 ± 0.055)	7.26 (0.861 ± 0.114)	1570 (3.196 ± 0.237)*	70.0 ± 7.9*

* Statistically different from receptor alone at p < 0.05 by Student's t test

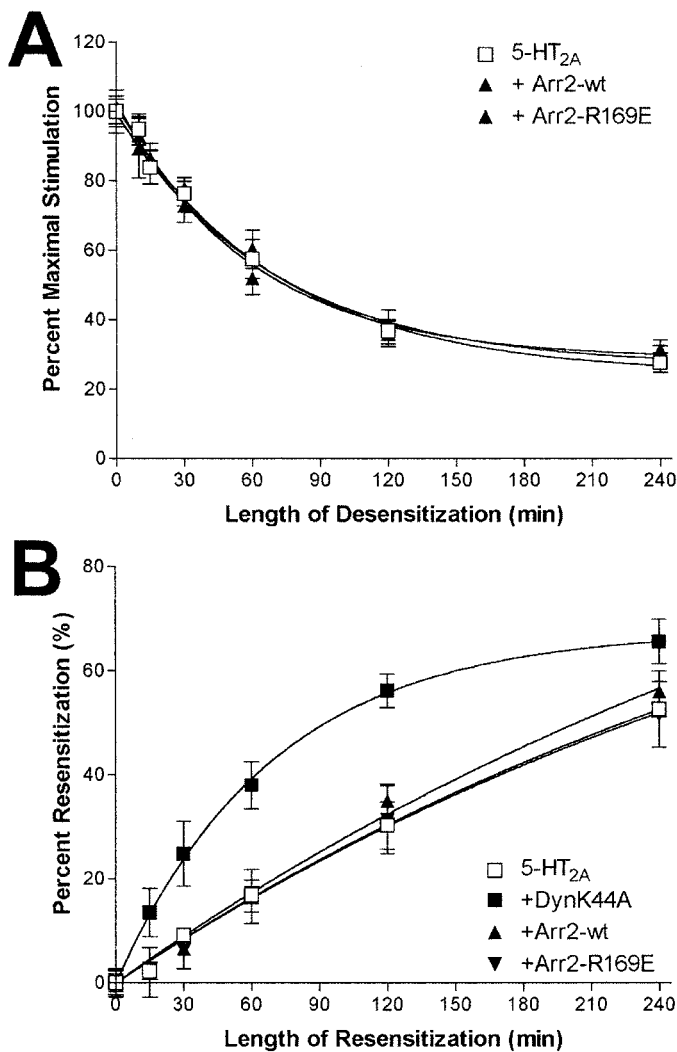


Fig. 9. Effect of Arr2-wt and Arr2-R169E on the time course of quipazine-induced desensitization (A) and resensitization (B) of 5-HT_{2A} receptor-mediated PI hydrolysis in HEK-293 cells. A, transiently transfected HEK-293 cells were preincubated with vehicle or 300 μ M quipazine for various times. Cells were then extensively washed, stimulated with 10 μ M 5-HT in the presence of 10 mM LiCl and the levels of PI hydrolysis were measured as described under *Materials and Methods*. The data are expressed as a percentage of the response observed in the control (vehicle-treated) group of cells. B, cells were desensitized with 300 μ M quipazine for 2 h, washed three times, and allowed to resensitize for various times. Cells were then stimulated with 10 μ M 5-HT in the presence of 10 mM LiCl and the levels of PI hydrolysis were measured as described under *Materials and Methods*. The data represent the mean \pm S.E.M. values from three independent experiments performed in triplicate.

through interactions with arrestins and 5-HT_{2A} receptors may be regulated by arrestins in novel ways.

We have also demonstrated that the association of Arr2-R169E with the 5-HT_{2A} receptor inactivates the receptor. Interestingly, the remaining population of signaling receptors is desensitized and resensitized by agonist at the same rate and to the same extent as receptors expressed alone. Additionally, upon Arr2-R169E binding, the receptor population becomes enriched in the agonist high-affinity (R*) conformation, suggesting that Arr2-R169E binds to and stabilizes the agonist high-affinity state of the receptor. We have recently shown that 5-HT_{2A} receptors exhibit about 20% constitutive activity (Shapiro et al., 2002), and it is possible that when the receptor spontaneously transitions to the R*

state, it is 'trapped' by Arr2-R169E. Thus, this basal level of constitutive activity may serve to facilitate the interaction of Arr2-R169E with the receptor in the absence of agonist. To this end, we demonstrated that treatment of the transfected cells with the potent 5-HT_{2A} receptor inverse agonist ritanerlin resulted in the redistribution of the cell-surface translocated Arr2-R169E back to a diffuse cytoplasmic distribution, suggesting that inverse agonists can inhibit the interaction between the receptor and Arr2-R169E. To our knowledge, this is the first demonstration that an inverse agonist can reverse a spontaneously formed receptor-arrestin interacting unit. Previous data have demonstrated that the ternary complex formed by agonist, phosphorylated receptor, and arrestin prefers that high agonist affinity state (Gurevich et al., 1997), although these prior studies did not address whether these arrestin-sensitive receptors were capable of activating second messenger production. Thus, with the 5-HT_{2A} receptor, Arr2-R169E favors the high-affinity agonist binding conformation even in the absence of agonist and detectable phosphorylation. It is feasible that the interaction of 5-HT_{2A} receptor with arrestins in neurons is a consequence of the high level of 5-HT_{2A} receptor constitutive activity and is a mechanism to prevent the potentially pathological over-signaling of this receptor.

In conclusion, the major findings of this study are that although 5-HT_{2A} receptors can be internalized and desensitized in an arrestin-independent manner, a constitutively active arrestin mutant can force receptor internalization and desensitization even in the absence of agonist. Thus, these results suggest that although 5-HT_{2A} receptors are capable of being regulated by arrestins, they are unable to 'activate' arrestins. In addition, the binding of a constitutively active arrestin to 5-HT_{2A} receptors reduces signaling and increases the proportion of receptors in the high-affinity agonist binding state (R*). The use of the constitutively active arrestin has allowed clear demonstration that arrestins can interact with 5-HT_{2A} receptors as suggested by immunohistochemistry in neurons (Gelber et al., 1999). Thus, taken together, these results indicate that arrestin interacts with 5-HT_{2A} receptors under certain circumstances, possibly maintaining an intracellular distribution of receptors in quiescent neurons. Additionally, the high level of 5-HT_{2A} receptor constitutive activity facilitates the interaction with Arr2-R169E in HEK-293 cells and may be important for their regulation by arrestins in neurons.

Acknowledgments

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