Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Structure and Function of the Third Intracellular Loop of the 5-Hydroxytryptamine$_{2A}$ Receptor: The Third Intracellular Loop Is α-Helical and Binds Purified Arrestins

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Abstract: Understanding the precise structure and function of the intracellular domains of G protein-coupled receptors is essential for understanding how receptors are regulated, and how they transduce their signals from the extracellular milieu to intracellular sites. To understand better the structure and function of the intracellular domain of the 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) receptor, a model $G_{\alpha q}$-coupled receptor, we overexpressed and purified to homogeneity the entire third intracellular loop (i3) of the 5-HT$_{2A}$ receptor, a region previously implicated in G-protein coupling. Circular dichroism spectroscopy of the purified i3 protein was consistent with α-helical and β-loop, -turn, and -sheet structure. Using random peptide phage libraries, we identified several arrestin-like sequences as i3-interacting peptides. We subsequently found that all three known arrestins (β-arrestin, arrestin-3, and visual arrestin) bound specifically to fusion proteins encoding the i3 loop of the 5-HT$_{2A}$ receptor. Competition binding studies with synthetic and recombinant peptides showed that the middle portion of the i3 loop, and not the extreme N and C termini, was likely to be involved in i3–arrestin interactions. Dual-label immunofluorescence confocal microscopic studies of rat cortex indicated that many cortical pyramidal neurons coexpressed arrestins (β-arrestin or arrestin-3) and 5-HT$_{2A}$ receptors, particularly in intracellular vesicles. Our results demonstrate (a) that the i3 loop of the 5-HT$_{2A}$ receptor represents a structurally ordered domain composed of α-helical and β-loop, -turn, and -sheet regions, (b) that this loop interacts with arrestins in vitro, and is hence active, and (c) that arrestins are colocalized with 5-HT$_{2A}$ receptors in vivo. **Key Words:** β-Arrestin—Structure–function—5-HT$_{2A}$ receptor—Receptor–effector coupling.
J. Neurochem. **72,** 2206–2214 (1999).

5-Hydroxytryptamine (5-HT; serotonin) is a biogenic amine neurotransmitter whose actions are mediated by seven major families of receptors: 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$, and 5-HT$_7$. The 5-HT$_2$-family is important for a variety of activities, including the regulation of perception, appetite, mood, and neuronal excitability (Hoyer et al., 1994; Roth et al., 1998*a*,*b*). In the 5-HT$_2$ family of receptors, the 5-HT$_{2A}$ receptor appears to be particularly important, because it mediates, at least in part, the actions of hallucinogens, some antidepressants, and many atypical antipsychotic drugs (Teitler et al., 1990; Roth et al., 1998*a*). The 5-HT$_{2A}$ receptor couples via $G_{\alpha q}$ to phospholipase C-mediated hydrolysis of polyphosphoinositides in native and heterologous expression systems (de Chaffoy de Courcelles et al., 1985; Roth et al., 1986, 1998*b*).

$G_{\alpha q}$-coupled receptors are characterized by rather long third intracellular loops (i3), which, in some cases, are important for $G_{\alpha q}$ coupling (Oksenberg et al., 1995; Hill-Eubanks et al., 1996) and arrestin binding (Wu et al., 1997). The structures of these loops are currently unknown as is their ultimate function. Several models of G protein-coupled receptor (GPCR) activation suggest that the i3 loops are, at least in part, α-helical in structure and that movements of these intracellular α-helical domains are involved in G-protein activation (Burstein et al., 1995; Farrens et al., 1996; Hill-Eubanks et al., 1996). With the exception of the i3 loop of rhodopsin, for which NMR structures are available (Yeagle et al., 1995, 1997), the structure of any i3 loop of other GPCRs is unknown.

Received October 21, 1998; revised manuscript received January 5, 1999; accepted January 6, 1999.

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Abbreviations used: CD, circular dichroism; GPCR, G protein-coupled receptor; GST, glutathione *S*-transferase; 5-HT, 5-hydroxytryptamine or serotonin; i3, third intracellular loop; IPTG, isopropyl thiogalactoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; v-arrestin, visual arrestin.

In this article, we demonstrate that the i3 loop of the 5-HT$_{2A}$ receptor has pronounced α-helical and β-loop and -turn secondary structure and that it interacts with arrestin in vitro. These results also imply that the α-helical structure of the transmembrane regions of GPCRs is continuous with the i3 loop. These results support models of GPCR structure and function that predict that domains of intracellular loops are α-helical in nature (Liu et al., 1996).

MATERIALS AND METHODS

Materials

A random peptide library in M13 phage and T4 DNA ligase, as well as the IMPACT 1 System, were obtained from New England Biolabs (Beverly, MA, U.S.A.). The Geneclean III Kit was obtained from Bio 101 (Vista, CA, U.S.A.). The glutathione *S*-transferase (GST) Gene Fusion System was obtained from Pharmacia Biotech (Uppsala, Sweden). *E. coli* JM109 cells and *Pfu* polymerase were obtained from Stratagene (La Jolla, CA, U.S.A.). Visual arrestin (v-arrestin), β-arrestin (arrestin-1), arrestin-3 (also known as arrestin-2), and a non–subtype-selective anti-arrestin rabbit polyclonal antibody (F4C1) were obtained as described previously (Wu et al., 1997). β-Arrestin and arrestin-3 murine monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Renaissance Western Blot Chemiluminescence Reagent was obtained from NEN Life Science Products (Boston, MA, U.S.A.). The T7 Sequenase dideoxy sequencing kit was obtained from Amersham Life Science (Cleveland, OH, U.S.A.). Trans-Blot nitrocellulose transfer medium was obtained from Bio-Rad (Hercules, CA, U.S.A.). The following peptides were from Research Genetics: peptide 40 (TYFLTIKSLQKE), which corresponds to the N terminus of the i3 loop of the 5-HT$_{2A}$ receptor; and peptide 41 (SNEQKACKVLGI), which corresponds to the C terminus of the 5-HT$_{2A}$ receptor. Both peptides were HPLC-purified and verified by mass spectrometry.

Preparation of i3-specific antibody

A synthetic peptide corresponding to amino acids 280–295 (prepared by the peptide synthesis core facility of the Beckman Research Institute at Stanford University) of the rat 5-HT$_{2A}$ receptor was coupled to keyhole limpet hemocyanin using glutaraldehyde and antibodies to this peptide prepared in two New Zealand white rabbits with complete Freund's adjuvant using the inoculation and boost schedule previously detailed (Roth et al., 1989). Serum from the fourth through the eighth bleeds (antibody IA) from one of the rabbits was used for western blot experiments. The antibodies displayed a titer of 1:30,000 against the synthetic peptide and at least 1:20,000 against the purified i3 peptide.

Western blot analysis

Western blots were performed as previously described (Roth et al., 1989). The F4C1 anti-arrestin antibody was used at 1:10,000 dilution; arrestin-3 and β-arrestin-1 antibodies were used at 1:2,000 dilution. Peroxidase-coupled goat anti-mouse secondary antibodies were used at 1:1,000 dilutions with visualization by the Renaissance Chemiluminescence System (NEN). In some experiments, rat brain medial prefrontal cortical homogenates were obtained, diluted 1:1 in sodium dodecyl sulfate (SDS) sample buffer, and used for western blot analysis.

Preparation of fusion proteins

Preparation of i3–GST fusion protein and GST. The putative i3 loop (FLTIKSLQKEATLCVSDLSTRAKLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKACKV) of the 5-HT$_{2A}$ receptor was amplified using *Pfu* polymerase and the following primers: BELOOP: 5′-AAC GGG ATC CCC ATC ACC TAC TTC CTG ACT ATC-3′, which corresponds to base pairs 815–834, and ENDLOOP: 5′-GCG GGA TCC TTA ACG ATG CCC AGC ACC TTG CAC-3′, which corresponds to base pairs 1,024–1,043 (both have *Bam*HI adaptors). The product was purified by agarose gel electrophoresis, followed by treatment with the Geneclean III Kit, digested with *Bam*HI, ligated into pGEX-3X, and transformed into *E. coli* JM109. GST was prepared by transforming JM109 with native pGEX-3X; subsequent steps in the preparation of GST are the same as those used to prepare i3–GST. The resultant transformants were screened by expression with 100 μM isopropyl thiogalactoside (IPTG: 37°C for 3 h) and western blot analysis using an i3-specific antibody (see Western blots). Positive colonies were then verified by double-stranded DNA sequencing and used for large-scale purification of i3–GST fusion proteins.

For large-scale purification, LB broth containing ampicillin (60 μg/ml; LB-Amp; 1 L) was inoculated with bacteria from the frozen stock, and the culture was grown to $A_{600} = 0.6$. Cultures at midlog phase were stimulated with 0.5 m*M* IPTG for 5 h. Bacteria were pelleted and washed with phosphate-buffered saline (14 m*M* NaCl, 2.6 m*M* KCl, 10 m*M* Na_2HPO_4, 1.7 m*M* KH_2PO_4, pH 7.4). Bacteria were pelleted again and resuspended in lysis buffer (50 m*M* Tris, 1 m*M* EDTA, 1 mg/ml lysozyme, pH 8.0). Phenylmethylsulfonyl fluoride was added to a final concentration of 100 μM. Bacteria were resuspended in lysis buffer, incubated for 1 h at 4°C with vigorous stirring, and then homogenized in a French press. The homogenate was clarified by centrifugation, and the supernatant was applied to a glutathione–Sepharose column. After the column was washed with phosphate-buffered saline, i3–GST was eluted with elution buffer (50 m*M* Tris-HCl, 10 m*M* glutathione, pH 8.0). Those fractions that were determined by western blot to be highly enriched in i3–GST were concentrated in a 10,000 MW spin filter (Millipore). The product was stored in 30% glycerol at −20°C.

Preparation of i3–intein–CBD–His^6 construct. A second i3 fusion protein construct was made, in which the i3 loop was fused to an intein fusion protein expression construct using the IMPACT 1 System. For construction of the i3–intein construct, the i3 loop sequence was amplified using the following primers: i3-pCYB-forward, ACCATC**CATATG**ATCACCTACTTCCTGACTATCAAG (*Nde*I site in bold); i3-pCYB-reverse, AAA**CCCGGG**CACGATGCCCAGCACCTTGCACGCCTTT (*Sma*I site in bold). It was purified, cut with *Nde*I and *Sma*I, and ligated into the *Nde*I and *Sma*I sites of pCYB2. This resulted in an insert with an additional N-terminal Met and C-terminal Pro-Gly-COOH sequence. Colonies were screened for expression of the i3 peptide by western blot analysis and verified by sequencing. The resulting insert was reamplified using the following primers to create an i3–intein–CBD–His^6 construct: CBDHISBH, TAT GGA TCC TTA **GTG GTG GTG GTG GTG GTG** TTG AAG CTG CCA CAA GGC AGG (6-His sequence in bold) and i3-pCYB-forward. This construct was purified and ligated into a pET11a vector for high-level expression. As before, colonies were screened for expression by western blot analysis and verified by sequencing.

Large-scale purification of i3 peptide

A 20-ml culture of LB-Amp was inoculated with bacteria expressing the i3–intein–CBD construct and incubated overnight at 37°C with constant agitation. The next day, the culture was diluted 1:100 into 2 L of M9 minimal medium supplemented with glucose, $MgSO_4$, and $CaCl_2$ (Sambrook et al., 1989) with incubation at 37°C until the OD_{600} was between 0.5 and 0.7. The culture was placed at room temperature with constant agitation in the presence of 30 μM IPTG with cells harvested as above using an alternative lysis buffer (20 m*M* HEPES, 0.5 *M* NaCl, 0.1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, pH 8.0) with the homogenate clarified by centrifugation (12,000 *g*, 45 min). The clarified supernatant was then added to a Ni-affinity resin (Qiagen Corp; 3-ml volume) and washed with 150–250 ml of lysis buffer without Triton X-100. After washing, the i3–INTEIN–CBD–His^6 fusion protein was eluted with a gradient of imidazole (0–500 m*M*)-containing lysis buffer without Triton X-100 and the fractions assayed by SDS-polyacrylamide gel electrophoresis (PAGE). Fractions of the highest purity were then combined and added to a chitin column (2-ml volume); the column was then washed extensively with lysis buffer containing 1 *M* NaCl and the i3 peptide liberated exactly as described by the manufacturer (New England Biolabs; IMPACT Purification System). The fractions with the highest purity were then combined, filtered through a 10-kDa filter to remove higher molecular weight contaminants, and then concentrated using a 5-kDa filter. Fractions were assayed for purity by 15% SDS-PAGE.

Circular dichroism (CD) spectra

CD spectra were obtained on a Jasco J-600 Spectropolarimeter using a 0.5-cm path-length cell. The protein was dissolved in double-distilled H_2O containing 250 μM Na-HEPES, pH 7.4, 625 μM NaCl, 0.0625% glycerol, and 0.00025% NaN_3. For a typical experiment, five scans were averaged and the data analyzed as described (Perczel et al., 1992).

Phage display

Sterile polystyrene 65 × 15 mm Falcon 1034 dishes were coated with 100 μg/ml i3–GST in 100 m*M* $NaHCO_3$, pH 8.6. Dishes were incubated overnight at 4°C in a humidified environment. The coating solution was replaced by blocking solution (5 mg/ml bovine serum albumin, 100 m*M* $NaHCO_3$, pH 8.6, 0.02% NaN_3) for a 1-h incubation at 4°C. Blocking buffer was washed away with six washes of Tris-buffered saline (TBS; 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl) containing Tween 20 (0.5%, vol/vol) and replaced by phage solution prepared as follows: M13 phage expressing random heptamers (New England Biolabs) were diluted to a final concentration of 2×10^{11} plaque-forming units per milliliter in TBS containing Tween 20 (0.5%, vol/vol). After a 1-h incubation, unbound phage were washed away. Specifically bound phage were eluted with TBS for 1 h at room temperature. A liquid culture of *E. coli* TG1 was infected with phage and amplified for 4.5 h at 37°C on an orbital shaker. The bacteria were pelleted by centrifugation, and the supernatant, containing phage, was removed. Phage were precipitated by incubating the supernatant at 4°C overnight with 1/6 volume of polyethylene glycol/NaCl [20% (wt/vol) polyethylene glycol-8000, 2.5 *M* NaCl]. Precipitated phage were pelleted and resuspended in 300 μl of TBS. Amplified phage were then used for the next round of biopanning. After three or four rounds of biopanning, individual plaques were sequenced using dideoxy sequencing technology (Tabor and Richardson, 1987).

Arrestin-i3 binding assay

Binding assays were performed at 4°C. Glutathione–Sepharose resin (Pharmacia) was equilibrated with buffer D (20 m*M* Tris-HCl, pH 6.9, 70 m*M* NaCl, 100 μg/ml soybean trypsin inhibitor). i3–GST was allowed to bind to the resin (0.4 ng of i3–GST/μl of resin) during a 30-min incubation with end-over-end mixing. Nonspecific binding was determined with GST (0.8 ng/μl of resin) bound to glutathione–Sepharose resin. Resin was divided into 100-μl aliquots in microcentrifuge tubes, and 10 μM competitors were then added. β-Arrestin, v-arrestin, and arrestin-3 were then added to give a final concentration of 0.2 μg/μl. The slurry containing the resin, i3–GST or GST, competitors, and arrestins was incubated for 2.5 h with end-over-end mixing. The resin was then washed twice with 500 μl of buffer D, and the pellet was treated with 100 μl of SDS sample buffer for 5 min at 95°C to release bound proteins. Samples were run on a 12% SDS-PAGE gel and transferred to nitrocellulose. Western blots were performed with F4C1 mouse monoclonal antibody, which detects v-arrestin, β-arrestin, and arrestin-3, and horseradish peroxidase anti-mouse secondary antibody. Blots were developed with Renaissance chemiluminescence kit. Relative amounts of protein were determined by densitometry.

Dual-label fluorescence confocal microscopy

For these studies, rat brains were prepared exactly as described previously (Willins et al., 1997). Sections of medial prefrontal cortex were incubated with β-arrestin-1 or β-arrestin-3 specific monoclonal antibodies (1:500 dilution) and a polyclonal 5-HT_{2A} antibody (1:3,000 dilution) as previously described (Berry et al., 1996; Willins et al., 1997, 1999). BODIPY-labeled goat anti-mouse (1:100) and Texas Red-labeled goat anti-rabbit (1:200) antibodies were used to visualize arrestin and 5-HT_{2A} antibodies, respectively, as previously described (Willins et al., 1997, 1999).

RESULTS

The i3 loop of the 5-HT_{2A} receptor has α-helical and β-sheet secondary structure

As the i3 loops of $G_{\alpha q}$-coupled GPCRs are generally quite large, attempts to predict secondary structure are likely to be inexact. Prior mutagenesis studies have been predicated on the assumption that at least the proximal portions of the N and C termini of the i3 region are α-helical in structure, although direct tests of this notion have not been performed. In general, these putative α-helical domains are thought to be essential for $G_{\alpha q}$ interactions, although the large size of these domains, which in some cases may be >100 amino acids in length, suggests that they may serve to interact with other intracellular proteins, in addition to G proteins.

Our initial studies were aimed at determining the secondary structure of the i3 region from the 5-HT_{2A} receptor. We used a two-step cloning procedure to subclone the i3 region into an appropriate expression vector. We initially subcloned the i3 region (1A) into pCYB2—a prokaryotic expression system that used a chitin binding domain for affinity purification and a dithiothreitol-sensitive self-cleaving protein (intein domain). Yields of pure i3 were quite low with this system (<0.1 mg/L; data not shown) because of low levels of induction of i3–intein–CBD fusion protein.

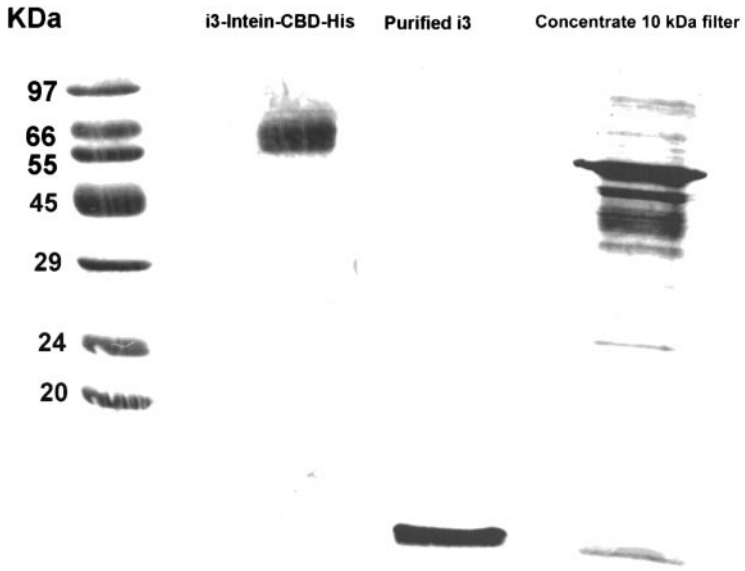

FIG. 1. Purification of i3 protein. Shown is a representative Coomassie Blue-stained gel of a typical purification of i3 protein. Lane 1, molecular mass markers in kilodaltons; lane 2, the purified i3–intein–CBD–His protein eluted from the Ni-affinity column; lane 3, the purified i3 peptide after cleavage, filtration through a 10-kDa filter, and concentration using a 5-kDa filter; lane 4, the concentrate from the 10-kDa filter.

We next subcloned the entire fusion protein construct with the addition of a His^6-tag at the C terminus of the CBD domain into the pET11a expression system, which uses the T7 promoter for high levels of expression. As is shown in Fig. 1, following Ni-affinity chromatography, essentially homogeneous i3–intein–CBD–His^6 protein was obtained at relatively high yields (2–10 mg/L). This fusion protein was then applied to a chitin affinity column and i3 liberated by self-cleavage overnight in the presence of dithiothreitol. A residual amount of high molecular weight material was removed by passing the eluate from the chitin column through a 10-kDa filter (Fig. 1). Homogeneous i3 was prepared in acceptable yields (~1 mg/L).

CD studies with purified i3 protein were performed next (Fig. 2). Classically (Van Holde, 1971), proteins with pure α-helical structure display maxima at 191 nm and minima at 208 and 222 nm. Proteins with pure β-sheet structure display a maximum at 195 nm and a minimum at 217 nm. Random coils display a minimum at 197 nm and a maximum at 217 nm. Visual inspection of the CD spectrum of the purified i3 protein (Fig. 2) suggested both α-helical and β-sheet structure with minimal random coils. Recently, computerized algorithms for the analysis of CD spectra have appeared (e.g., Perczel et al., 1992). Analysis with this algorithm disclosed that the i3 protein was 48% α-helical in structure, with the remainder being β-turns, -loops, and potentially β-sheet. These results indicate that the i3 loop, as suggested by mutagenesis and modeling studies, has predominantly an α-helical structure. What was not predicted is that significant β-turn, -loop, and potentially β-sheet structure is also apparent. Direct structural studies (e.g., x-ray crystallography) are currently in progress to solve the structure of the i3 loop (E. Hyde et al., work in progress).

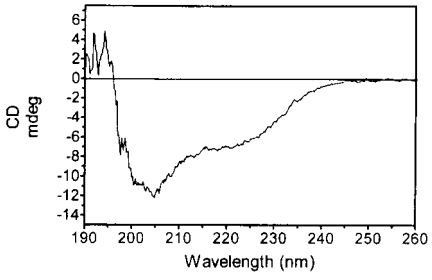

FIG. 2. CD spectra of the purified i3 protein. Shown is the mean of five separate CD scans of the i3 protein. The maximum at 190–195 nm and minimum at 208–210 nm predict that the protein has α-helical and β-sheet structure and is not a random coil (Van Holde, 1971). Computerized analysis of the spectrum using the method of Perczel et al. (1992) revealed that the i3 loop is composed of 48% α-helical structure with the remainder being β-loop, -turn, and possibly β-sheet structures.

Identification of arrestin as a potential 5-HT$_{2A}$ receptor interacting protein

After solving the gross secondary structure of the i3 loop, we next identified proteins that interact with the i3 loop. To do this, we used a novel strategy that uses random peptide libraries to identify consensus sequences for the interaction of the 5-HT$_{2A}$ receptor i3 loop and other proteins. For these studies, we used as bait an i3–GST fusion protein.

Several sequences were discovered that shared considerable homologies with various arrestin sequences (see Fig. 3). The arrestin sequences represented 18–21% of the total sequences identified (Table 1). A number of other sequences were found that did not yield homology with G proteins or other proteins thought to interact with i3 domains (protein kinases and phosphatases). These results suggest that other, unidentified proteins may also interact with the i3 peptide. Experiments are currently in progress using the yeast two-hybrid system to identify

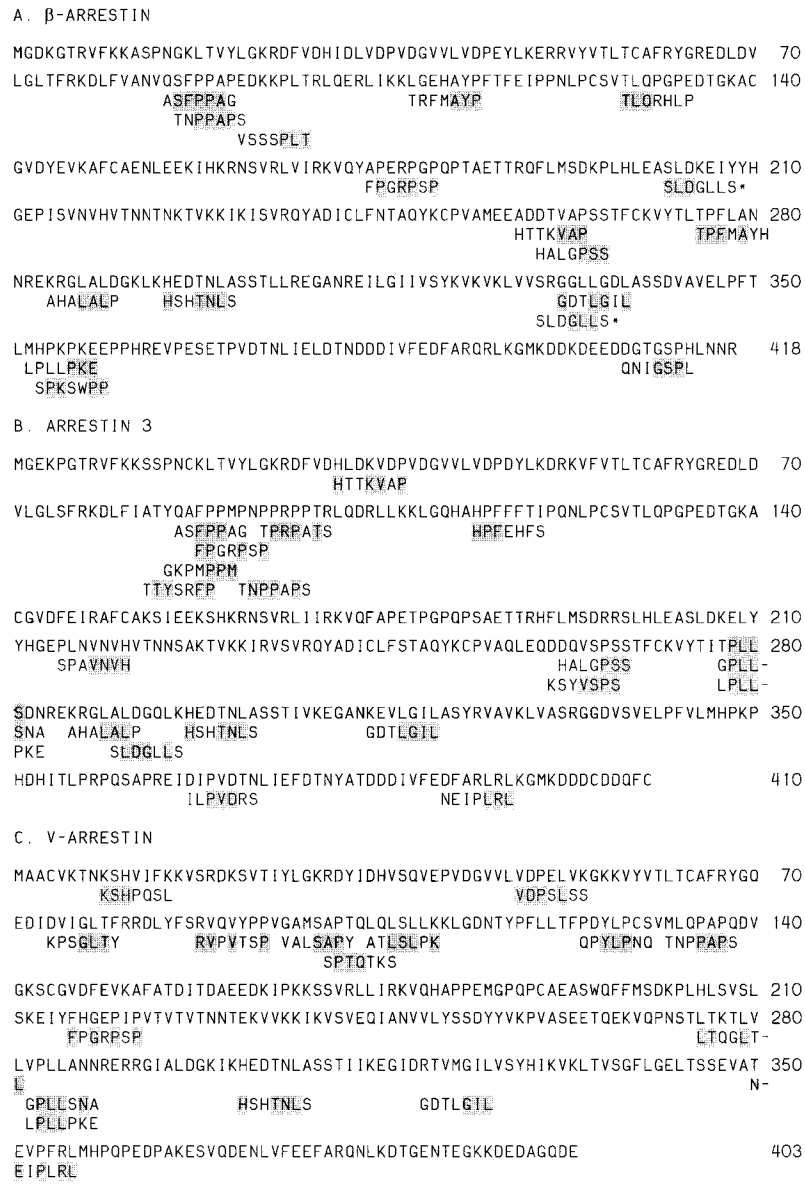

FIG. 3. Phage display using random peptides that bind to the i3 loop reveals sequences with homologies to arrestin. Alignment of phage display heptapeptide sequences with various arrestins was produced using the CLUSTAL algorithm: (**A**) β-arrestin, (**B**) arrestin-3, and (**C**) v-arrestin. Significant matches were defined as either (a) at least three consecutive identical residues or (b) at least four nonconsecutive identical matches. Highlighted residues in the phage display sequences indicate identical matches with the corresponding arrestin sequences. The asterisks indicate a sequence (SLDGLLS) that aligns equally well at two positions with β-arrestin. Significant alignments were also found with $G_{\alpha q}$, 13 protein kinases, and eight protein phosphatases.

these potential interacting proteins (W. K. Kroeze and B. L. Roth, work in progress). It is interesting that several of these arrestin sequences identified are in the putative interaction domain where arrestins are thought to bind to GPCRs (Granzin et al., 1998).

Arrestins bind directly to the i3 loop of the 5-HT_{2A} receptor

These results suggested to us that various arrestins might serve as 5-HT_{2A} interacting proteins. To test this hypothesis, we incubated purified β-arrestin, arrestin-3, and v-arrestin with an i3–GST fusion protein construct. To measure the degree of specific binding, we used an excess of homogeneous i3 protein. As shown in Figs. 4 and 5, the i3 construct specifically bound β-arrestin, arrestin-3, and v-arrestin. As a control for specificity, neither excess GST nor soybean trypsin inhibitor (10 μM each) inhibited the binding of any of the tested arrestins (data not shown). These results suggest that arrestins specifically bind to the i3 loop of the 5-HT_{2A} receptor.

β-Arrestin and arrestin-3 are colocalized with 5-HT_{2A} receptors in rat prefrontal cortex

The 5-HT_{2A} receptor is found primarily in cortical pyramidal neurons, and a prior study of β-arrestin and arrestin-3 distribution found particularly high concentrations of both arrestins in the cortex (Attramadal et al., 1992), although a quite heterogeneous distribution of both arrestins was seen. These results suggested to us that either arrestin-3 or β-arrestin might be colocalized with 5-HT_{2A} receptors in rat cortex.

To test this hypothesis, we performed dual-label fluorescence confocal microscopic studies of rat prefrontal cortex using a newly developed 5-HT_{2A}-specific anti-

TABLE 1. *Number of significant alignments of phage display sequences and arrestin target protein sequences*

Protein	Alignments (n)	% of total
β-Arrestin	16	18
Arrestin-3	19	21
v-Arrestin	16	18

Data represent the number of sequences that could be aligned with the target sequences, as well as the percentage of total sequences. Significant alignments were also found with $G_{\alpha q}$, 13 protein kinases, and eight protein phosphatases. Significant matches were defined as either (a) at least three consecutive identical residues or (b) at least four nonconsecutive identical matches.

body. To verify the specificity of the arrestin subtype-specific antibodies, we performed western blots with purified arrestins and arrestin-specific antibodies. As shown in Fig. 6A, the β-arrestin and arrestin-3 antibodies cross-reacted with only purified arrestin-3 and β-arrestin. Likewise, the arrestin-3-specific antibody labeled a single band of the appropriate molecular weight in cortical homogenates (Fig. 6B, lanes 1 and 2). Likewise, the β-arrestin-specific antibody labeled only a band of the appropriate molecular weight in cortical homogenates (Fig. 6B, lanes 3 and 4).

As shown in Fig. 7A–C, β-arrestin and 5-HT$_{2A}$ receptors were coexpressed in a number of pyramidal neurons in the prefrontal cortex. Arrestin-3 and 5-HT$_{2A}$ receptors were also colocalized in a number of pyramidal neurons (Fig. 7D–F). These results represent the first demonstration that GPCRs are endogenously coexpressed with arrestins in the brain.

As can also be seen, although many cortical pyramidal neurons showed extensive coexpression of 5-HT$_{2A}$ receptors and arrestin-3, some did not. Figure 8A–C, from a neighboring section of medial prefrontal cortex at a lower power, shows more clearly that β-arrestin and 5-HT$_{2A}$ receptors are coexpressed in some, but not all, cortical pyramidal neurons. As can be seen (Fig. 8C), several neurons express substantial quantities of 5-HT$_{2A}$ receptors (Fig. 8A, red channel) and little detectable β-arrestin (Fig. 8B, green channel).

As arrestins have been implicated in receptor trafficking (Ferguson et al., 1996; Goodman et al., 1996), we also investigated whether arrestins are coexpressed in intracellular vesicles. As seen in Fig. 8A′–C′, extensive

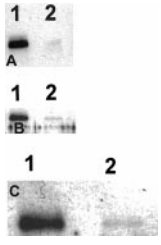

FIG. 4. Arrestins bind to an i3–GST fusion protein. Shown are typical results in which β-arrestin (**A**), v-arrestin (**B**), and arrestin-3 (**C**) binding was determined for the i3–GST fusion protein. A: Lane 1, total binding of β-arrestin to i3–GST; lane 2, inhibition of β-arrestin binding to i3–GST by 10 μ*M* purified i3. B: Lane 1, total binding of v-arrestin to i3–GST; lane 2, inhibition by 10 μ*M* purified i3. C: Lane 1, total binding of arrestin-3; lane 2, inhibition by 10 μ*M* purified i3 peptide. Results are from typical experiments, which were replicated at least three times.

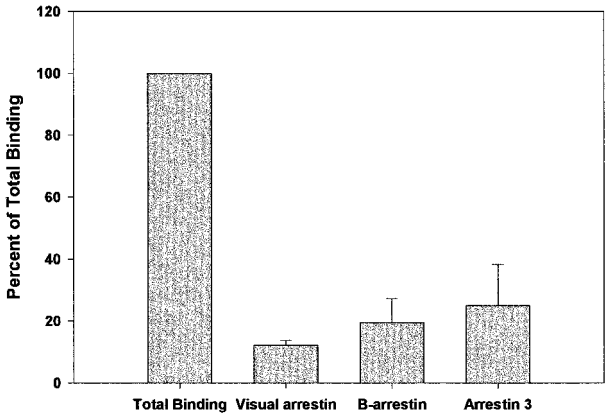

FIG. 5. Inhibition of arrestin binding to i3–GST by purified i3 peptide. The effect of 10 μ*M* purified i3 peptide on the binding of v-arrestin, β-arrestin, and arrestin-3 to i3–GST fusion protein is shown. Data are expressed as means ± SEM of total arrestin binding for three to five separate experiments. Data were normalized to represent the percentage of maximal binding of each arrestin based on scanning densitometry of western blot analysis.

colocalization of β-arrestin and 5-HT$_{2A}$ receptors was found in intracellular vesicles in some, but not all, medial prefrontal cortical pyramidal neurons. As seen in these high-power confocal images, several neurons showed extensive colocalization (large arrows), whereas others did not (small arrows). These results suggest that al-

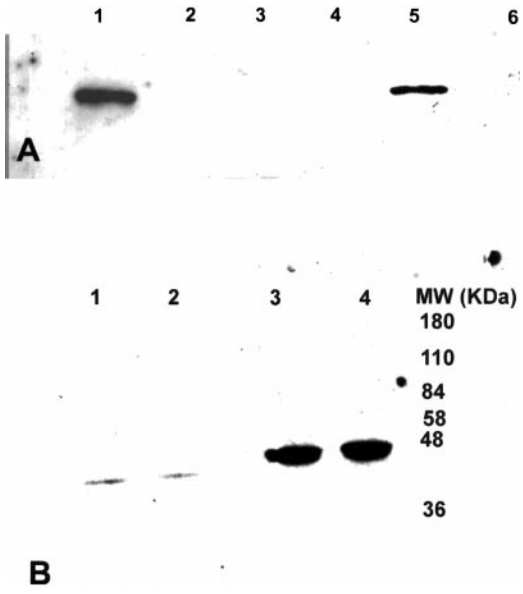

FIG. 6. Characterization of subtype-selective arrestin antibodies. **A:** Representative western blots in which β-arrestin (lanes 1–3) and arrestin-3 (lanes 4–6) specific antibodies were used to visualize β-arrestin (lanes 1 and 4), arrestin-3 (lanes 2 and 5), and v-arrestin (lanes 3 and 6). **B:** A typical western blot in which homogenates obtained from rat medial prefrontal cortex (10 μg of total protein) were probed with arrestin-3-specific antibodies (lanes 1 and 2) and β-arrestin-specific antibodies (lanes 3 and 4).

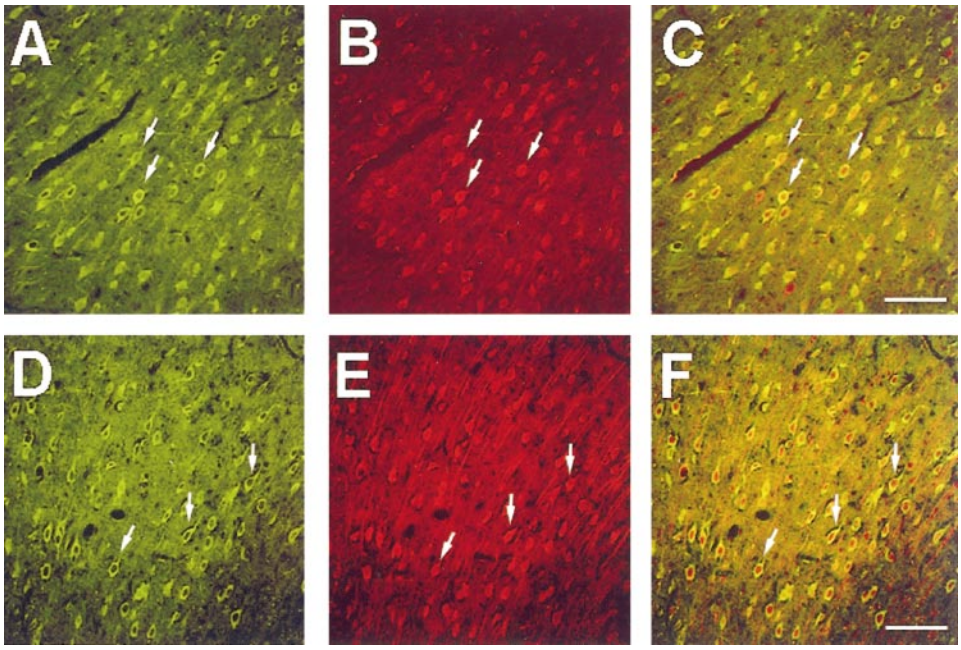

FIG. 7. β-Arrestin and arrestin-3 are colocalized with 5-HT_{2A} receptors in prefrontal cortical neurons. Shown are typical dual-label fluorescence confocal micrographs of rat medial prefrontal cortical sections. **A:** β-Arrestin immunofluorescence, in green. **B:** 5-HT_{2A} receptor immunofluorescence, in red. **C:** The red and green channels from A and B are superimposed. Arrows represent cells expressing both β-arrestin and 5-HT_{2A} receptors. **D:** Arrestin-3 immunoreactivity, in green. **E:** 5-HT_{2A} immunoreactivity, in red. **F:** The red and green channels from D and E are superimposed. Arrows represent cells expressing both arrestin-3 and 5-HT_{2A} receptors. Incubation with preimmune sera resulted in no specific staining (data not shown). Note that occasional cortical pyramidal neurons express 5-HT_{2A} receptors, but no detectable β-arrestin and that arrestin immunoreactivity does not extend into the apical dendrites.

though β-arrestin is extensively colocalized with 5-HT_{2A} receptors in intracellular vesicles, this colocalization is by no means obligatory.

DISCUSSION

This report has three major findings, all of which are consistent with the notion that the i3 loop of the 5-HT_{2A} receptor has a defined secondary structure and that this allows it to interact with the arrestin family of proteins. Our first observation was that the i3 loop of the 5-HT_{2A} receptor has considerable secondary structure, being composed primarily of α-helical regions and β-loops, -turns, and, potentially, β-sheets. These are the first studies in which the entire putative i3 domain of a $G_{\alpha q}$-coupled receptor has been shown to have a defined secondary structure. Secondly, using purified i3 proteins, we identified a family of interacting proteins, the arrestins, which bind to the i3 loop. Thirdly, we showed that arrestin-3 and β-arrestin are colocalized with 5-HT_{2A} receptors in many prefrontal cortical neurons and some intracellular vesicles. These are also the first studies to demonstrate that *any* arrestin colocalizes with *any* GPCR in the brain. Each of these findings will be discussed in turn.

Prior structure–function studies of the intracellular domains of GPCRs have focused on relatively small intracellular loops. Recent studies, in which the entire intracellular loops of rhodopsin were solved by NMR, show that the i3 loop has a turn-helix-turn motif (Yeagle et al., 1997). Yeagle et al. (1997) have published recently a model for the entire intracellular surface of rhodopsin, incorporating their prior NMR findings of rhodopsin-derived peptides, in which the i3 loop has a short α-helical region in the N-terminal region. The current studies, in which the i3 loop of the 5-HT_{2A} receptor was shown to be composed primarily of α-helices and β-sheets, imply that the larger loops seen in other $G_{\alpha q}$-coupled receptors will have greater α-helical character than the rhodopsin i3 loop. These results are important, because they imply that these relatively large intracellular domains have a defined structure. Our arrestin binding assays suggest that this could be essential for the binding of accessory proteins, in addition to G proteins.

Our results support recent models of GPCR activation that suggest that the i3 loop has α-helical structure and that this facilitates a ligand-induced conformational change leading to G-protein coupling (Farrens et al., 1996; Hill-Eubanks et al., 1996; Liu et al., 1996). The availability of large quantities of purified i3 regions of the 5-HT_{2A} receptor, facilitated by our construction of a new expression cassette, will facilitate direct structural studies (e.g., crystallography) of the i3 loop of the 5-HT_{2A} receptor. Such studies are currently in progress (E. Hyde et al., work in progress).

Exploiting the availability of large amounts of purified i3 protein, we were able to use a random peptide phage

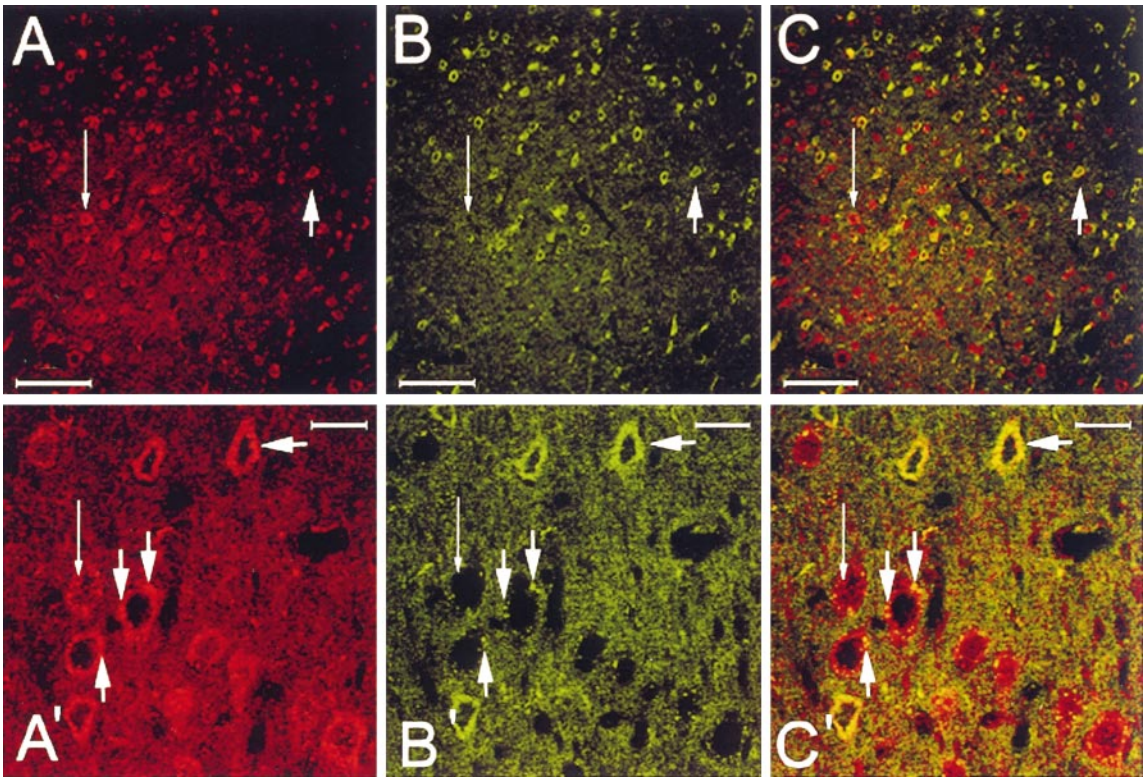

FIG. 8. β-Arrestin and 5-HT$_{2A}$ receptors are colocalized in intracellular vesicles in prefrontal cortical cells. Shown are dual-label fluorescence photomicrographs from slices incubated with β-arrestin-specific monoclonal antibodies (**A** and **A′**) and 5-HT$_{2A}$-specific polyclonal antibodies (**B** and **B′**) at ×60 (A–C) and ×600 (A′–C′). **C** and **C′:** The red and green channels from A and A′ and B and B′ are superimposed. A–C: Although most medial prefrontal cortical pyramidal neurons coexpress 5-HT$_{2A}$ receptors and β-arrestin (large arrows), several express only 5-HT$_{2A}$ receptors (small arrows). Higher power views (A′–C′) clearly demonstrate that 5-HT$_{2A}$ receptors and β-arrestin are colocalized in intracellular vesicles (large arrows). It is also evident that several neurons express abundant amounts of intracellular 5-HT$_{2A}$ receptors and minimal quantities of vesicular β-arrestin.

library to search for sequences that interact with various i3 domains. We discovered several sequences that had homologies for β-arrestin. It is interesting that several of the sequences appear to be in the putative receptor interaction domain of v-arrestin (Granzin et al., 1998). To test the possibility that one of the functions of the i3 loop is to bind arrestins, we incubated purified arrestins with an i3–GST fusion protein and discovered that v-arrestin, β-arrestin, and arrestin-3 all specifically bound to the i3 loop. These results are reminiscent of recent findings by Wu et al. (1997), who used an analogous approach to demonstrate that arrestins can bind to the i3 loops of muscarinic and α_2-adrenergic receptors. These results are important, because they imply that the i3 loop may be a common binding or interaction region of arrestins, and this brings to three the number of GPCRs that can bind arrestins via the i3 loop. We are currently attempting to identify the regions of the i3 loop important for arrestin interactions by using synthetic peptides and i3 mutant proteins.

Finally, we found that arrestins and 5-HT$_{2A}$ receptors are colocalized in vivo in many individual prefrontal cortical pyramidal neurons. One preliminary study of arrestin-3 and β-arrestin localization showed that both arrestins were found in a large number of neurons, particularly in the cortex (Attramadal et al., 1992). It should be emphasized, however, that this preliminary study appeared also to show a quite heterogeneous distribution of arrestin-3 and β-arrestin. Given the fact that 5-HT$_{2A}$ receptors were demonstrated recently to be enriched in the same neuronal population (Willins et al., 1997), we investigated the possibility that these two proteins may be colocalized in vivo. We discovered that both arrestin-3 and β-arrestin show extensive colocalization with 5-HT$_{2A}$ receptors in individual cortical neurons. Our results also showed that although β-arrestin and arrestin-3 were found in many cortical neurons, their distribution was by no means ubiquitous. On the ultrastructural level, high-magnification confocal sections revealed arrestin and 5-HT$_{2A}$ receptor colocalization in intracellular vesicles. These findings are important, because they represent the first demonstration that GPCRs and arrestins are colocalized in neurons. Additionally, these studies support notions derived from previous in vitro studies that suggest that arrestins are involved in regulating the trafficking of GPCRs (Ferguson et al., 1996; Goodman et al., 1996). Whether arrestins regulate the internalization of 5-HT$_{2A}$ receptors or other 5-HT$_{2A}$ re-

ceptor functions is currently under investigation. In this regard, we have obtained preliminary data that β-arrestin affects 5-HT_{2A} receptor desensitization (E. I. Gelber and B. L. Roth, unpublished observation).

In conclusion, we demonstrated that the i3 loop of the 5-HT_{2A} receptor has 48% α-helical structure with the remainder being β-loop, -turn, and -sheet structure and that the i3 loop serves as an arrestin binding domain. We also found that arrestins and 5-HT_{2A} receptors are colocalized in neurons in vivo. Future studies on the domains of arrestins and the i3 loop responsible for arrestin binding, as well as the effects of phosphorylation of the i3 loop on arrestin binding, should clarify the role(s) of arrestin–i3 interactions.

Acknowledgment: This work was supported in part by RO1MH57635, a Research Career Development Award KO2 MH01366, and a NARSAD Independent Investigator Award to B.L.R. During the time period in which some of this work was performed, B.L.R. was the 1998 Stagelin Music Festival Investigator for NARSAD. D.L.W. was supported in part by a NARSAD Young Investigator Award, and C.A.S. was supported in part by a grant from the Keck Foundation to John Carroll University. We are grateful to Vernon Anderson (Department of Biochemistry, Case Western Reserve University) for assistance with analysis of the CD spectra.

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