Structure and Function of the Third Intracellular Loop of the 5-Hydroxytryptamine$_{2A}$ Receptor: The Third Intracellular Loop Is $\alpha$-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 $\alpha$-helical and $\beta$-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. 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, 1998b).

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, $\alpha$-helical in structure and that movements of these intracellular $\alpha$-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.

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., 1998a,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., 1998a). 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, 1998b).

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, $\alpha$-helical in structure and that movements of these intracellular $\alpha$-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.

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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 (Stratagene, La Jolla, CA, U.S.A.) was obtained from Amersham Life Science (Cleveland, OH, U.S.A.). Trans-Blot nitrocellulose transfer medium was obtained from Bio-Rad Laboratories (Heracles, CA, U.S.A.). The following peptides were from Research Genetics: peptide 40 (TYFLTIKSLQKE), which corresponds to the C terminus of the i3 loop of the 5-HT_{2A} receptor; and peptide 41 (SNEQKACKVLGI), which corresponds to the N terminus of the i3 loop of the 5-HT_{2A} receptor. These results support models of GPCR structure and function that predict that domains of intracellular loops are α-helical in nature.

**Preparation of fusion proteins**

**Preparation of i3–GST fusion protein and GST.** The putative i3 loop (FLTIKSLQKEATLCVSDLSTRAKLASFSFLPQSS-LSSEKLFQRSHPGSGYAGRRTMQSISNNEQKACKV) of the 5-HT_{2A} receptor was amplified using Pfu polymerase and the following primers: BELOOP: 5′-AAC GGG ATC CCC ATC ACC TAC TTT CTT GCT ACT ATC-3′, which corresponds to base pairs 815–834, and ENDOLOOP: 5′-GGG GGC TTA ACG ATG CCC AGC ACC TTG CAC-3′, which corresponds to base pairs 1,024–1,043 (both have BamHI adaptors). The product was purified by agarose gel electrophoresis. 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 mM IPTG for 5 h. Bacteria were pelleted and washed with phosphate-buffered saline (14 mM NaCl, 2.6 mM KCl, 10 mM NaHPO_4, 1.7 mM KH_2PO_4, pH 7.4). Bacteria were pelleted again and resuspended in lysis buffer (50 mM Tris, 1 mM 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 mM Tris, 1 mM EDTA, 1 mg/ml lysozyme, pH 8.0). The putative i3 loop was 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 fusion proteins. 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, ACCATCCCATATGATCCACTAATCTCTGACTATCAAG (Ndel site in bold); i3-pCBY-reverse, AACCCGGGACAGATGCCAGAACCTTGACGCG-CCTT (SmaI site in bold). It was purified, cut with Ndel and SmaI, and ligated into the Ndel and SmaI sites of pCBY2. 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: CBDDHSBH, TAT GGA TCC TTA GTG GTG GTG GTG GTG GTG GTG TGT GTG AGG CTC CCA CCA GGC AGAG (6-His sequence in bold) and i3-pCBY-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.

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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, MgSO₄, and CaCl₂ (Sambrook et al., 1989) with incubation at 37°C until the OD₆₀₀ 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 mM 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–His⁶ fusion protein was eluted with a gradient of imidazole (0–500 mM)-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 H₂O containing 250 mM Na-HEPES, pH 7.4, 625 µM NaCl, 0.0625% glycerol, and 0.00025% NaN₃. 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 mM NaHCO₃, 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 mM NaCl, 0.1% Triton X-100, 0.02% NaN₃) to block non-specific binding. 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, β-arrestin-1, 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 F4Cl mouse monoclonal antibody, which detects v-arrestin, β-arrestin, and arrestin-3, and horseradish peroxidase antimouse 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-HT₂A 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-HT₂A antibodies, respectively, as previously described (Willins et al., 1997, 1999).

RESULTS
The i3 loop of the 5-HT₂A receptor has α-helical and β-sheet secondary structure
As the i3 loops of Gαq-coupled GPCRs are generally quite large, attempts to predict secondary structure are likely to be inexact. Prior mutagenesis studies have been predominately conducted 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 Gαq 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-HT₂A 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.
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 \(\alpha\)-helical structure display maxima at 191 nm and minima at 208 and 222 nm. Proteins with pure \(\beta\)-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 \(\alpha\)-helical and \(\beta\)-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% \(\alpha\)-helical in structure, with the remainder being \(\beta\)-turns, -loops, and potentially \(\beta\)-sheet. These results indicate that the i3 loop, as suggested by mutagenesis and modeling studies, has predominantly an \(\alpha\)-helical structure. What was not predicted is that significant \(\beta\)-turn, -loop, and potentially \(\beta\)-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).

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...
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-HT2A receptor

These results suggested to us that various arrestins might serve as 5-HT2A 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-HT2A receptor.

β-Arrestin and arrestin-3 are colocalized with 5-HT2A receptors in rat prefrontal cortex

The 5-HT2A 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-HT2A 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-HT2A-specific anti-

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 (SLDGGLS) that aligns equally well at two positions with β-arrestin. Significant alignments were also found with Gαq, 13 protein kinases, and eight protein phosphatases.
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 \(\beta\)-arrestin and arrestin-3 antibodies cross-reacted with only purified arrestin-3 and \(\beta\)-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 \(\beta\)-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, \(\beta\)-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 \(\beta\)-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 \(\beta\)-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 these high-power confocal images, several neurons showed extensive colocalization (large arrows), whereas others did not (small arrows). These results suggest that al-

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<tr>
<th>Protein</th>
<th>Alignments (n)</th>
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<tr>
<td>(\beta)-Arrestin</td>
<td>16</td>
<td>18</td>
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<tr>
<td>Arrestin-3</td>
<td>19</td>
<td>21</td>
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<td>v-Arrestin</td>
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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 \(G_{out}\) 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.
though β-arrestin is extensively colocalized with 5-HT₂A 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-HT₂A 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-HT₂A 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 Gα⁰-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-HT₂A 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-HT₂A receptor was shown to be composed primarily of α-helices and β-sheets, imply that the larger loops seen in other Gα⁰-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-HT₂A 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-HT₂A 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
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 α3-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-

![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.](image-url)
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.

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