# Human 5-$HT_1$ Receptor Subtypes Exhibit Distinct G Protein Coupling Behaviors in Membranes from Sf9 Cells†

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ABSTRACT: The G protein coupling behavior of four human 5-hydroxytryptamine receptor subtypes (5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$, and 5-$HT_{1E}$) has been studied in membranes from Sf9 cells expressing the individual receptors. The 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors exhibited both high- and low-affinity states for agonist, with the majority of the receptors in a low-affinity state. Addition of purified G protein subunits to membranes expressing either 5-$HT_{1A}$ or 5-$HT_{1B}$ receptors shifted the majority of the receptors to a high-affinity state in the absence, but not in the presence, of guanine nucleotides. The $\alpha_{i1}$, $\alpha_{i2}$, $\alpha_{i3}$, and $\alpha_o$ subunits were able to shift the receptors to a high-affinity state with either $\beta\gamma_{brain}$ or $\beta\gamma_{retina}$ while $\alpha_t$ subunits were inactive regardless of which $\beta\gamma$ preparation was used. A significantly higher affinity for agonist was observed with both receptors in the presence of $\alpha_{i3}$ subunits compared with either $\alpha_{i2}$ or $\alpha_o$ subunits, while a significantly lower concentration of $\alpha$ subunits was required for a maximal affinity shift of 5-$HT_{1A}$ receptors compared with 5-$HT_{1B}$ receptors ($EC_{50}$ values of 6.4 and 12.0 nM, respectively). The 5-$HT_{1D}$ and 5-$HT_{1E}$ receptors exhibited only a single affinity state for agonist. Addition of purified G protein subunits to membranes containing 5-$HT_{1D}$ receptors caused a small increase in affinity for agonist that was only partially reversed by guanine nucleotides while the addition of purified G protein subunits to membranes containing 5-$HT_{1E}$ receptors had no affect on agonist binding. Thus when expressed in an identical membrane environment these four closely related 5-$HT_1$ receptor subtypes exhibit different G protein coupling behaviors.

The heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins)[1] mediate signaling from a large number of diverse heptahelical cell surface receptors to a variety of intracellular effectors. These pathways control numerous essential functions in all tissues and are ubiquitous throughout the animal kingdom. The components of these pathways, the receptors, the G proteins, and the effectors, may be classified into major families on the basis of structural and functional criteria. However, a striking feature of G protein-coupled signaling pathways is the abundance of distinct subtypes identified within the major families for each of these components. The functional significance of so many closely related molecules is largely unappreciated. At the biochemical level, significant contributions in this area might be made by developing the ability to examine the behavior of closely related members of specific signal transduction pathways in a reconstitution paradigm. Toward this end, the ability of four subtypes from one family of receptors for serotonin (5-hydroxytryptamine, or 5-HT) to interact with specific G protein heterotrimers in a membrane environment has been examined.

Seven distinct families of 5-HT receptors have been classified, with many of the families containing multiple members (Humphrey et al., 1993). With the exception of the 5-$HT_3$ receptor, which is a ligand-gated ion channel, all known 5-HT receptors are members of the G protein-coupled heptahelical receptor superfamily (Boess & Martin, 1994). The 5-$HT_1$ family has proven particularly diverse, containing five distinct subtypes denoted 5-$HT_{1A}$, 5-$HT_{1B}$ (formerly 5-$HT_{1D\beta}$), 5-$HT_{1D}$ (formerly 5-$HT_{1D\alpha}$), 5-$HT_{1E}$, and 5-$HT_{1F}$ (Hartig et al., 1996; Humphrey et al., 1993). All five have been shown to mediate the inhibition of adenyl cyclase in a pertussis toxin sensitive fashion (Adham et al., 1993, 1994; Zgombick et al., 1993; De Vivo & Maayani, 1986). Members of the 5-$HT_1$ receptor family have also been shown to mediate a pertussis toxin sensitive activation of $K^+$ channels, stimulation of phospholipase C, and elevation of cytosolic $Ca^{2+}$, in a cell-type and receptor-density, dependent manner [reviewed in Boess and Martin (1994)]. The pertussis toxin sensitivity of these responses implicates members of the $\alpha_{i/o}$ family of G proteins (Gilman, 1987), which includes the pertussis toxin substrates $\alpha_{i1}$, $\alpha_{i2}$, $\alpha_{i3}$, $\alpha_o$, and $\alpha_t$ and the non-pertussis toxin substrate $\alpha_z$ (Strathmann & Simon, 1990). Nevertheless, a direct examination of the specific nature of the G proteins coupling these receptors has only been reported for the 5-$HT_{1A}$ receptor (Butkerait et al., 1995; Mulheron et al., 1994; Raymond et al., 1993; Bertin et al., 1992).

The baculovirus expression system, using *Spodoptera frugiperda* (Sf9) cells, combines a very abundant expression

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[1] Abbreviations: G proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; $\alpha_x$, a specific subtype of G protein $\alpha$ subunit; $\beta_x\gamma_x$, a specific subtype of G protein $\beta\gamma$ dimer; 5-HT, 5-hydroxytryptamine or serotonin; 5-$HT_x$, a specific subtype of 5-HT receptor; GTP$\gamma$S, guanosine 5′-3-*O*-(thiotriphosphate); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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of recombinant proteins with the protein modification, processing, and transport systems of higher eukaryotes (Luckow & Summers, 1988) and has been used to express distinct subtypes of heptahelical receptors, G proteins, and intracellular effectors shown to be functionally equivalent to their native counterparts (Sutkowski et al., 1994; Taussig et al., 1993; Hepler et al., 1993; Iniguez-Lluhi et al., 1992; Graber et al., 1992a,b; Tang et al., 1991; Parker et al., 1991). A particular advantage of the Sf9 cell system has been the absence of homologs of most mammalian heptahelical receptors examined. Although most mammalian heptahelical receptors expressed in this system have been shown to functionally interact with endogenous Sf9 cell G proteins and are capable of modulating intracellular effector activities (Harteneck et al., 1995; Kwatra et al., 1993; Mulheron et al., 1994; Ng et al., 1993; Richardson & Hosey, 1992; Parker et al., 1991), the coupled receptors generally represent a small proportion of those expressed such that the majority of receptors expressed at high levels are not coupled with endogenous G proteins (Butkerait et al., 1995; Parker et al., 1991, 1994; Boundy et al., 1993; Quehenberger et al., 1992). Thus the Sf9 cell can provide an environment in which the G protein coupling of expressed receptors can be examined in the absence of competing interactions with either related receptors or significant levels of G proteins. Recently such interactions were studied using the co-infection of receptor and various G protein subunits in Sf9 cells (Butkerait et al., 1995). However, since the relative levels of expression of the various components cannot be controlled with such an approach, it is useful for assessing functional interactions in a qualitative fashion only. With the demonstration that 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$, and 5-$HT_{1E}$ receptors expressed in Sf9 cells exhibit the pharmacological properties of their native counterparts while remaining largely uncoupled from endogenous G proteins (Parker et al., 1994), we sought to develop a membrane-based reconstitution system in which the relative amounts and identities of G protein heterotrimers that might interact with these receptors could be controlled precisely. Such an approach has recently been taken with a single subtype of adenosine receptors (Figler et al., 1996). We report here the development of a membrane-based reconstitution system in which the functional coupling, as assayed by restoration of high-affinity agonist binding, of two of the expressed 5-$HT_1$ receptor subtypes with exogenously supplied, purified, G protein subunits can be observed. Reconstitution is achieved with nM concentrations of G protein subunits and requires from 3−8 mol of G protein subunits per mole of 5-$HT_1$ receptor. Moreover, we provide evidence that these four closely related subtypes of 5-$HT_1$ receptors may be distinguished on the basis of their G protein coupling behavior in an identical membrane environment.

## EXPERIMENTAL PROCEDURES

*Preparation of Sf9 Cell Membranes Containing Expressed 5-$HT_1$ Receptors.* Construction of the recombinant baculoviruses expressing the 5-$HT_1$ receptor subtypes has been described (Parker et al., 1994). Sf9 cells were infected with viruses, cultured and harvested as previously described (Graber et al., 1992a). Infections were allowed to proceed for 35−52 h, and membranes prepared from infected cells typically contained from 2−10 pmol of receptor/mg of membrane protein. To prepare membranes, harvested cells were thawed in 15× their wet weight of ice-cold homogenization buffer (10 mM Tris-HCl, pH 8.0 at 4 °C, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 20 $\mu$g/mL of benzamidine, and 2 $\mu$g/mL of each of aprotinin, leupeptin, and pepstatin A) and burst by $N_2$ cavitation (600 psi, 20 min). Cavitated cells were centrifuged at 4 °C for 10 min at 500*g* to remove the unbroken nuclei and cell debris. The supernatant from the low-speed spin was centrifuged at 4 °C for 30 min at 28000*g*. The supernatant was discarded, and the pellets were resuspended and pooled in 35 mL of 5 mM NaHEPES, 1 mM EDTA, pH 7.5, containing the same protease inhibitors as used in the homogenization buffer. The membranes were washed twice, resuspended in the same buffer at a concentration of 1−3 mg of protein/mL, aliquoted, snap frozen in liquid nitrogen, and stored at −70 °C.

*[$^3$H]-5-HT Binding Assays.* [$^3$H]-5-HT binding to 20−50 $\mu$g of membrane protein was determined in binding assay buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5) in the presence of the indicated additional components. Nonspecific binding was determined by addition of 10 $\mu$M 5-hydroxytryptamine. Incubations were for 1.5 h in a room temperature shaker and were terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters were rinsed with 3 × 4 mL of ice-cold 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.01% sodium azide, pH 7.5, at 4 °C, placed in 4.5 mL of CytoScint (ICN Pharmaceuticals, Costa Mesa, CA), and counted to constant error in a scintillation counter. For saturation binding isotherms, concentrations of [$^3$H]-5-HT were from 0.07 to 250 nM in a final volume of 500 $\mu$L. For reconstitution of high-affinity agonist binding a single concentration (0.4−1.2 nM, depending on the receptor) of [$^3$H]-5-HT was used in a final volume of 150 $\mu$L. Radioligand purity was monitored by chromatography on a Zorbax ODS column (4.6 × 150 mm) using 1% triethylamine acetate, pH 4:methanol (95:5) as the mobile phase. Radioligand was repurified or replaced when the radiochemical purity fell below 85%.

*Purification of G Protein Subunits.* Recombinant $\alpha_{i/o}$ subunits were expressed in Sf9 cells and purified as described (Graber et al., 1992a) except that the final chromatography step was performed on 15 $\mu$ Waters Protein-Pak QHR (Waters Chromatography, Milford, MA). Native $\beta\gamma$ dimers purified from bovine brain were kindly provided by Dr. Paul Sternweis of the University of Texas Southwestern Medical Center. Holotransducin and resolved $\alpha_t$ and $\beta\gamma_t$ purified from bovine retina were kindly provided by Dr. Thomas Sakmar of The Rockefeller University.

*Reconstitution of Receptors with Exogenous G Proteins.* Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge (10 min), and resuspended at approximately 10 mg/mL in a reconstitution buffer consisting of 5 mM NaHEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 500 nM GDP, 0.04% CHAPS, pH 7.5. G protein subunits were diluted in the same buffer such that the desired amount of subunit was contained in 1−5 $\mu$L. When small concentrations (less than 0.2 mg/mL) of G proteins were used, 0.1 mg/mL BSA was added to the buffer to minimize absorptive loss of G protein subunits. Typically, 1−2 $\mu$L of G protein subunits was added to 40 $\mu$L of membrane suspension, and the mixture was incubated at 25 °C for 15 min and held on ice until the start of the [$^3$H]-5-HT binding assay. Just prior to the start of the binding assay (described above) the reconstitution mixture was diluted with a 10−15-fold excess

Table 1: Binding Parameters of Expressed 5-$HT_1$ Receptors[a]

| binding parameters | receptor subtype | | | |
|---|---|---|---|---|
| | 5-$HT_{1A}$ | 5-$HT_{1B}$ | 5-$HT_{1D}$ | 5-$HT_{1E}$ |
| $K_D$-H (nM) | 0.36 ± 0.06 (4) | 0.62 ± 0.13 (5) | 1.6 ± 0.18 (3) | 4.8 ± 0.17 (3) |
| $B_{max}$-H (fmol/mg) | 584 ± 10 (2) | 2577 ± 215 (2) | 2052 ± 192 (2) | 5449 ± 917 (2) |
| $K_D$-L (nM) | 86.1 ± 38.1 (4) | 39.5 ± 2.9 (5) | | |
| $B_{max}$-L (fmol/mg) | 15976 ± 3393 (2) | 5737 ± 276 (2) | | |

[a] Saturation binding isotherms were obtained using concentrations of [$^3$H]-5-HT from 0.07−250 nM. Data were fit to one-site and two-site models, and the best-fit model was chosen by *F*-test. $K_D$ values are the means from different membrane preparations, while $B_{max}$ values are the mean from a representative membrane preparation. Results are expressed as the mean ± SEM, and the number of determinations is shown in parentheses.

of binding assay buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5) such that the desired amount of membranes (20−40 $\mu$g of protein) was contained in 50 $\mu$L.

*Miscellaneous Procedures.* Amounts of protein were determined by the bicinchonic acid method (Pierce Chemicals, Chicago, IL) using BSA as the standard. Curve fitting, evaluation of one- and two-site models, and statistical analyses were done using GraphPad PRISM (GraphPad Software, San Diego, CA). When appropriate, multiple comparisons were made using Tukey's method as provided in GraphPad PRISM. Data in all figures are from representative experiments that were repeated exactly as described a minimum of three times with nearly identical results. Many similar experiments, with multiple preparations of membranes and G protein subunits, have yielded results similar to those depicted.

## RESULTS

Recombinant baculoviruses containing the genes for the human 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$, or 5-$HT_{1E}$ receptors were used to express the receptors in Sf9 cells. Radioligand binding to crude membrane preparations using the agonist [$^3$H]-5-HT was used to evaluate the expressed receptors and the results are summarized in Table 1. The 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors exhibited two affinity states, with the majority of the receptors in a low-affinity state, while the 5-$HT_{1D}$ and 5-$HT_{1E}$ receptors exhibited only a single affinity state in the Sf9 cell membranes. These results are in agreement with a previous study which demonstrated that all four receptors have the expected pharmacological properties and functionally couple to the inhibition of adenyl cyclase when expressed in Sf9 cells (Parker et al., 1994).

Since the majority of the 5-$HT_{1A}$ receptors expressed in the Sf9 cell membranes bound [$^3$H]-5-HT with low affinity it was of interest to determine if they could be converted to a high-affinity state for agonist by the addition of purified G protein $\alpha$ and $\beta\gamma$ subunits. A 120-fold molar excess of $\alpha$ subunits, $\beta\gamma$ subunits, or $\alpha\beta\gamma$ heterotrimers was added to Sf9 cell membranes containing expressed 5-$HT_{1A}$ receptors. The reconstituted membranes were then used in a radioligand binding assay with a low (0.4 nM) concentration of the agonist [$^3$H]-5-HT. This concentration is slightly above the $K_D$ for the high-affinity 5-$HT_{1A}$ site but is well below the $K_D$ for the low-affinity 5-$HT_{1A}$ site, and would thus readily detect formation of high-affinity, G protein-coupled receptors from the low-affinity, uncoupled receptors. As shown in Figure 1, addition of complete G protein heterotrimers produced more than a 4-fold increase in specific binding of agonist in membranes containing expressed 5-$HT_{1A}$ receptors. Significantly, the increase in binding is completely abolished

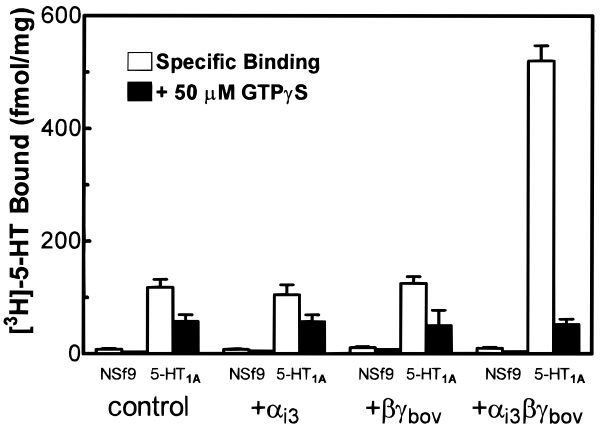


FIGURE 1: [$^3$H]-5-HT binding in membranes from Sf9 cells reconstituted with G protein subunits. Membranes (20 $\mu$g of protein) from normal Sf9 cells (NSf9) or Sf9 cells expressing 5-$HT_{1A}$ receptors (5-$HT_{1A}$) were reconstituted with buffer (control), purified $\alpha_{i3}$ subunits (+$\alpha_{i3}$), purified bovine brain $\beta\gamma$ subunits (+$\beta\gamma_{bov}$), or purified $\alpha_{i3}$ subunits plus bovine brain $\beta\gamma$ subunits (+$\alpha_{i3}\beta\gamma_{bov}$), and used in a [$^3$H]-5-HT binding assay as described in Materials and Methods. The concentration of [$^3$H]-5-HT used was 0.4 nM and the G protein subunits were present at a concentration of 40 nM which was approximately a 120-fold molar excess over the expressed 5-$HT_1$ receptors. Bars represent specific binding as the mean ± SD of triplicate determinations from a representative experiment.

by the addition of 50 $\mu$M GTP$\gamma$S, consistent with the interpretation that the added G proteins are coupling the expressed receptor in the accepted fashion. The small decrease in agonist binding produced by GTP$\gamma$S in the absence of added G proteins suggests that a small proportion of the expressed receptors are coupled with endogenous Sf9 cell G proteins. As would be expected, there is no increase in binding when either $\alpha$ or $\beta\gamma$ subunits alone are added to membranes expressing 5-$HT_{1A}$ receptors. Figure 1 also demonstrates that binding of [$^3$H]-5-HT to membranes from uninfected Sf9 cells (NSf9) is virtually undetectable.

To determine if the other expressed 5-$HT_1$ receptor subtypes were able to couple with exogenously supplied G protein subunits similar experiments were performed using membranes containing 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$, or 5-$HT_{1E}$ receptors. Representative results are summarized in Figure 2. Only the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors coupled with the exogenously supplied G proteins in the expected fashion. In both cases, addition of exogenous G proteins resulted in significantly increased agonist binding with a low concentration of agonist (1.2 nM) which was completely reversed by the addition of 50 $\mu$M GTP$\gamma$S. Interestingly, only these receptor subtypes exhibited two affinity states for agonist after expression in Sf9 cells (Table 1). The 5-$HT_{1D}$ receptors exhibited a small increase in agonist binding after addition

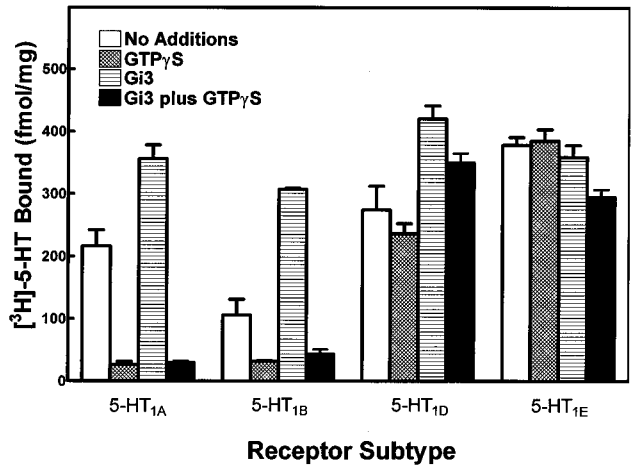


FIGURE 2: [$^3$H]-5-HT binding to 5-HT$_1$ receptor subtypes with and without reconstitution of exogenous G proteins. [$^3$H]-5-HT binding to the indicated 5-HT$_1$ receptor subtypes was determined in membranes from Sf9 cells with the indicated additions. The concentration of GTP$\gamma$S, when present, was 50 $\mu$M. Reconstituted membranes contained purified $\alpha_{i3}$ and bovine brain $\beta\gamma$ subunits in at least a 30-fold molar excess over expressed receptors. The concentration of [$^3$H]-5-HT used was 1.2 nM. Bars represent specific binding as the mean ± SD of triplicate determinations from a representative experiment.

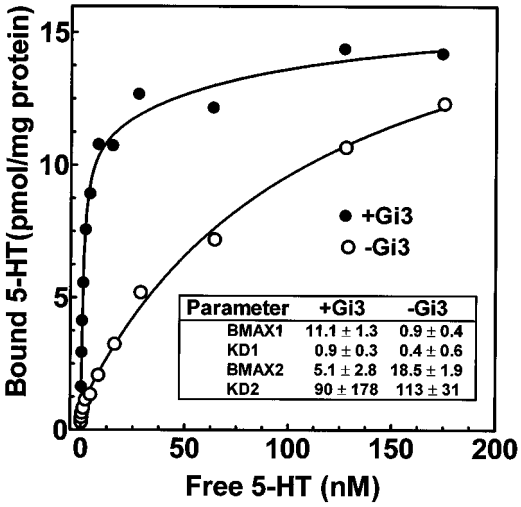


FIGURE 3: Saturation binding isotherm of 5-HT$_{1A}$ receptors with and without reconstitution of exogenous G proteins. [$^3$H]-5-HT binding was determined in membranes (19 $\mu$g of protein) containing expressed 5-HT$_{1A}$ receptors as described in Materials and Methods. The solid circles (+Gi3) indicate specific binding in membranes reconstituted with a 12-fold molar excess of G protein heterotrimers (containing an $\alpha_{i3}$ subunit) relative to 5-HT$_{1A}$ receptors. The open circles (−Gi3) indicate specific binding in membranes without exogenous G proteins. Data points are the mean of triplicate determinations from a representative experiment. The average SD for all data points was 7.8% of the mean values so error bars have been omitted for clarity. The solid lines indicate the best fits to the data of a two-site model governed by the law of mass action. In each case the two-site fit was superior to a one-site fit as determined by the F ratio. The inset table presents the parameters obtained by nonlinear regression analysis.

of exogenous G proteins. Curiously, an increase in agonist binding occurred even in the presence of 50 $\mu$M GTP$\gamma$S, suggesting that agonist binding to 5-HT$_{1D}$ receptors is less sensitive to GTP$\gamma$S. Agonist binding to the 5-HT$_{1E}$ receptors was not affected by the addition of exogenous G proteins. Identical results were obtained with the 5-HT$_{1D}$ and 5-HT$_{1E}$ receptors regardless of which G protein $\alpha_{i/o}$ subunit was used in the reconstitution paradigm (data not shown). The basis of these observations is not presently understood but is likely to be related to the fact that these receptors exhibit only a single affinity state for agonist when they are expressed in Sf9 cells [Table 1 and Parker et al. (1994)].

It was important to verify that the exogenous G proteins were actually increasing the proportion of receptors in a high-affinity state rather than altering either receptor affinity or number. As shown in Figure 3, a complete saturation binding isotherm was obtained for 5-HT$_{1A}$ receptors in the absence or presence of a 12-fold molar excess of exogenous G protein heterotrimers. Both sets of data are best described by a two-site model (as determined by *F*-test), and the inset table depicts the parameters obtained by nonlinear regression analysis. The small changes in the agonist affinities are not statistically significant. However, the proportion of receptors in the high-affinity state increases from 5% in the absence of exogenous G proteins to 69% in the presence of exogenous G proteins without a significant change in the total number of receptors (19.4 ± 2.33 pmol/mg in the absence of $G_{i3}$ vs 16.1 ± 4.10 pmol/mg in the presence of $G_{i3}$). Similar results were obtained with 5-HT$_{1B}$ receptors (data not shown). Thus, the most straightforward interpretation of the data is that the exogenous G protein heterotrimers are coupling with over-expressed receptors and shifting them from a low-affinity to a high-affinity state for agonists.

For the system to be useful in studying receptor−G protein coupling it must be capable of distinguishing among specific G protein heterotrimers. The retinal specific G protein (transducin) is perhaps the least likely heterotrimer to functionally couple with serotonin receptors and would therefore not be expected to enhance the binding of low concentrations of [$^3$H]-5-HT to serotonin receptors expressed in Sf9 cell membranes. Increasing amounts of $\alpha_{i3}$ or $\alpha_t$ in combination with a molar excess of $\beta\gamma$ subunits purified from either bovine brain or retina were added to Sf9 cell membranes containing 5-HT$_{1B}$ receptors. The reconstituted membranes were then used in a radioligand binding assay with a low (0.8 nM) concentration of the agonist [$^3$H]-5-HT. As shown in Figure 4, $\alpha_{i3}$ is equally effective in coupling the 5-HT$_{1B}$ receptor with either bovine brain $\beta\gamma$ ($\beta\gamma_{brain}$) or transducin $\beta\gamma$ ($\beta\gamma_t$) subunits, while the $\alpha_t$ subunit is not capable of functional interactions with the 5-HT$_{1B}$ receptors regardless of which $\beta\gamma$ subunit is present. Identical results were obtained with the 5-HT$_{1A}$ receptor (data not shown). Thus, the $\alpha$ subunit appears to be the major determinant of the ability of G protein heterotrimers to couple with 5-HT$_1$ receptors.

To further examine the selective coupling of G protein $\alpha$ subunits to specific receptor subtypes, the ability of increasing amounts of $\alpha_{i1}$, $\alpha_{i2}$, $\alpha_{i3}$, and $\alpha_o$ in combination with $\beta\gamma_{brain}$, to restore high-affinity agonist binding to 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors was compared. These data fit well to a model for a single-site interaction governed by the law of mass action and can be used to estimate the maximal level of agonist binding achieved with each $\alpha$ subunit and the concentration of each $\alpha$ subunit required for the half-maximal level of binding ($EC_{50}$) with each receptor. Figure 5 shows representative results with the 5-HT$_{1A}$ receptor while Figure 6 shows representative results with the 5-HT$_{1B}$ receptor. Reconstitution of high-affinity agonist binding for both receptors was achieved with nM concentrations of $\alpha$ subunits ranging from a 2−25-fold excess over the expressed recep-

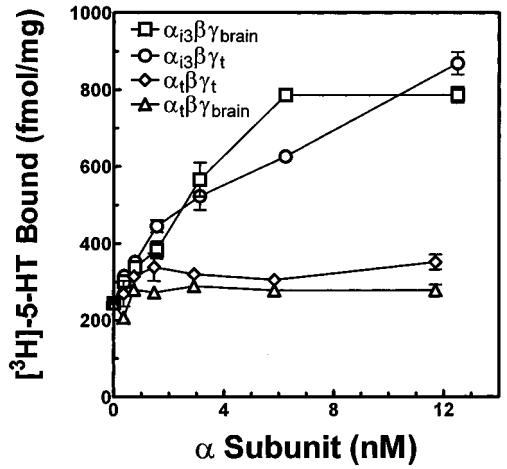


FIGURE 4: Concentration dependence of brain and retinal G protein subunits in the reconstitution of high-affinity agonist binding in membranes from Sf9 cells expressing 5-$HT_{1B}$ receptors. [$^3$H]-5-HT binding was determined in membranes (24 $\mu$g of protein) from Sf9 cells containing expressed 5-$HT_{1B}$ receptors reconstituted with the indicated combinations of G protein subunits purified from bovine retina ($\alpha_t$ and $\beta\gamma_t$), bovine brain ($\beta\gamma_{brain}$), or infected Sf9 cells ($\alpha_{i3}$). The $\beta\gamma$ subunits were present in excess of $\alpha$ subunits, and the highest concentration of $\alpha$ subunits is an estimated 17-fold molar excess over the expressed receptors. The concentration of [$^3$H]-5-HT used was 0.76 nM. Data represent specific binding expressed as the mean ± SD of triplicate determinations from a representative experiment. Identical results were obtained with the 5-$HT_{1A}$ receptor.

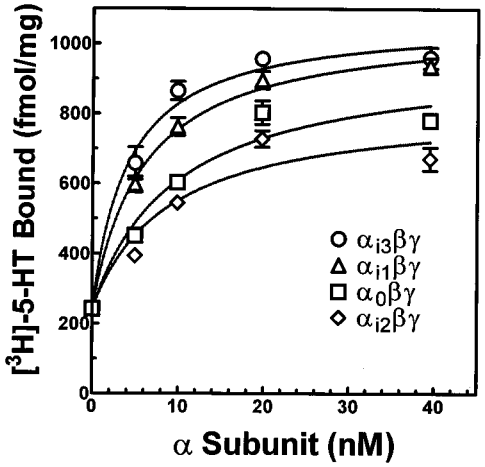


FIGURE 5: Concentration dependence of $\alpha_{i/o}$ subunits in the reconstitution of high-affinity agonist binding in membranes from Sf9 cells expressing 5-$HT_{1A}$ receptors. [$^3$H]-5-HT binding was determined in membranes (24 $\mu$g of protein) containing expressed 5-$HT_{1A}$ receptors reconstituted with the indicated concentrations of specific $\alpha_{i/o}$ subunits in combination with an excess of bovine brain $\beta\gamma$ subunits. The highest concentration of $\alpha$ subunits represents an estimated 20-fold molar excess over expressed receptors. The concentration of [$^3$H]-5-HT used was 0.35 nM. Data points represent specific binding expressed as the mean ± SD of triplicate determinations from a representative experiment. The solid lines indicate the best fit to the data of a single-site interaction between receptor and G protein governed by the law of mass action.

tors. Because the actual concentration of agonist varied slightly in separate experiments and because small variations in agonist concentration at the low concentrations used in these coupling experiments result in significant differences in the absolute levels of binding observed, it was necessary to normalize the data in order to make comparisons among separate experiments. This was accomplished by normalizing the maximal level of binding determined for each $\alpha$ subunit by least squares nonlinear regression with the greatest level of binding determined within that experiment. The normalized data are presented in Table 2. Two-way analysis of variance on normalized data from six separate experiments revealed that the differences in the levels of [$^3$H]-5-HT binding observed after reconstitution with specific G protein heterotrimers were highly significant ($p < 0.001$) but not dependent on which receptor (i.e., 5-$HT_{1A}$ or 5-$HT_{1B}$) was coupled. Thus the level of [$^3$H]-5-HT binding was greatest to 5-$HT_{1A}$ or 5-$HT_{1B}$ receptors coupled with $\alpha_{i3}$. However, when individual comparisons are made, only the differences between $\alpha_{i3}$ and $\alpha_{i2}$ or $\alpha_o$ reach statistical significance ($p < 0.05$). The concentrations of the $\alpha$ subunits required for the half-maximal increase in [$^3$H]-5-HT binding are presented in Table 3. While the differences among the various $\alpha$ subunits for the 5-$HT_{1B}$ receptor were not significant, the $EC_{50}$ value of $\alpha_{i3}$ with the 5-$HT_{1A}$ receptor was significantly lower than the values with either $\alpha_{i2}$ or $\alpha_o$ ($p < 0.05$). Overall, the difference between the 5-$HT_{1A}$ and 5-$HT_{1B}$

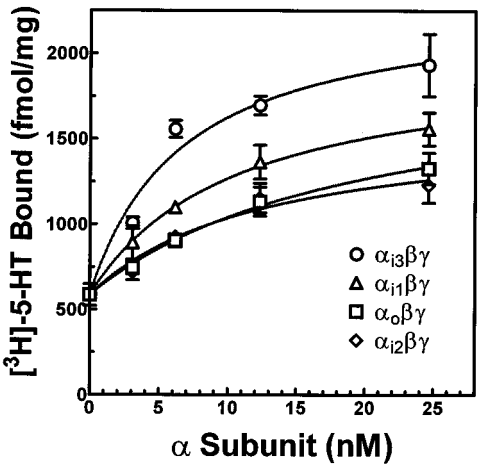


FIGURE 6: Concentration dependence of $\alpha_{i/o}$ subunits in the reconstitution of high-affinity agonist binding in membranes from Sf9 cells expressing 5-$HT_{1B}$ receptors. [$^3$H]-5-HT binding was determined in membranes (17 $\mu$g of protein) containing expressed 5-$HT_{1B}$ receptors reconstituted with the indicated concentrations of specific $\alpha_{i/o}$ subunits in combination with an excess of bovine brain $\beta\gamma$ subunits. The highest concentration of $\alpha$ subunits represents an estimated 25-fold molar excess over expressed receptors. The concentration of [$^3$H]-5-HT used was 0.6 nM. Data points represent specific binding expressed as the mean ± SD of triplicate determinations from a representative experiment. The solid lines indicate the best fit to the data of a single-site interaction between receptor and G protein governed by the law of mass action.

Table 2: Normalized Levels of Maximal [$^3$H]-5-HT Binding[a]

| G protein | 5-$HT_{1A}$ receptor | 5-$HT_{1B}$ receptor | combined receptors |
|---|---|---|---|
| $G_{i3}$ | 1.00 ± 0.000 (3) | 0.97 ± 0.029 (3) | 0.98 ± 0.015 (6) |
| $G_{i1}$ | 0.91 ± 0.039 (3) | 0.85 ± 0.075 (3) | 0.88 ± 0.040 (6) |
| $G_o$ | 0.85 ± 0.075 (3) | 0.74 ± 0.030 (3) | 0.80 ± 0.043 (6) |
| $G_{i2}$ | 0.68 ± 0.045 (3) | 0.67 ± 0.096 (3) | 0.67 ± 0.048 (6) |

[a] Membranes containing either 5-$HT_{1A}$ or 5-$HT_{1B}$ receptors were reconstituted with increasing amounts of the indicated G proteins as shown in Figures 4 and 5. The maximal level of binding achieved with each G protein was obtained by fitting the data to a model for a single-site interaction between receptor and G protein. For each experiment, maximal levels of binding obtained with each G protein were normalized by dividing by the greatest maximal level of binding obtained in that experiment. In 5 of 6 experiments the maximal level of binding was achieved with $G_{i3}$. Values represent the mean ± SEM, and the number of independent experiments is shown in parentheses.

Table 3: $EC_{50}$ Values for Reconstitution of High-Affinity Agonist Binding[a]

| G protein | 5-$HT_{1A}$ receptor | 5-$HT_{1B}$ receptor |
|---|---|---|
| $G_{i3}$ | 3.74 ± 0.409 nM (3) | 6.49 ± 0.179 nM (3) |
| $G_{i2}$ | 7.56 ± 0.971 nM (3) | 13.90 ± 2.993 nM (3) |
| $G_{i1}$ | 4.43 ± 0.759 nM (3) | 10.21 ± 2.645 nM (3) |
| $G_o$ | 9.77 ± 1.028 nM (3) | 17.51 ± 3.760 nM (3) |
| combined | 6.38 ± 0.814 nM (12) | 12.03 ± 1.704 nM (12)[b] |

[a] Membranes containing either 5-$HT_{1A}$ or 5-$HT_{1B}$ receptors were reconstituted with increasing amounts of the indicated G proteins as shown in Figures 4 and 5. The concentration of G protein producing the half-maximal level of binding ($EC_{50}$ value) was obtained for each G protein by fitting the data to a model for a single-site interaction between receptor and G protein. Values represent the mean ± SEM, and the number of independent experiments is shown in parentheses. The combined means for each receptor were compared with Student's unpaired *t*-test using Welch's correction for non-homogeneity of variances, and the difference is highly significant ($p < 0.01$). [b] $p < 0.05$.

receptor with respect to the concentration of α subunit required for a half-maximal increase in agonist binding was highly significant ($p < 0.01$). Taken together the data suggest that the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors interact with G proteins with different affinities while the G proteins that interact with these receptors are each capable of inducing slightly different affinity states in the receptors.

## DISCUSSION

In order to study potentially small differences in the interactions among highly similar components of G protein-mediated signal transduction pathways, a membrane-based reconstitution system has been developed by expression of 5-$HT_1$ receptor subtypes in Sf9 cells using recombinant baculoviruses. An advantage of the Sf9 cell is the absence of most mammalian heptahelical receptors that have been examined, which allows comparisons of individual subtypes of receptors in a common cellular environment. Although all four of the 5-$HT_1$ receptor subtypes expressed in this study are capable of agonist dependent inhibition of an endogenous adenyl cyclase activity (Ng et al., 1993; Parker et al., 1994) and are therefore presumably coupled to an endogenous $G_{i/o}$-like protein, only the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptor subtypes exhibited high- and low-affinity states for agonist in membranes prepared from infected Sf9 cells [Table 1 and Parker et al. (1994)]. Because only a small proportion of these receptors existed in the high-affinity, G protein-coupled state it was possible to examine the ability of known concentrations of highly purified preparations of G proteins of defined subunit composition to shift the low-affinity, uncoupled receptors to a high-affinity, G protein-coupled state. Receptor coupling in the Sf9 cell membranes required both α and $\beta\gamma$ subunits, which is consistent with previous determinations in other systems (Hekman et al., 1987; Florio & Sternweis, 1985; Fung, 1983) and the generally accepted model of G protein action (Gilman, 1987). The absence of any coupling activity by either α or $\beta\gamma$ subunits when added alone (Figure 1) suggests that the endogenous Sf9 G protein pools are not appreciably perturbed by the addition of exogenous G protein subunits. Therefore, the changes in high-affinity agonist binding are solely due to the addition of the exogenous G protein subunits. This is in contrast to a recent study by Manning and co-workers in which G protein−receptor coupling was examined by changes in high-affinity agonist binding in membranes from Sf9 cells co-expressing 5-$HT_{1A}$ receptors and various G protein subunits (Butkerait et al., 1995). While this co-infection approach revealed the expected selectivity across families of α subunits, and the greatest enhancement of high-affinity agonist binding required the co-expression of both α and $\beta\gamma$ subunits, significant increases in high-affinity agonist binding were observed when specific α or $\beta\gamma$ subunits were co-expressed alone with 5-$HT_{1A}$ receptors. Presumably this resulted from the increased expression of complementary endogenous Sf9 cell G protein subunits caused by the expression of the exogenous mammalian subunits as described for some other expression paradigms (Hermouet et al., 1993). Alternatively, there may have been a shuffling among expressed G protein subunits and the endogenous Sf9 cell G protein subunits. Combined with the difficulties of controlling both expression levels and the proportions of cells infected with each of four viruses from infection to infection, the co-infection approach is not likely to reveal small differences in the extent or stoichiometry of receptor coupling.

As evidenced by the inability of transducin heterotrimers to couple with expressed 5-$HT_1$ receptors, the reconstitution system in Sf9 cell membranes is capable of revealing selectivity in coupling. Discrimination among major families of α subunits by receptors has been reported in a variety of systems (Butkerait et al., 1995; Raymond et al., 1993; Bertin et al., 1992; Freissmuth et al., 1991; Parker et al., 1991; Cerione et al., 1985, 1986); however, such discrimination is certainly not a general property of heptahelical receptors [reviewed in Neer (1995)]. In view of the recent report that the human thyrotropin receptor is capable of functional interactions with each of the four major families of G protein α subunits extreme caution should be adopted in generalizing about the coupling selectivity of heptahelical receptors (Laugwitz et al., 1996). Several aspects of the ability of the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors to discriminate between G protein heterotrimers containing $\alpha_{i/o}$ or $\alpha_t$ subunits is of interest. First, rhodopsin, the photon receptor coupled to transducin in retina, does not distinguish between such heterotrimers (Cerione et al., 1986; Kanaho et al., 1984). Second, the discrimination is entirely at the level of the $\alpha_t$ subunit. Regardless of which $\beta\gamma$ preparation (bovine brain or retina) was used, $\alpha_t$ was not capable of reconstitution of high-affinity agonist binding, while both preparations of $\beta\gamma$ equally supported the ability of $\alpha_{i/o}$ to reconstitute high-affinity agonist binding. This is consistent with the reports that pertussis toxin-catalyzed ADP-ribosylation of α subunits is supported equivalently by preparations of $\beta\gamma$ from either brain or retina, but in clear contrast with the differences in these $\beta\gamma$ preparations that have been observed in their interactions with β-adrenoceptors, $\alpha_s$, and adenyl cyclase (Casey et al., 1989; Hekman et al., 1987; Cerione et al., 1987). More recently, several studies have demonstrated a lowered activity of recombinantly produced $\beta_1\gamma_1$, compared with other combinations of $\beta\gamma$, in interactions with effectors (Ueda et al., 1994; Iniguez-Lluhi et al., 1992) and in coupling adenosine $A_1$ (Figler et al., 1996) and 5-$HT_{1A}$ receptors (Butkerait et al., 1995). Although retinal $\beta\gamma$ has been assumed to contain exclusively $\beta_1\gamma_1$, this is certainly not the case as Fung and co-workers have clearly demonstrated the presence of $\beta_3\gamma_8$ in retinal $\beta\gamma$ preparations, although it was roughly an order of magnitude less abundant than $\beta_1\gamma_1$ (Ong et al., 1995; Lee et al., 1992). Thus the presence of $\beta\gamma$ combinations other than $\beta_1\gamma_1$ in the retinal preparation used

in this study is unlikely to account for the ability of the retinal $\beta\gamma$ to couple with 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors compared with the greatly reduced ability of recombinant $\beta_1\gamma_1$ to couple 5-$HT_{1A}$ (Butkerait et al., 1995) and adenosine $A_1$ receptors (Figler et al., 1996). Resolution of this issue will have to await direct comparison of the activities of these preparations.

Despite the increasing awareness that the efficacy and selectivity of G protein coupled signaling pathways are critically dependent on the cellular context of the components of such pathways, the ability to employ reconstitution paradigms using preparations containing defined molecular species allows comparisons among closely related components of these pathways in a single environment. Thus it is of interest to compare the properties of four closely related subtypes of human 5-$HT_1$ receptors expressed in Sf9 cells. All four receptors are known to mediate the inhibition of adenyl cyclase in both a wide variety of tissues where they occur naturally [reviewed in Boess and Martin (1994)] and following expression in Sf9 cells (Parker et al., 1994; Ng et al., 1993). Nonetheless, these four human 5-$HT_1$ receptor subtypes exhibited significantly different behavior following reconstitution with exogenous G proteins in membranes from Sf9 cells containing expressed receptors. Only the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors exhibited enhanced high-affinity agonist binding that was fully sensitive to guanine nucleotides (Figure 2).

Although the 5-$HT_{1D}$ receptor exhibited a 1.5-fold increase in high-affinity agonist binding after reconstitution with exogenous G proteins, agonist binding to these receptors was much less sensitive to GTP$\gamma$S than agonist binding to either the 5-$HT_{1A}$ or 5-$HT_{1B}$ receptors (Figure 2). A similar lack of sensitivity of binding to guanine nucleotides for 5-$HT_{1D}$ receptors expressed in Sf9 cells has been reported (Parker et al., 1994). This is in contrast to the report of high-affinity [$^3$H]-5-HT binding sensitive to guanine nucleotides following expression in CHO cells (Hamblin & Metcalf, 1991). Furthermore, the observed pharmacology and effector coupling of the human 5-$HT_{1D}$ and 5-$HT_{1B}$ receptors are highly similar to each other following stable expression in murine fibroblasts (Zgombick et al., 1993; Weinshank et al., 1992). Clearly the 5-$HT_{1D}$ receptors expressed in Sf9 cells can couple to G proteins as shown by their ability to inhibit the endogenous adenyl cyclase (Parker et al., 1994) and the enhancement of binding produced by reconstitution with exogenous G proteins (Figure 2), and yet this coupling in Sf9 cells is fundamentally different than that exhibited by 5-$HT_{1B}$ receptors in the same environment. Recently the existence of cell type specific factors capable of augmenting receptor−G protein coupling have been reported (Sato et al., 1995) and it may be that such a factor is required for appropriate coupling of 5-$HT_{1D}$ receptors but missing from Sf9 cells. It will thus be of interest to determine the basis for the different behavior of these highly similar receptors when expressed in Sf9 cells.

The 5-$HT_{1E}$ receptor showed no sensitivity of binding to guanine nucleotides, consistent with an earlier report following expression in Sf9 cells (Parker et al., 1994). A similar lack of guanine nucleotide sensitivity of binding was reported for the 5-$HT_{1E}$ receptor following stable or transient expression in HEK 293 cells, despite a functional coupling to adenyl cyclase (McAllister et al., 1992). This is in contrast to the guanine nucleotide sensitive high-affinity binding reported for 5-$HT_{1E}$ receptors in human brain after pharmacological blockade of non-5-$HT_{1E}$ sites (Leonhardt et al., 1989). Guanine nucleotide sensitivity was also reported for human 5-$HT_{1E}$ receptors stably expressed in murine fibroblasts, although the maximal inhibition was only 21% (Zgombick et al., 1992). Clearly this aspect of receptor coupling is highly dependent on the cellular environment of the receptor. It is also of interest that the addition of exogenous G proteins had no effect on [$^3$H]-5-HT binding to the expressed 5-$HT_{1E}$ receptors (Figure 2). Since the 5-$HT_{1E}$ receptors were expressed at similar levels as the other 5-$HT_1$ subtypes, it seems unlikely that they were all coupled with endogenous G proteins. However, considering the report that the 5-$HT_{1E}$ receptor is capable of both inhibition and stimulation of adenyl cyclase when transfected into African green monkey kidney cells (Adham et al., 1994), it may be that this receptor functionally interacts with multiple families of G proteins in Sf9 cells and as a result is completely coupled. Further study will be required to explain the seemingly atypical behavior of this receptor in Sf9 cells, but it is nonetheless clear that the 5-$HT_{1E}$ receptor exhibits different coupling behavior that the other 5-$HT_1$ receptor subtypes in an identical environment.

Following the addition of exogenous G protein heterotrimers, both the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors exhibited enhanced high-affinity agonist binding that was sensitive to guanine nucleotides (Figure 2) and it was therefore possible to compare the abilities of known amounts of distinct $\alpha$ subunits to functionally couple these 5-$HT_1$ receptor subtypes in an identical membrane environment. Using a single, low (0.4−1.2 nM) concentration of agonist, the reconstitution of high-affinity binding by increasing concentrations of G proteins was a saturable process that produced different levels of binding depending on which specific $\alpha_{i/o}$ subunit was used (Figures 4 and 5). Notably, an identical rank order was observed for the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors, and while the largest difference observed among $\alpha_{i/o}$ subunits was a modest 1.5-fold, the differences between $\alpha_{i3}$ and $\alpha_o$ or $\alpha_{i2}$ are statistically significant (Table 2). These results are consistent with the conclusion that these 5-$HT_1$ receptor subtypes are capable of multiple affinity states for agonist depending on the nature of the G protein with which they are coupled. Even larger differences in apparent agonist affinities have been observed in reconstitution experiments using a series of chimeric $\alpha_t/\alpha_{i1}$ subunits.[2] While the molecular mechanism responsible for these observed differences in receptor affinity have not been determined, the data are consistent with distinct $\alpha$ subunits having either different abilities to "traffic" the receptor between high- and low-affinity states, or the ability to "induce" different affinity states in the receptor. While the 5-$HT_{1B}$ receptor does not distinguish among the $\alpha_{i/o}$ subunits with respect to the $EC_{50}$ values for the reconstitution of high-affinity binding in a statistically significant fashion, the $EC_{50}$ values for the 5-$HT_{1A}$ receptor were significantly lower with $\alpha_{i3}$ than with $\alpha_{i2}$ or $\alpha_o$. Overall, however, the $EC_{50}$ values for the 5-$HT_{1A}$ receptor are lower than those for the 5-$HT_{1B}$ receptor, and the difference between the receptors is highly significant (Table 3). Thus it appears that these closely related 5-$HT_1$ receptor subtypes distinguish themselves by the affinity with which they interact with G proteins. These results are

[2] H. Hamm and S. Graber, submitted.

reminiscent of earlier studies which demonstrated differences in both physical and functional coupling to $\alpha_s$ by $\beta_1$- and $\beta_2$-adrenergic receptor subtypes (Levy et al., 1993; Green et al., 1992). Both studies examined receptor behavior in stably transformed cell lines, and receptor coupling was therefore dependent on the endogenous complement of G proteins which was not precisely defined. Nonetheless, the high-affinity agonist binding state of the $\beta_2$-adrenergic receptor was significantly greater than that of the $\beta_1$-adrenergic receptor (Green et al., 1992), and the $\beta_2$-adrenergic receptor was significantly more efficient in stimulating adenyl cyclase than was the $\beta_1$-adrenergic receptor (Levy et al., 1993; Green et al., 1992). Moreover, one of these studies co-expressed the receptors in an identical biochemical environment, thus demonstrating that the differences are inherent properties of the receptors (Levy et al., 1993).

Although this is the first study to examine the selectivity of G protein coupling for 5-$HT_{1B}$ receptors, several earlier studies have examined the coupling of 5-$HT_{1A}$ receptors with specific G protein heterotrimers. While the approach of co-expressing various G proteins and 5-$HT_{1A}$ receptors in Sf9 cells with multiple recombinant baculoviruses used by Butkerait et al. (1995) was not capable of precisely controlling the relative amounts of expressed proteins (or, for that matter, of insuring that every Sf9 cell was infected with each virus) it is notable that they reported an identical, though not statistically significant, rank order in the levels of high affinity agonist binding achieved with the $\alpha_{i/o}$ subunits to that shown in Table 2. Their finding that $\alpha_z$ is capable of coupling with 5-$HT_{1A}$ receptors (Butkerait et al., 1995) is intriguing in view of the fact that $\alpha_z$ is less related to the other members of the $\alpha_{i/o}$ family than is $\alpha_t$ (Strathmann & Simon, 1990), which is not capable of interacting with either the 5-$HT_{1A}$ or the 5-$HT_{1B}$ receptors (Figure 4). Using reconstitution of 5-$HT_{1A}$ receptors expressed in *Escherichia coli*, (Bertin et al., 1992) observed a strong preference for coupling with $\alpha_{i3}$ and essentially no interaction with $\alpha_o$ in contrast with the present study and others in Sf9 cells (Butkerait et al., 1995; Mulheron et al., 1994). Presumably these differences are related to either variations in the membrane environments or post-translational modifications of the expressed proteins. Using an immunological approach, Raymond et al. reported agonist preferentially induced coupling with an endogenous $\alpha_{i3}$ in both HeLa and CHO-K1 cells heterologously expressing 5-$HT_{1A}$ receptors (Raymond et al., 1993). Taken together, these studies suggest that 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors couple most efficiently with $\alpha_{i3}$ subunits.

In summary, expression of four subtypes of 5-$HT_1$ receptors in Sf9 cells has provided a common cellular environment in which receptor−G protein coupling has been characterized. Functional coupling of 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors can be achieved by reconstitution of membranes prepared from receptor expressing cells with purified G protein heterotrimers of defined subunit composition. Little or no mixing of the exogenous G protein subunits with the endogenous G proteins is observed, and this reconstitution system is capable of revealing selectivity among different $\alpha$ subunits. The ability to precisely control the concentrations and stoichiometries of components of signal transduction pathways is a major advantage of reconstitution paradigms. Examination of four closely related subtypes of 5-$HT_1$ receptors has revealed differences in their coupling with specific G proteins in an identical membrane environment. On the basis of the ability of these receptors to mediate inhibition of adenyl cyclase in Sf9 cells and numerous other tissues, they would be expected to functionally interact with members of the $\alpha_{i/o}$ family of G proteins. In this system, only the 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors interacted with exogenous $\alpha_{i/o}$ subunits in a manner that was completely reversible by GTP$\gamma$S as predicted by the widely accepted ternary complex model of G protein−receptor coupling. While all members of the $\alpha_{i/o}$ family interacted with both of these receptors, the highest affinity for agonist was achieved with $\alpha_{i3}$ subunits (significantly greater than with either $\alpha_{i2}$ or $\alpha_o$). The 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors are distinguished from one another by the affinity of their interactions with G protein heterotrimers. Overall, the 5-$HT_{1A}$ receptor interacted with the G protein heterotrimers with a significantly lower $EC_{50}$ than did the 5-$HT_{1B}$ receptor (6.4 vs 12.0 nM). Thus the four closely related 5-$HT_1$ receptors exhibit significant differences in the manner in which they are coupled with G proteins. It will be of interest to determine if similar differences exist among other heptahelical receptor families composed of multiple closely related subtypes. Additionally, further development of the Sf9 cell membrane system may allow reconstitution of complete receptor−G protein effector pathways and reveal a functional significance to the many closely related subtypes that exist at each step of these pathways.

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