A Conservative, Single-Amino Acid Substitution in the Second Cytoplasmic Domain of the Human Serotonin$_{2C}$ Receptor Alters Both Ligand-Dependent and -Independent Receptor Signaling

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ABSTRACT

The post-transcriptional process of mRNA editing changes up to three amino acids in the second intracellular domain (i2) of the serotonin$_{2C}$ (5-HT$_{2C}$) receptor and alters some signaling characteristics of the receptor. Here, we report that the substitution of valine for isoleucine (I156V; 5-HT$_{2C}$-VNI), which occurs naturally as a result of mRNA editing, alters functional selectivity at the 5-HT$_{2C}$-VNI receptor differed from both ligand-dependent and -independent signaling. Agonist functional selectivity at the 5-HT$_{2C}$-VNI receptor differed from that for the fully edited receptors, suggesting that the capacity of the agonist-occupied receptor to couple to $G_{q/11}$ proteins was not different. Ligand-independent (i.e., constitutive) receptor activity toward PLC for the 5-HT$_{2C}$-VNI receptor was markedly reduced to a level similar to that for the fully edited 5-HT$_{2C}$-VSV isofrom. However, there was no difference in the thermal stability of the edited receptors, suggesting that mRNA editing does not alter the capacity of receptors to adopt active conformations. These results indicate that a conservative change in one amino acid (156V) located in i2 of the 5-HT$_{2C}$ receptor produces profound changes in receptor function that differ depending upon whether the receptor is unoccupied or occupied by agonist.

The serotonin$_{2C}$ (5-HT$_{2C}$) receptor is a member of the 5-HT2 seven transmembrane-spanning (7-TMS) receptor family, also known as G protein-coupled receptors. The 5-HT$_{2C}$ receptor is widely expressed in numerous brain regions and plays significant roles in many physiological functions and behaviors, such as sleep, affective state, feeding behavior, and temperature regulation. In addition, the 5-HT$_{2C}$ receptor is a focus for the development of drugs to treat schizophrenia, depression, Parkinson’s disease, and obesity and is also a likely target for hallucinogenic drugs of abuse (Di Giovanni et al., 2006).

It is noteworthy that the 5-HT$_{2C}$ receptor is the only 7-TMS receptor whose mRNA undergoes adenosine-inosine editing events that change the coding for amino acids located within the putative second intracellular domain (i2) of the receptor. In human brain, the nonedited receptor contains the amino acids isoleucine, asparagine, and isoleucine (INI) at positions 156, 158, and 160, respectively, whereas the principle fully edited isoforms express valine, serine, and valine (VSV) or valine, glycine, and valine (VGV). Partially edited receptor isoforms also exist where alteration of one or two amino acids within i2 occurs. Several groups have reported differences in function of some edited receptor isoforms (Burns et al., 1997; Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Berg et al., 2001), and it has been suggested that alterations in RNA editing efficiency may be involved in the etiology of schizophrenia (Sodhi et al., 2001) and affective disorders (Niswender et al., 2001; Gurevich et al., 2002b).

ABBREVIATIONS: 5-HT, serotonin; 7-TMS, 7-transmembrane spanning; i2, second intracellular domain; PLC, phospholipase C; PL2A2, phospholipase A2; DOI, 2,5-dimethoxy-4-iodophenylisopropylamine; bufotenin, 3-(2-dimethylaminoethyl)-1H-indol-5-ol; quipazine maleate, 2-(1-piperaziny1)quinoline dimaleate; TFMPP, 3-trifluoromethylphenyl-piperazine; Ro 60-0175, (S)-(2-(6-chloro-5-fluorindol-1-yl)-1-methyllethylamine; WAY-161503, (4R)-8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinolaxin-5(6H)-one; CHO, Chinese hamster ovary; HEK, human embryonic kidney; IP, inositol phosphate; AA, arachidonic acid; LSD, lysergic acid diethylamide.
The i2 of 7-TMS receptors plays an important role in receptor function. The highly conserved E/DRY motif is located in i2 at the cytosolic end of transmembrane helix 3 and has been linked strongly to mechanisms of receptor activation and G protein coupling (see Flanagan, 2005). Many studies have provided evidence that the E/DRY motif and other residues within i2 are involved with direct coupling to G proteins (Moro et al., 1993; Burstein et al., 1998; Sugimoto et al., 2004) or other signaling molecules (Laghmani et al., 2005). In addition, i2 may participate in desensitization mechanisms such as β-arrestin binding, receptor internalization, and down-regulation (Marion et al., 2006). Evidence also suggests that residues within i2 regulate the capacity of receptors to isomerize, thereby controlling the formation of active receptor configurations and constitutive receptor activity (Burstein et al., 1998; Raasch et al., 1999; Alewijnse et al., 2000; Scheer et al., 2000; Flanagan, 2005).

Editing of the 5-HT2C receptor mRNA leads to changes in amino acids starting just two residues downstream from the E/DRY motif. Pharmacological characterization of the predominant fully edited isoforms (5-HT2C-VGV and 5-HT2C-VSV) has revealed decreases in agonist affinity (Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Quirk et al., 2001), potency (Burns et al., 1997; Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Wang et al., 2000; McGrew et al., 2004), constitutive receptor activity (Herrick-Davis et al., 1999; Niswender et al., 1999), and receptor-arrestin binding and internalization (Marion et al., 2004) compared with the nonedited receptor (5-HT2C-INI). It is possible that the mechanism for the reduced function of the fully edited receptors involves reduced capacity to isomerize to an active conformation(s) and/or reduced ability of active receptor conformations to couple to G proteins (Herrick-Davis et al., 1999; Niswender et al., 1999). In addition, agonists acting at the fully edited receptor isoforms do not display preferential activity toward the phospholipase C (PLC) and phospholipase A2 (PLA2) signal transduction pathways (functional selectivity) as they do when acting at the nonedited receptor (Berg et al., 1998, 2001). In this study, we examined the effect of the conservative substitution of valine for methionine within i2 at the cytosolic end of transmembrane helix 3 and has some affinity for the 5-HT1B receptor, which is naturally expressed in CHO-K1 cells (Berg et al., 1998), all functional selectivity experiments were done in cells previously treated with pertussis toxin (24 h, 50 ng/ml) during the serum-free culture period.

For transient receptor expression, HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% CO2 at 37°C. Cells were seeded into 12-well plates at 60 to 80% confluence and transfected with various amounts of cDNA for 5-HT2C-INI, 5-HT2C-VNI, 5-HT2C-VSV, or 5-HT2C-VGV and Lipofectamine 2000 using the manufacturer's protocol. pcDNA3 (empty vector) was used to keep total cDNA constant at 4 μg/well. Forty-eight hours after transfection, cells were plated in serum-free medium, and experiments were done 12 to 16 h later.

**Radioligand Binding.** Saturation binding assays using [3H]-mesulergine in cell membrane preparations (50 μg protein/tube) were done using 15 concentrations of radioligand in duplicate over a 3-log unit range as described previously (Berg et al., 2001). Incubations were carried out for 1 h at 37°C followed by rapid filtration through 0.3% polyethyleneimine-coated filters using a Brandel cell harvester. Nonspecific binding was determined in the presence of 1 μM mianserin.

Competition binding assays were done with cell membrane preparations (50 μg protein/tube) using half-log unit concentrations of the competing ligand over a range from 1 × 10^-10 to 1 × 10^-3 M in the presence of [3H]mesulergine (0.5 nM). Incubations were carried out for 1 h at 37°C followed by rapid filtration through 0.3% polyethyleneimine-coated filters using a Brandel cell harvester.

Thermostability binding experiments were done following the procedure of Claeyse et al. (2001). Membranes prepared from HEK cells expressing 5-HT2C receptor isoforms were preincubated at 55°C for 0 to 90 min in the presence of protease inhibitors (protease inhibitor cocktail; Sigma-Aldrich). After preincubation, the membranes were incubated with [3H]mesulergine (10 nM) for 1 h at 37°C followed by rapid filtration through 0.3% polyethyleneimine-coated filters using a Brandel cell harvester.

**Inositol Phosphate Accumulation and Arachidonic Acid Release Measurements.** CHO cells were labeled with 1 μCi/ml myo-[3H]inositol in serum-free medium for 24 h and with 0.1 μCi/ml [3H]arachidonic acid for 4 h at 37°C before experiments. HEK cells were labeled with 1 μCi/ml myo-[3H]inositol in serum free medium for 12 h. For experiments in which both PLC-inositol phosphate (IP) and PLA2-arachidonic acid (AA) pathways were measured, total [3H]IP accumulation and [3H]AA release were measured from the same multwell (simultaneously) after 10 min of agonist exposure as described previously (Berg et al., 1998, 2001). For measurement of constitutive receptor activity, basal IP accumulation was measured over a 30-min period.

**Assessment of Functional Selectivity.** To assess the capacity of agonists to differentially activate PLC-IP versus the PLA2-AA signaling pathways, relative efficacies of the test agonists were calculated for each response, using the ratio of the response to maximal concentrations of the test agonist to that of the reference agonist 5-HT, as we have done before (Berg et al., 1998, 2001). Measurement of relative efficacy removes the system dependence (e.g., receptor density, efficiency of signal transduction) of efficacy and permits assessment of the capacity of ligands to alter receptor activation (i.e., intrinsic efficacy). A difference in agonist relative efficacy between

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**Materials and Methods**

**Materials.** The myo-[3H]inositol and [3H]arachidonic acid were purchased from New England Nuclear (Boston, MA). 5-HT HCl, 2,5-dimethoxy-4-iodophenylisopropylamine (DOI), butifenprox, quipazine maleate, and 3-trifluoromethylphenyl-piperazine (TFMPP) were purchased from Sigma RBI (Natick, MA). Ro 60-0175 and WAY-161503 were synthesized at Wyeth-Ayerst Research (Princeton, NJ). All other drugs and chemicals (reagent grade) were purchased from Sigma A-Z (St. Louis, MO). Fetal bovine serum was from Gemini Bioproducts (Calabasas, CA). All other tissue culture reagents were purchased from Invitrogen (Grand Island, NY).

**Cell Culture.** Cells stably expressing the nonedited, human 5-HT2C-INI receptor (250 fmol/mg protein) or the edited, human 5-HT2C-VNI receptor (50 fmol/mg protein) were derived from transfection of CHO-K1 cells as described previously (Berg et al., 1998; Zhang et al., 2006). Cells were maintained in α-minimal essential medium supplemented with 5% fetal bovine serum and seeded into 12- or 24-well tissue culture vessels for functional studies or 15-cm dishes for radioligand binding studies at a density of 4 × 10^5 cells/cm². After a 24-h plating period, cells were washed with Hanks' balanced salt solution and placed into Dulbecco's modified Eagle's medium/F-12 (1:1) with 5 μg/ml insulin, 5 μg/ml transferrin, 30 nM selenium, 20 nM progesterone, and 100 μM putrescine (serum-free media) and cultured for an additional 24 h before experimentation. Because some of the agonists used in this study (e.g., TFMPP) have some affinity for the 5-HT1A receptor, which is naturally expressed in CHO-K1 cells (Berg et al., 1998), all functional selectivity experiments were done in cells previously treated with pertussis toxin (24 h, 50 ng/ml) during the serum-free culture period.

**Assessment of Functional Selectivity.** To assess the capacity of agonists to differentially activate PLC-IP versus the PLA2-AA signaling pathways, relative efficacies of the test agonists were calculated for each response, using the ratio of the response to maximal concentrations of the test agonist to that of the reference agonist 5-HT, as we have done before (Berg et al., 1998, 2001). Measurement of relative efficacy removes the system dependence (e.g., receptor density, efficiency of signal transduction) of efficacy and permits assessment of the capacity of ligands to alter receptor activation (i.e., intrinsic efficacy). A difference in agonist relative efficacy between
responses is evidence for functional agonist selectivity (Berg et al., 1998; Berg and Clarke, 2006).

**Data Analysis.** For saturation binding experiments, data were fit with nonlinear regression to eq. 1:

\[
B = \frac{B_{\text{max}}}{1 + \left(\frac{K_d}{D}\right)^m} 
\]

where \(B\) is the measured amount of radioligand bound (femtomoles per milligram of protein) in the presence of various concentrations of radioligand \(D\), \(B_{\text{max}}\) is the maximal amount bound, \(K_d\) is the concentration of \(D\) that produces half-maximal binding, \(n\) is the slope factor, and \(m\) is the slope of the linear regression line for nonspecific binding.

IC\(_{50}\) values were derived from nonlinear regression analysis of competition binding data using the program Prism 4.0 for Macintosh (GraphPad Software, Inc., San Diego, CA). Data were fit with nonlinear regression analysis to one- and two-site models, and the best fit was determined with an \(F\) test by Prism software. The competition curves for all of the test ligands used were best fit with a one-site model.

\(K_i\) values were calculated from IC\(_{50}\) values using the transformation of Cheng and Prusoff (1973).

Concentration response data were fit with nonlinear regression to eq. 2:

\[
R = \frac{R_{\text{max}}}{1 + \left(\frac{\text{EC}_{50}}{A}\right)^n} 
\]

where \(R\) is the measured response at a given agonist concentration \(A\), \(R_{\text{max}}\) is the maximal response, \(\text{EC}_{50}\) is the concentration of agonist that produces half-maximal response, and \(n\) is the slope index.

Loss of binding due to thermal instability was measured by fitting the data to eq. 3:

\[
B = B_0 - B_{\text{max}} e^{-k_{\text{obs}} \cdot \text{time}} 
\]

where \(B\) is the measured binding at a given time point, \(B_0\) is the binding at time 0, \(B_{\text{max}}\) is the maximal loss of binding, and \(k_{\text{obs}}\) is the observed rate constant for binding loss.

Receptor-effector coupling efficiency was assessed by measuring the slope of the regression line for receptor density-IP response curves across a wide range of receptor densities (Fathy et al., 1999). A runs test for each of the regression lines was performed with Prism (GraphPad Software, Inc.) to verify linearity.

Where appropriate, two-way analysis of variance followed by Dunnett's post hoc or Student's \(t\) test (paired) were used for statistical comparisons using Prism (GraphPad Software, Inc.).

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>5-HT(_{\text{SC-INI}})</th>
<th>5-HT(_{\text{SC-VNI}})</th>
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</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>7.32 ± 0.16</td>
<td>7.23 ± 0.17</td>
</tr>
<tr>
<td>Bufotenin</td>
<td>7.40 ± 0.07</td>
<td>7.42 ± 0.12</td>
</tr>
<tr>
<td>DOI</td>
<td>7.84 ± 0.05</td>
<td>7.75 ± 0.14</td>
</tr>
<tr>
<td>LSD</td>
<td>7.98 ± 0.07</td>
<td>8.14 ± 0.04</td>
</tr>
<tr>
<td>Quipazine</td>
<td>8.33 ± 0.06</td>
<td>6.28 ± 0.13</td>
</tr>
<tr>
<td>Ro 60-0175</td>
<td>7.42 ± 0.07</td>
<td>7.22 ± 0.13</td>
</tr>
<tr>
<td>TFMPPP</td>
<td>7.19 ± 0.08</td>
<td>7.06 ± 0.14</td>
</tr>
<tr>
<td>WAY-161503</td>
<td>7.78 ± 0.07</td>
<td>7.60 ± 0.11</td>
</tr>
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were repeated at least three times. *p* Values < 0.05 were considered statistically significant.

**Results**

**Effect of I156V Substitution on Agonist-Dependent Signaling.** Figure 1 shows the effect of 5-HT on activation of the PLC and PLA2 signaling cascades in CHO cells stably expressing the 5-HT\(_{2C-INI}\) (nonedited) or the 5-HT\(_{2C-VNI}\) receptor. The maximal responses to 5-HT for AA release (Fig. 1A) and IP accumulation (Fig. 1B) in 5-HT\(_{2C-VNI}\) cells were approximately 70% less than those in 5-HT\(_{2C-INI}\) cells. The potency of 5-HT did not differ between the 5-HT\(_{2C-INI}\) and the 5-HT\(_{2C-VNI}\) isoforms (Fig. 1, C and D), which is consistent with the similar affinity of 5-HT for these receptor isoforms (Table 1). To determine whether the lower maximal responses to 5-HT in the cells expressing the 5-HT\(_{2C-VNI}\) receptor versus the 5-HT\(_{2C-INI}\) receptor were due to lower receptor expression (50 versus 250 fmol/mg protein for 5-HT\(_{2C-VNI}\) versus 5-HT\(_{2C-INI}\)) or to reduced receptor-effector coupling efficiency, we examined the PLC-IP response to maximal concentrations of the full agonist, 5-HT, or the partial agonists, LSD or DOI, applied to HEK-293 cells transiently expressing 5-HT\(_{2C}\) receptor isoforms over a range of receptor densities. As shown in Fig. 2, when occupied by agonist, coupling efficiency of the 5-HT\(_{2C-VNI}\) receptor to the PLC-IP pathway was not less than that of the 5-HT\(_{2C-INI}\) receptor. The slope of the regression line between receptor density and agonist-stimulated IP accumulation was 14,712 ± 3205 versus 11,599 ± 1583 for 5-HT (*p* > 0.05) and 10,362 ± 1712 versus 4216 ± 1492 for LSD (*p* < 0.05) and 12,337 ± 935 versus 15,948 ± 935 (*p* > 0.05) for DOI for the 5-HT\(_{2C-VNI}\) versus 5-HT\(_{2C-INI}\) isoforms, respectively. Likewise, with the exception of LSD, which has been shown not to activate the 5-HT\(_{2C-VGV}\) isoform (Backstrom et al., 1999; Fitzgerald et al., 1999; Berg et al., 2001), the slopes of the regression line between receptor density and agonist-stimulated IP accumulation for two fully edited receptor isoforms, 5-HT\(_{2C-VSV}\) and 5-HT\(_{2C-VGV}\), were also not less than that of the 5-HT\(_{2C-INI}\) isoform.

We have previously shown that ligands traffic the 5-HT\(_{2C-INI}\) receptor stimulus differentially to PLA2 and PLC signaling pathways (Berg et al., 1998; Moya et al., 2007) and that this functional selectivity is lost at two fully RNA-edited isoforms, 5-HT\(_{2C-VSV}\) and 5-HT\(_{2C-VGV}\) (Berg et al., 2001). Figure 3 shows concentration-response curves for two agonists, Ro 60-0175 and WAY-161503, for AA release and IP accumulation obtained from cells expressing the nonedited 5-HT\(_{2C-INI}\) and the 5-HT\(_{2C-VNI}\) receptor isoforms. The data are expressed as the percentage of the maximal response to 5-HT, for which full concentration-response curves were run in each experiment. When data are expressed in this manner, the plateaus of the concentration-response curves reflect the relative efficacies of the ligands with respect to 5-HT. At the 5-HT\(_{2C-INI}\) receptor, both compounds were strong agonists, relative to 5-HT, containing \(\text{myo-}[^{3}\text{H}]\text{inositol for 12 h. After loading, cells were washed, incubated with 20 mM LiCl with or without 5-HT (100 \mu M; A), LSD (1 \mu M; B), or DOI (10 \mu M; C), and the amount of IP accumulated for 30 min was measured. Receptor density was determined from radioligand binding using a saturating concentration of \([^{3}\text{H}]\text{mesulergine (10 nM). Data shown are from three independent experiments, with each point performed in triplicate.\text{}}\right)

![Fig. 2. Coupling efficiency of agonist-occupied 5-HT\(_{2C}\) receptor isoforms to the PLC-IP pathway. HEK-293 cells were transfected with various amounts 5-HT\(_{2C-INI}\), 5-HT\(_{2C-VNI}\), 5-HT\(_{2C-VSV}\) or 5-HT\(_{2C-VGV}\) receptor cDNA. Forty-eight hours after transfection, cells were placed in serum-free medium containing \(\text{myo-}[^{3}\text{H}]\text{inositol for 12 h. After loading, cells were washed, incubated with 20 mM LiCl with or without 5-HT (100 \mu M; A), LSD (1 \mu M; B), or DOI (10 \mu M; C), and the amount of IP accumulated for 30 min was measured. Receptor density was determined from radioligand binding using a saturating concentration of \([^{3}\text{H}]\text{mesulergine (10 nM). Data shown are from three independent experiments, with each point performed in triplicate.}\)
for each response, and there was no difference in the relative efficiencies of the ligands between responses (i.e., no functional selectivity). For the 5-HT2C-VNI isoform, both ligands were also strong agonists for IP accumulation; however, relative efficacy was markedly less for the AA response such that both ligands were functionally selective for the PLC-IP pathway.

Figure 4 shows the relative efficacies of a series of drugs for the PLC-IP and PLA2-AA responses obtained in 5-HT2C-INI versus 5-HT2C-VNI-expressing cells. At the 5-HT2C-INI Receptor, bufotenin, DOI and LSD have greater relative efficacy for AA release than for IP accumulation, whereas quipazine and TFMPP preferentially activate the PLC-IP response. For the 5-HT2C-VNI isoform, although quipazine and TFMPP still preferentially activated the PLC-IP response, the relative efficacies of bufotenin, DOI, and LSD did not differ for either response. It is interesting to note that Ro 60-0175 and WAY 161503, which were response neutral when tested at the 5-HT2C-INI receptor, preferentially activated the PLC response in the 5-HT2C-VNI cells. Closer examination of the differences in pathway-selective relative efficacy between the 5-HT2C-INI and the 5-HT2C-VNI cells revealed that, with the exception of LSD, relative efficacy for the PLA2 response was reduced for those agonists that preferentially activated the PLA2-AA response in 5-HT2C-INI cells, whereas relative efficacy for the PLA2 response was not changed for those drugs that preferred the PLC response (Table 2). The relative efficacies of the Ro 60-0175 and WAY 161503 compounds for PLC did not differ between the receptor isoforms, but for the PLA2 response, relative efficacy was reduced compared with the 5-HT2C-INI isoform.

Effect of I156V Substitution on Ligand-Independent Signaling. Figure 5 shows that the receptor-effector coupling efficiency for the unoccupied 5-HT2C-VNI isoform to the PLC-IP pathway is approximately 2-fold lower than that for the nonedited 5-HT2C-INI Receptor. The slope of the regression line between receptor density and basal IP accumulation, a quantitative measure of constitutive receptor activity, was 7185 ± 1412 for the 5-HT2C-INI receptor and was
14,056 ± 1650 for the 5-HT\textsubscript{2C-INI} isoform (p < 0.05). Likewise, the slopes for the fully edited receptor isoforms, 5-HT\textsubscript{2C-VSV} (4488 ± 774) and 5-HT\textsubscript{2C-VGV} (238 ± 1573), were also lower than that for the nonedited receptor (p < 0.05).

**Effect of I156V Substitution on Receptor Thermal Stability.** It has been suggested that structural instability may be a property of constitutively active receptors as a result of a reduced level of stabilizing intramolecular constraints (Gether et al., 1997; Samama et al., 1997). At elevated temperatures (45–55°C), the active receptor denatures more readily than the inactive receptor, which can be measured by the kinetics of the loss of ligand binding (Gether et al., 1997; Alewijnse et al., 2000; Claeyesen et al., 2001). Figure 6 shows a comparison of thermal stability of the nonedited 5-HT\textsubscript{2C-INI} receptor with the 5-HT\textsubscript{2C-VNI} and fully edited receptors, 5-HT\textsubscript{2C-VSV} and 5-HT\textsubscript{2C-VGV}. Preincubation of the receptors at 55°C produced a rapid loss of \[^3H\]mesulergine binding for all receptor isoforms. There was no difference in either the rate or magnitude of the thermostability between the receptor isoforms. There was no loss of binding when membranes were preincubated at 4 or at 55°C in the presence of mesulergine.

### Discussion

As a result of mRNA editing, the coding sequence of the 5-HT\textsubscript{2C} receptor can be post-translationally modified such that up to three amino acids located in the i2 domain can be altered. The results of this study indicate that a single, conservative change in one amino acid (I156V) located in i2 of the 5-HT\textsubscript{2C} receptor produces profound changes in receptor function that differ depending upon whether the receptor is unoccupied or occupied by agonist.

Many studies have shown that, in addition to selectivity for receptor subtypes, agonists have selectivity for different signaling pathways coupled to a single receptor subtype, a process known as “functional selectivity” (Berg and Clarke, 2006; Urban et al., 2007). Agonists at the 5-HT\textsubscript{2C-INI} isoform display functional selectivity for PLC versus PLA\textsubscript{2} signaling pathways (Berg et al., 1998, 2001; Moya et al., 2007); however, this response selectivity is absent for two fully edited isoforms, 5-HT\textsubscript{2C-VSV} and 5-HT\textsubscript{2C-VGV} (Berg et al., 2001). In contrast, a single amino acid change in i2 of the 5-HT\textsubscript{2C-VNI} receptor alters, but does not abolish, functional selectivity. Agonists with greater relative efficacy for PLC versus PLA\textsubscript{2} at the nonedited, 5-HT\textsubscript{2C-INI} lost preferential efficacy toward PLA\textsubscript{2} in cells expressing the 5-HT\textsubscript{2C-VNI} receptor (agonist relative efficacies for PLC and PLA\textsubscript{2} were not different). However, agonists with preferential activity toward PLC still retained their PLC signaling preference. Two drugs that were nonselective for PLC versus PLA\textsubscript{2} at the 5-HT\textsubscript{2C-INI} receptor became selective at the 5-HT\textsubscript{2C-VNI} isoform for the PLC response due to reduced relative efficacy toward the PLA\textsubscript{2}. Thus, in general, the overall effect of the I156V substitution of the 5-HT\textsubscript{2C-VNI} receptor seems to be a loss of functional selectivity for the PLA\textsubscript{2} pathway. Taken together, these data suggest that the i2 domain of the 5-HT\textsubscript{2C} receptor plays a role in providing agonist-specific information to the signal transduction machinery of the cell, perhaps with an emphasis on the PLA\textsubscript{2} signaling cascade.

Changes in the selectivity of receptor coupling to cellular signaling pathways as a result of RNA editing may provide a novel approach for regulation of neurotransmission via the 5-HT\textsubscript{2C} Receptor. The efficiency of RNA editing and thus the expression of RNA-edited receptor isoforms differs in different brain regions (Burns et al., 1997; Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Wang et al., 2000), suggesting that cells differ in their capacity to process 5-HT\textsubscript{2C} receptor mRNA. Moreover, editing efficiency and isoform expression seem to be regulated in a brain region- and perhaps cell-dependent manner. Two studies have shown increased RNA editing in the prefrontal cortex of depressed suicide victims (Niswender et al., 2001; Gurevich...
et al., 2002b). In contrast, editing efficiency is decreased in the brains of schizophrenic patients (Sodhi et al., 2001). Likewise, changes in RNA editing occur in various animal models of anxiety (Hackler et al., 2006; Du et al., 2007) and depression (Iwamoto et al., 2005). Moreover, drugs that alter serotonin signaling and other psychoactive drugs also alter RNA editing efficiency (Gurevich et al., 2002a,b; Yang et al., 2004). These studies suggest that regulation of RNA editing may be highly dynamic. Furthermore, the marked differences in signaling between the nonedited and the various edited 5-HT2C receptor isoforms suggest that disease- or drug-induced changes in RNA editing efficiency consequently would alter serotonergic neurotransmission. Disease-related changes in expression of 5-HT2C receptor isoforms with altered profiles of cellular signaling highlight the importance of using disease-appropriate cellular models to study the pharmacology of drugs.

The I156V substitution of the 5-HT2C-VNI isoform reduced ligand-independent activity toward the PLC-IP pathway. The slope of the regression line between receptor density and basal IP accumulation, which is a quantitative measure of constitutive receptor activity, was 2-fold less for the 5-HT2C-VNI isoform versus the nonedited 5-HT2C-INI receptor. This level of reduction of constitutive activity was similar to that of the fully edited 5-HT2C-VSV isoform. Consistent with the reports of others (Herrick-Davis et al., 1999; Niswender et al., 1999; Wang et al., 2000), the constitutive activity of the 5-HT2C-VGV isoform was almost completely abolished.

Reduction in the magnitude of constitutive activity as a result of mRNA editing could arise from reduced capacity of

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TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>5-HT2C-INI (pmol/mg protein)</th>
<th>5-HT2C-VNI (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bufotenin</td>
<td>1.03 ± 0.08</td>
<td>0.77 ± 0.06</td>
</tr>
<tr>
<td>DOI</td>
<td>0.97 ± 0.08</td>
<td>0.69 ± 0.05</td>
</tr>
<tr>
<td>Ro 60-0175</td>
<td>0.39 ± 0.06</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>WAY-161503</td>
<td>1.07 ± 0.12</td>
<td>0.45 ± 0.09**</td>
</tr>
<tr>
<td>Quipazine</td>
<td>0.59 ± 0.07</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td>TFMPP</td>
<td>0.66 ± 0.06</td>
<td>0.56 ± 0.12</td>
</tr>
</tbody>
</table>

*p < 0.05 compared with 5-HT2C-INI isoform.

**p < 0.01 compared with 5-HT2C-INI isoform.

***p < 0.001 compared with 5-HT2C-INI isoform.
the edited receptors to isomerize to an active conformation(s) or to reduced G protein coupling of the active receptor conformation(s). The latter possibility is supported by the evidence that amino acids in the i2 region of 7-TMS receptors close to the conserved DRY sequence are involved in G protein coupling (Moro et al., 1993; Burstein et al., 1998; Sugimoto et al., 2004). On the other hand, it has been suggested that the G protein coupling capacity of some edited receptors is not altered on the basis that maximal PLC-IP responses to 5-HT are not different from the nonedited receptor (Burns et al., 1997; Herrick-Davis et al., 1999; Niswender et al., 1999). However these experiments may be confounded by the use of a full agonist (5-HT) in cells expressing relatively high levels of the receptors. Under such conditions, alterations in agonist efficacy can be masked by receptor reserve. We compared the G_{s/\alpha}-PLC-IP coupling efficiency of the 5-HT_{2C-VNI} receptor occupied by the full agonist, 5-HT, or the partial agonists, LSD and DOI, to that of the nonedited 5-HT_{2C-INI} and the fully edited 5-HT_{2C-VSV} and 5-HT_{2C-VGV} isoforms over a range of receptor densities. The slopes of the regression line between receptor density and response to maximal occupancy by agonist (full or partial) were not reduced for the 5-HT_{2C-VNI} or the fully edited 5-HT_{2C-VSV} and 5-HT_{2C-VGV} receptors compared with the nonedited 5-HT_{2C-INI} isoform. The notable exception was LSD, which is known not to activate the 5-HT_{2C-VGV} isoform (Backstrom et al., 1999; Fitzgerald et al., 1999; Berg et al., 2001). These results are consistent with the conclusions of others (Niswender et al., 1999) that the coupling efficiency of the agonist-occupied edited receptors was not less than that of the 5-HT_{2C-INI} isoform and suggested that a reduced capacity to isomerize to an active conformation(s) may underlie the reduced constitutive activity of the edited receptors.

The DRY sequence (especially the arginine residue) in i2 of 7-TMS receptors plays an important role in regulating the ability of a receptor to isomerize between inactive and active conformations (Burstein et al., 1998; Rasmussen et al., 1999; Alewijnse et al., 2000; Scheer et al., 2000; Flanagan, 2005). Given the proximity to the DRY sequence of the amino acids in i2 of the 5-HT_{2C} receptor modified by RNA editing, it is possible that RNA editing could produce receptor isoforms with reduced ability to isomerize. To assess this, we measured the thermal stability of the receptors. Constitutively active receptors with a high capacity to isomerize are structurally unstable as a consequence of reduced stabilizing intramolecular constraints and therefore denature more readily at elevated temperature (Gether et al., 1997; Samama et al., 1997; Alewijnse et al., 2000). This instability can be measured by the kinetics of the loss of ligand binding at elevated temperature (Claeysen et al., 2001). We were surprised to find that there was no difference in the thermal stability of the 5-HT_{2C-VNI}, 5-HT_{2C-VSV}, or 5-HT_{2C-VGV} isoforms from the highly constitutively active nonedited 5-HT_{2C-INI} receptor. This suggests that the capacity of the edited isoforms to isomerize is similar to that of the nonedited receptor.

If there is no difference in the G protein coupling efficiency or the capacity to isomerize between the edited and nonedited 5-HT_{2C} isoforms, how can the difference in constitutive activity be explained? We suggest that the reduced constitutive activity of the edited 5-HT_{2C} isoforms is caused by reduced efficiency of G protein coupling of the unoccupied receptor. However, agonist occupancy could promote an active receptor conformation(s) that differs from that of the unoccupied receptor such that agonist-occupied edited and nonedited receptors have equal ability to couple to the G_{s/\alpha}-PLC-IP signaling pathway. Differences in signaling between agonist-occupied and unoccupied receptors (possibly due to different active conformations) have been demonstrated previously for the 5-HT_{2C-INI} receptor (Westphal and Sanders-Bush, 1996). In addition, Kobilka and colleagues (Gether et al., 2007) using a conformationally sensitive fluorescent probe demonstrated that agonists produce distinct conformational populations of the solubilized β2-adrenergic receptor.

In summary, RNA editing can produce different 5-HT_{2C} receptor isoforms with markedly different signaling profiles of agonist-stimulated activity and markedly reduced levels of ligand-independent receptor activity. Our results suggest that reduced constitutive activity of the edited receptor isoforms is due to reduced G protein coupling efficiency and not to a reduced ability to isomerize to an active conformation(s). However, agonist occupancy of the receptors is capable of promoting active conformations that do not differ in G protein coupling ability. Changes in receptor function as a result of mRNA editing would be expected to alter serotonergic neurotransmission as well as responses to drugs that act via the 5-HT_{2C} receptor system. As a result, in vitro study of the pharmacological properties of drugs should be done using cells that express 5-HT_{2C} receptor isoforms that reflect those present in vivo.

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