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Characterisation of agonist binding on human 5-HT_{2C} receptor isoforms

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Abstract

The 5-HT_{2C} receptor is expressed in different isoforms as a result of mRNA editing. Both INI (unedited) and VSV (a fully edited version) isoforms are abundant in rat brain. The VSV isoform lacks the high affinity recognition site for 5-HT, which may be caused by low efficiency coupling to G-proteins. In this study we have investigated the pharmacology of the agonist binding site of these two isoforms of the 5-HT_{2C} receptor. The VSV isoform was expressed in Chinese hamster ovary cells (CHO) and the INI isoform in both Chinese hamster ovary cells and human embryonic kidney cells (HEK-293). Saturation analysis using [^{3}H]5-HT revealed high and low affinity recognition sites on the INI isoform in both cell types whilst the VSV isoform did not have the high affinity binding site for [^{3}H]5-HT. Displacement studies were undertaken using [^{3}H]5-HT to label the receptors. In these studies the affinity of agonists (5-HT, Ro600175 ((*S*)-2-(6-Chloro-5-fluoroindol-1-yl)-1-methylethylamine), MK212 (6-Chloro-2-(piperazinyl) pyrazine), mCPP (1-(*m*-chlorophenyl)-piperazine), TfMPP (*N*-(*m*-trifluoromethylphenyl)piperazine), DOI (1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane), DOB (1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane) and 8OH-DPAT (8-hydroxy-2-(di-*N*-propylamino)tetralin) was higher at the INI isoform, whilst antagonist affinity (ketanserin and mesulergine) did not change between the two receptor isoforms. There were no differences between the INI isoform expressed in the CHO and HEK-293. This suggests that the INI isoform of the 5-HT_{2C} receptor is pharmacologically similar to the VSV form of the 5-HT_{2C} receptor but that it couples more efficiently to G-proteins.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT_{2C} receptor INI; 5-HT_{2C} receptor VSV; Isoform; G-protein

1. Introduction

5-HT (5-hydroxytryptamine, serotonin) is a major neurotransmitter that is thought to be involved in many CNS (central nervous system) processes including feeding, anxiety, aggression, sexual behaviour, mood and pain (Roth, 1994; Wilkinson and Dourish, 1991). 5-HT exerts its physiological effects via an action on a diverse family of cell surface receptor proteins. Currently, there are thought to be seven families of 5-HT receptor and at least 14 subtypes, as well as splice variants and different edited isoforms. A subgroup of these is the 5-HT_2 receptors that belong to a large group of seven transmembrane spanning G-protein coupled receptors. Three subtypes (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}) of 5-HT_2 receptor have been identified on the basis of molecular cloning (Boess and Martin, 1994). The three subtypes of the 5-HT_2 receptor have a high degree of sequence homology, which is greater than 80% in the transmembrane spanning regions.

The 5-HT_{2C} receptor was first discovered in the choroid plexus as a highly expressed protein, which was labelled by [^{3}H]mesulergine and [^{3}H]5-HT (Pazos et al., 1984) and coupled to phosphoinositide turnover (Conn et al., 1986). 5-HT_{2C} receptors have subsequently been found in many regions in both rat and human brain (Hoyer et al., 1986; Pazos and Palacios, 1992). 5-HT_{2C} receptors are G-protein coupled receptors that are linked to the activation of phospholipase C, resulting in the increased production of inositol phosphates and diacylglycerol, and to phospholipase A_2, resulting in an increased release of arachadonic acid.

The 5-HT_{2C} receptor has recently been shown to undergo mRNA editing. RNA editing is a post-transcriptional modification that results in the primary nucleotide sequence of the RNA transcripts being altered. Double stranded RNA-dependent adenosine deaminase catalyses

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the conversion of adenosine to inosine. The genomic transcript undergoes adenosine to inosine RNA editing at five positions, resulting in an alteration of amino acids 156, 158 and 160 in the putative second intracellular loop (Burns et al., 1997). There are 14 possible receptor isoforms (Burns et al., 1997; Niswender et al., 1999; Fitzgerald et al., 1999) and mRNA for seven major isoforms of the 5-HT_{2C} receptor have been shown to be present in the mammalian brain (Burns et al., 1997) with INI being the unedited version of the receptor whilst VSV is one of the extensively edited versions.

The editing occurs in the putative second intracellular loop, a region thought to be important for the G-protein coupling of receptors (Wang et al., 1993; Blin et al., 1995; Moro et al., 1993; Herrick-Davis et al., 1997). The editing therefore is associated with different G-protein coupling efficiencies of the isoforms. It has been shown that the INI version, which is unedited, is 10–15 times more potent in stimulating phosphatidylinositol hydrolysis than some of the edited versions of the receptor (Burns et al., 1997; Niswender et al., 1999). The INI isoform has also been shown to have high and low affinity binding sites for 5-HT whilst the VGV and VSV isoforms, which are fully edited, only have one site that is of the lower affinity state (Niswender et al., 1999). These studies have been carried out using the 5-HT_{2C} receptor antagonist [^{3}H]mesulergine, which labels both sites with equal affinity. Here we compare the binding profiles of the INI and VSV isoforms of the 5-HT_{2C} receptor using [^{3}H]5-HT to label the agonist binding site. We examined the profile of the INI isoform expressed in two different cell lines and at two different expression levels to determine whether these variables affect agonist pharmacology.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary cells (CHO) stably transfected with the VSV isoform of the human 5-HT_{2C} receptor were purchased from Euroscreen. The cells were grown in a monolayer in 50 ml UltraCHO media supplemented with 1% (v/v) dialysed fetal bovine serum, 1% (v/v) penicillin/streptomycin and 0.4 mg/ml Geneticin G418. Chinese hamster ovary cell and human embryonic kidney cells (HEK-293) were stably transfected with the INI isoform of the human 5-HT_{2C} receptor as described by Porter et al. (1999). The cells were grown in a monolayer in 50 ml Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) dialysed fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1% L-glutamine, 1% non-essential amino acids and 5 μg/ml Blasticidin. Cells were grown in a humidified incubator at 37°C in 5% CO_2 until they were 80% confluent. Cells were used for up to 30 passages. Confluent cells were harvested into growth media, centrifuged at $1000 \times g$ for 20 min and then washed once with Dulbecco's phosphate buffered saline before being stored at −80°C.

2.2. Radioligand binding assays

Radioligand binding assays were carried out using [^{3}H]5-HT and [^{3}H]mesulergine. For saturation analysis, both radioligands were used at 0.01–40 nM, and 100 μM mianserin was used to define non-specific binding. For competition curves [^{3}H]5-HT was used at 8 nM for VSV and 0.8 nM for INI. Compounds were assayed at a range of concentrations from 10 μM to 0.1 nM. Assays were carried out in a final volume of 250 μl in 50 mM Tris pH 7.5 containing 0.1% ascorbic acid. Incubations were carried out for 60 min at 37°C followed by filtration through either a Packard Filtermate 196 or a Brandel (MB48R) 48-well cell harvester followed by 3×3 ml washes in ice-cold 50 mM Tris pH 7.5. GF/B filters were used and were pre-soaked in 0.1% polyethylenimine. Filters were then either counted on a Packard Topcount or a Beckman LS6500. All curves were fitted using Graphpad Prism. K_i values were calculated according to the Cheng–Prusoff equation (Cheng and Prusoff, 1973). The K_d values used to determine the K_i values were 1.76 nM for the INI isoform expressed in the CHO cells, 3.40 nM for the INI isoform expressed in the HEK cells and 15.34 nM for the VSV isoform expressed in the CHO cells. R^2 values have been calculated by Prism using the Pearson correlation coefficient. Protein levels were determined using Biorad colorimetric reagent with bovine serum albumin as standard (Bradford, 1979).

2.3. Materials

UltraCHO media was purchased from Boehringer Mannheim. Dialysed fetal bovine serum, penicillin/streptomycin, geneticin, DMEM, L-glutamine, phosphate buffered saline (PBS), 5-HT, tryptamine, polyethyenimine, GppNHp (5′-guanylylimidodiphoaphate trisodium salt) and ascorbic acid were purchased from Sigma. Blasticidin was purchased from Invitrogen. 5-Hydroxy[^{3}H]tryptamine trifluoroacetate (127 Ci/mmol) and [N^6-methyl-^{3}H] mesulergine (78 Ci/mmol) were both purchased from Amersham Life Science. Mianserin, DOI (1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane), DOB (1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane), 8-OH-DPAT (8-hydroxy-2-(di-*N*-propylamino)tetralin), ketanserin and mesulergine were all purchased from RBI. MK212 (6-Chloro-2-(piperazinyl) pyrazine) and Ru24969 (5-Methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1*H*-indole) were purchased from Tocris Cookson. TfMPP (*N*-(*m*-trifluoromethylphenyl)piperazine), mCPP (1-(*m*-chlorophenyl)-piperazine) and Ro 600175 ((*S*)-2-(6-Chloro-5-fluoroindol-1-yl)-1-methylethylamine) were all synthesised in-house.

3. Results

3.1. Saturation analysis

Saturation analysis were carried out using [^{3}H]mesulergine to determine the expression levels of the cell lines.

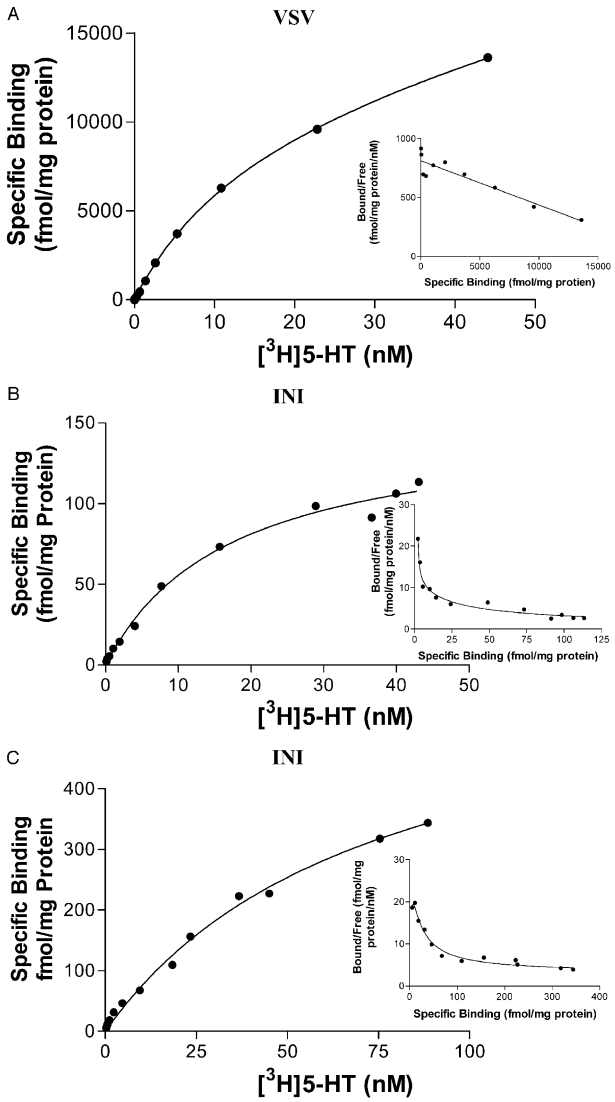

Fig. 1. Agonist radioligand binding profiles of 5-HT$_{2C}$ receptors. (A) Saturation binding isotherm for [^{3}H]5-HT binding to CHO cells expressing the VSV isoform of the h5-HT$_{2C}$ receptor. B_{max} is 13.8 ± 1.2 pmol/mg protein and K_d is 15.34 ± 1.9 nM (mean ± S.E.M., $n = 3$). (B) Saturation binding isotherm for [^{3}H]5-HT binding to CHO cells expressing the INI isoform of the h5HT$_{2C}$ receptor. Two binding sites were seen in this cell line. $B_{max}1$ is 42.0 ± 13.2 fmol/mg protein and K_d1 is 1.76 ± 0.4 nM. $B_{max}2$ is 160.5 ± 31.3 fmol/mg protein and K_d2 is 13.7 ± 2.3 nM. (C) Saturation binding isotherm for [^{3}H]5-HT binding to HEK-293 cells expressing the INI isoform of the h5-HT$_{2C}$ receptor. Two binding sites were seen in this cell line. $B_{max}1$ is 71.0 ± 8.3 fmol/mg protein and K_d1 is 3.4 ± 0.5 nM. $B_{max}2$ is 1561 ± 101 fmol/mg protein and K_d2 is 30.8 ± 1.3 nM. Non-specific binding was determined using 10 μM mianserin. Experiments were performed as described in Section 2. The insets are Scatchard analysis of the same points. Each diagram represents data from a single representative experiment.

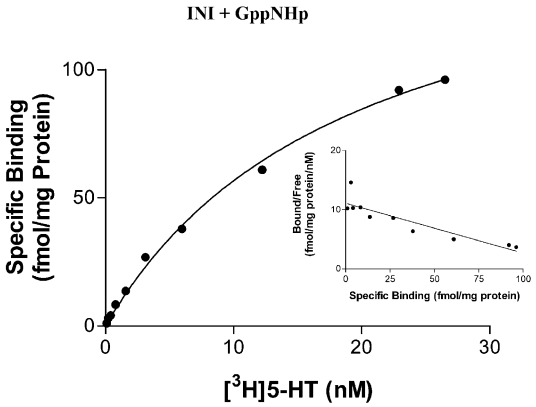

Fig. 2. Saturation binding isotherm for [^{3}H]5-HT binding to CHO cells expressing the INI isoform of the h5-HT$_{2C}$ receptor in the presence of 100 μM GppNHp. B_{max} is 182 ± 30.8 fmol/mg protein and K_d is 27.1 ± 10.4 nM. Experiments were performed as described in Section 2. The insets are Scatchard analysis of the same points. The graph shows data from a single representative experiment.

[^{3}H]Mesulergine binding to both isoforms of the receptor was saturable and of high affinity. The VSV cell line had a B_{max} of 34.9 ± 1.5 pmol/mg protein and a K_d of 4.9 ± 0.25 nM, whilst the INI had a B_{max} of 141 ± 35.4 fmol/mg protein in CHO cells and a B_{max} of 1.35 ± 0.41 pmol/mg protein in HEK cells. The affinity of [^{3}H]mesulergine in both cell lines containing the INI isoform of the receptor was similar to the affinity in the VSV containing cell line, i.e. K_d of 3.2 ± 0.06 nM for the CHO cell line and 5.6 ± 0.7 nM for the HEK cell line.

[^{3}H]5-HT bound to a single saturable site on the h5-HT$_{2C}$ VSV cells with a K_d of 15.34 ± 1.9 nM and a B_{max} of 13.8 ± 1.2 pmol/mg protein (Fig. 1A). [^{3}H]5-HT bound to

Table 1
Displacement of [^{3}H]5-HT binding in Chinese hamster ovary and HEK-293 cells containing the INI and VSV versions of the h5-HT$_{2C}$ receptor Receptors were labelled with 0.8 nM [^{3}H]5-HT for the INI cells and 8 nM [^{3}H]5-HT for the VSV cells. Experiments were carried out as described in Section 2. Each measurement is the average and the S.E.M. of three independent experiments.

	K_i (nM)		
	INI (CHO)	INI (HEK)	VSV (CHO)
Full agonists			
5-HT	2.45 ± 0.38	4.34 ± 1.32	18.10 ± 1.49
Ro600175	5.26 ± 2.49	2.20 ± 0.39	12.30 ± 2.9
MK212	62.05 ± 9.86	48.82 ± 15.34	262.0 ± 72.1
Partial Agonists			
mCPP	10.49 ± 2.48	8.13 ± 3.33	40.10 ± 5.27
TfMPP	10.79 ± 2.34	13.20 ± 6.96	41.70 ± 5.1
Ru24969	93.22 ± 9.45	218.57 ± 49.15	307.0 ± 84.4
DOI	5.96 ± 0.71	5.06 ± 1.87	12.90 ± 1.23
DOB	3.15 ± 0.32	3.54 ± 0.86	22.90 ± 2.9
8OH-DPAT	7983.48 ± 907	9016 ± 4043	13,700 ± 5030
Antagonists			
Ketanserin	31.07 ± 8.55	30.42 ± 3.55	39.66 ± 13.42
Mesulergine	2.11 ± 0.88	1.76 ± 0.24	2.56 ± 0.29

two sites on the h5-HT_{2C} INI cells expressed in CHO cells with affinities of 1.76 ± 0.4 nM and 13.7 ± 2.3 nM and B_{max} values of 42.0 ± 13.2 and 160.5 ± 31.3 fmol/mg protein (Fig. 1B). [^{3}H]5-HT bound to two sites on the h5-HT_{2C} INI cells expressed in HEK293 cells with affinities of 3.4 ± 0.5 nM and 30.8 ± 1.3 nM and B_{max} values of 71.0 ± 8.3 and 561 ± 101 fmol/mg protein (Fig. 1C). The K_d for the low affinity site on the INI isoform in both cell lines being similar to the K_d for [^{3}H]5-HT on the VSV receptors.

It has been shown that analogues of GTP can convert the high affinity binding site for 5-HT to the lower affinity binding site. Here we have carried out saturation analysis with [^{3}H]5-HT in the presence of 100 μM GppNHp, a non-hydrolyzable analogue of GTP, on the INI isoform of the receptor expressed in the CHO cells. As shown in Fig. 2 the high affinity site is abolished. The receptors have a single saturable site with a K_d of 27.1 ± 10.4 nM and a B_{max} of 182 ± 30.8 fmol/mg protein.

3.2. Pharmacology

The pharmacology of the two isoforms of the receptor was investigated using [^{3}H]5-HT. The results in Table 1 summarise the affinities obtained using [^{3}H]5-HT as ligand in all three cell lines. The rank order of potency is maintained in all three cell lines. The INI isoform gives comparable affinities for compounds in both the CHO and the HEK-293 cell lines, despite the different receptor expression levels and different cell lines. The full agonists showed the greatest differences between the two isoforms by being four to six times weaker in affinity for the VSV isoform. The partial agonists were also affected by the editing, showing a two to three-fold reduction in affinity in the VSV isoform. The affinities of the compounds were plotted for comparison in Fig. 3. The antagonists showed no significant difference in affinity between the two isoforms of the receptor.

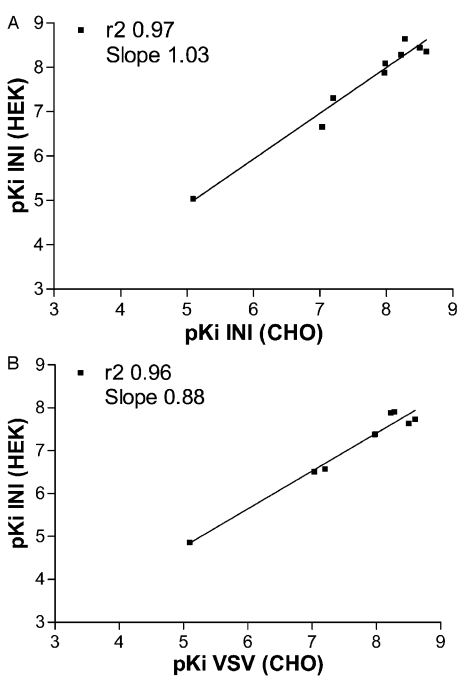

Fig. 3. The potency of compounds as displacers of [^{3}H]5-HT binding in different cell lines and against different isoforms of the receptor was compared. (A) Correlation plot of pK_i vs. [^{3}H]5-HT binding in INI (CHO) and INI (HEK). (B) Correlation plot of pK_i vs. [^{3}H]5-HT binding in INI (CHO) and VSV (CHO). Experiments were performed as described in Section 2. Data is an n of three to four experiments.

4. Discussion

[^{3}H]Mesulergine labelled one binding site on both isoforms of the receptor. The K_d obtained was consistent across all three cell lines tested. This suggests that antagonist binding is not affected by mRNA editing of the 5-HT_{2C} receptor in the second intracellular loop region.

The INI isoform of the 5-HT_{2C} receptor contained both a high and low affinity site for 5-HT in both the HEK-293 and CHO cell lines. This is consistent with data reported by other groups (Fitzgerald et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999). The high affinity site is thought to represent the G-protein coupled receptors whilst the lower affinity site is thought to be uncoupled receptors. The high affinity binding site for 5-HT was abolished in the presence of GppNHp. This is expected since the non-hydrolysable analogues of GTP disrupt the receptor–G-protein complex, resulting in the rapid dissociation of agonists from the uncoupled receptor (Gilman, 1987). The overall number of receptors was similar in the presence and absence of GppNHp, but in the presence of GppNHp all were in the lower affinity state.

The VSV isoform only had one binding site for 5-HT. The K_d of 5-HT for this site was similar to the lower affinity binding site on the INI isoform of the receptor. This has also been observed by Niswender et al. (1999), although Fitzgerald et al. (1999) saw two affinity sites for 5-HT on the VSV isoform. The lack of a high affinity binding site for 5-HT on the VSV isoform suggests that the G-protein coupling of this isoform has been affected by the RNA editing in this intracellular loop region. Previous results from our group (Quirk et al., 1999) have indicated that the VSV isoform of the 5-HT_{2C} receptor is coupled to G-proteins by showing that this cell line is sensitive to [^{35}S]GTPγS binding. However, the EC_{50} values obtained in stimulating GTPγS binding were 10-fold weaker than the K_i of the compounds for displacing [^{3}H]5-HT. This further suggests that the VSV isoform of the 5-HT_{2C} receptor is coupled to G-proteins but that the coupling is of lower efficiency than in the unedited version of the 5-HT_{2C} receptor.

[^{3}H]5-HT was used to investigate the agonist pharmacology of the INI and VSV isoforms. The binding to the

INI isoform with [^{3}H]5-HT was carried out at ligand concentrations of less than 1 nM to ensure that only the high affinity site was labelled. Comparing the CHO and the HEK-293 cells expressing the INI isoform of the h5-HT_{2C} receptor (Fig. 3A), all compounds tested displace [^{3}H]5-HT with similar affinities, giving a good correlation with an r^2 of 0.97 and a slope of 1.03. This suggests that the 10-fold difference in expression level and the use of two different cell lines did not affect the pharmacology of the receptor. Comparing the affinity of agonists in the INI cells with their affinity in the VSV cells, the compounds gave a good correlation with an r^2 of 0.96 (Fig. 3B), although compounds had a higher affinity for the INI isoform, resulting in a slope of 0.88. The full agonists, 5-HT, Ro600175 and MK-212 (Quirk et al., 1999; Porter et al., 1999; Alberts et al., 1999) showed the greatest shift in affinity by being four to six times more potent in the INI isoform. The partial agonists, mCPP, TfMPP, Ru24969 and DOI (Quirk et al., 1999; Porter et al., 1999; Alberts et al., 1999) showed a smaller shift in affinity by being two to three times more potent in the INI isoform. The partial agonist DOB (Quirk et al., 1999; Porter et al., 1999; Alberts et al., 1999) was an exception to this, showing a sixfold shift in affinity between the two isoforms. 8-OH-DPAT showed no significant difference in affinity between the INI and VSV isoform; however, this could be due to the fact that it is a very weak partial agonist, showing about 30% efficacy (Quirk et al., 1999).

The antagonists, ketanserin and mesulergine (Alberts et al., 1999), showed similar affinity for [^{3}H]5-HT binding at both the INI and VSV isoforms of the receptor. This further suggests that antagonist binding is unaffected by mRNA editing of the 5-HT_{2C} receptor. Previous studies have also shown no change in antagonist affinity as a result of mRNA editing of the 5-HT_{2C} receptor (Herrick-Davis et al., 1999; Fitzgerald et al., 1999). This may be expected since the editing occurs in the second intracellular loop region. This region is thought to be important for G-protein coupling and therefore it is more likely that agonist binding affinities will be affected by amino acid changes in this region.

In this study, we have demonstrated that mRNA editing in the second intracellular loop region of the human 5-HT_{2C} receptor affects agonist binding affinity. Also, the more efficacious the agonist, the greater the change in affinity of the compound between the edited and unedited versions of the receptor. Antagonist binding affinity is unaffected by this editing. These results point to the mRNA editing in this region of the receptor as being important in the efficiency of G-protein coupling. Other studies have shown that the degree of editing alters the efficacy of agonists in stimulating phosphotidyl inositol hydrolysis and also has effects on the constitutive activity of the 5-HT_{2C} receptor (Herrick-Davis et al., 1999; Niswender et al., 1999). The loss in efficiency of G-protein coupling appears to have physiologically relevant effects on signal transduction.

All studies so far on binding and efficacy of compounds on the different isoforms have been carried out in cell expression systems. mRNA coding for the different isoforms of the 5-HT_{2C} receptor is distributed heterogenously in human and rat brain. VSV is the most prevalent isoform of 5-HT_{2C} mRNA present in human brain accounting for 32%, whilst the INI accounts for 7% (Burns et al., 1997; Fitzgerald et al., 1999). It remains unclear whether the mRNA for different receptor isoforms is translated into cell surface receptor proteins and which physiological processes, if any, are facilitated by mRNA editing.

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