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[^{3}H]-8-OH-DPAT binding in the rat brain raphe area: involvement of 5-HT$_{1A}$ and non-5-HT$_{1A}$ receptors

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1 The 5-HT$_{1A}$ agonist 8-OH-DPAT has been shown to have additional 5-HT uptake inhibiting properties. The present work was undertaken to examine further the binding of [^{3}H]-8-OH-DPAT in the raphe area of the rat brain, a region rich in 5-HT$_{1A}$ receptors and 5-HT uptake sites.

2 5-HT inhibited [^{3}H]-8-OH-DPAT binding in a biphasic manner (pK$_{i1}$: 8.82±0.01, pK$_{i2}$: 6.07±0.05, $n=4$) with the low affinity site representing 36±4% of the total population. A biphasic inhibition curve was found also with the 5-HT$_{1A}$ antagonist, WAY 100635 (pK$_{i1}$: 8.65±0.17, pK$_{i2}$: 4.26±0.38, $n=3$). In the presence of 1 μM WAY 100635 to mask 5-HT$_{1A}$ receptors, 5-HT inhibited [^{3}H]-8-OH-DPAT binding in a monophasic manner (pK$_i$: 6.04±0.07, $n=3$).

3 The affinities of various compounds for sites labelled by [^{3}H]-8-OH-DPAT in the presence of 1 μM WAY 100635 and for sites labelled by [^{3}H]-citalopram (a selective 5-HT uptake inhibitor) were determined. There was a significant correlation between pK$_i$ values at 5-HT uptake sites and at non-5HT$_{1A}$ sites labelled by [^{3}H]-8-OH-DPAT ($r=0.80$, $P<0.001$, $n=17$), suggesting these latter sites to be 5-HT uptake sites.

4 Whereas the affinities of R(+) and S(−) enantiomers of 8-OH-DPAT for the 5-HT uptake site are similar, R(+)8-OH-DPAT has 10 times higher affinity for the non-5-HT$_{1A}$ site than S(−)8-OH-DPAT and was considered as an outlier in the correlation. It is suggested that [^{3}H]-8-OH-DPAT labels other, as yet unknown binding sites in the raphe.

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Keywords: 5-HT; 5-HT$_{1A}$ receptors; 8-OH-DPAT; 5-HT uptake; raphe; [^{3}H]-8-OH-DPAT binding

Abbreviations: 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, (±)-8-hydroxy-2-(di-n-propylamino)tetralin; RU 24969, 5-methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1H-indole; TFMPP, N-(3-trifluoromethylphenyl)piperazine; WAY 100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide

Introduction

The prototypical 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) agonist (±)-8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) has been used, as a tritiated ligand, to label 5HT$_{1A}$ receptors for more than 20 years (Gozlan *et al*., 1983). 8-OH-DPAT, however, is not devoid of other properties. Notably, 8-OH-DPAT has been reported to inhibit 5-HT uptake *in vitro* at concentrations 700 times higher than those necessary to inhibit [^{3}H]-5-HT binding to 5-HT$_{1A}$ receptors (Hamon *et al*., 1984) and *in vivo* at doses 60 times higher than those that produce 5-HT$_{1A}$ receptor-mediated effects at somatodendritic receptors in the raphe (Assié & Koek, 1996). [^{3}H]-8-OH-DPAT has also been reported to label 5-HT uptake sites, in rat striatum (Schoemaker & Langer, 1986; Alexander & Wood, 1988) and in bovine dorsal raphe (Sprouse *et al*., 1993).

The present work was undertaken to examine further the binding of [^{3}H]-8-OH-DPAT in the raphe area of the rat brain. The affinity of a series of compounds for non-5-HT$_{1A}$ binding sites labelled by [^{3}H]-8-OH-DPAT in the presence of a saturating concentration of the selective 5-HT$_{1A}$ antagonist, WAY 100635 (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide), was compared with their affinity for 5-HT uptake sites labelled by the selective 5-HT uptake inhibitor, [^{3}H]-citalopram.

In addition to its affinity for 5-HT uptake sites, 8-OH-DPAT has moderate, stereospecific affinity for human cloned 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors (Pauwels & Colpaert, 1996). 8-OH-DPAT has also been shown to have low affinity for α_1- and α_2-adrenoceptors and for dopamine D$_2$ receptors (e.g., Brown *et al*., 1990; Kleven *et al*., 1997). The possible involvement of these different binding sites in the non-5-HT$_{1A}$ effects of 8-OH-DPAT studied here was investigated by examining the ability of selective ligands at these sites to inhibit [^{3}H]-8-OH-DPAT binding in the presence of WAY 100635. Part of this work has been presented to the British Pharmacological Society in a preliminary form (Assié & Koek, 1999).

Methods

Tissue preparation

Frozen brains of male Sprague Dawley rats [Ico: OFA SD (I.O.P.S. Caw); Iffa Credo, France], weighing 180–200 g, were stored at −70°C prior to use in binding assays.

[^{3}H]-8-OH-DPAT binding assays were carried out in membrane preparations from rat raphe area or hippocampus. Frozen tissues were thawed, dissected and homogenized in 20 volumes of ice-cold Tris-HCl (50 mM, pH 7.4 at 25°C). For each membrane preparation, the homogenate was centrifuged at 39,000×*g* for 10 min, the pellet was resuspended in the same volume of buffer and was recentrifuged as before. Following a further resuspension, the tissue was incubated for 10 min at 37°C and centrifuged once again. The final pellet was suspended in the same buffer. The final tissue concentration was 5 mg per assay tube for raphe area and 3 mg per assay tube for hippocampus.

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[^{3}H]-Citalopram binding assays were carried out in membrane homogenates from the raphe area of rat brains as described by D'Amato *et al*. (1987). Thawed tissue was homogenized in 25 volumes of ice-cold Tris-HCl (50 mM, pH 7.4 at 25°C). The homogenate was centrifuged at 48,000 × *g* for 10 min, the pellet was resuspended in the same volume of buffer and was recentrifuged as before. Following a further resuspension, the tissue was centrifuged once again. The final pellet was suspended in an assay buffer Tris-HCl (50 mM, pH 7.4 at 25°C) containing 120 mM NaCl and 5 mM KCl. The final tissue concentration was 0.5 mg per assay tube.

Binding assays

For saturation experiments, the incubation medium consisted of 0.1 ml of different concentrations of either [^{3}H]-8-OH-DPAT (ranging from 0.25 to 8 nM, or from 1 to 480 nM in the presence of 1 μM WAY 100635) or [^{3}H]-citalopram (ranging from 0.125 to 16 nM), 0.1 ml of buffer or drug to define non-specific binding (10 μM 5-HT for [^{3}H]-8-OH-DPAT or 0.5 μM paroxetine for [^{3}H]-citalopram), and 0.8 ml of membrane preparation. For competition experiments, the incubation medium consisted of 0.1 ml of [^{3}H]-8-OH-DPAT (0.25 nM alone or in the presence of 1 μM WAY 100635, or 8 nM in the presence of 1 μM WAY 100635) or [^{3}H]-citalopram (0.5 nM), 0.1 ml of different concentrations of the test compound, and 0.8 ml of membrane preparation. The assay tubes were incubated for 30 min ([^{3}H]-8-OH-DPAT) or 60 min ([^{3}H]-citalopram) at room temperature. The reaction was terminated by rapid filtration, using a Brandel harvester, through GF/B glass fibre filters with two 4 ml washes of Tris buffer. The radioactivity retained on the filters was counted by scintillation spectroscopy in 4 ml of scintillation fluid (Emulsifier safe, Packard). [^{3}H]-citalopram binding experiments were performed in triplicate, and [^{3}H]-8-OH-DPAT binding experiments were performed in duplicate in the raphe and in triplicate in the hippocampus.

Data analysis

Results were analysed using the non-linear curve fitting program KELL, Radlig (Biosoft, Cambridge, U.K.). The dissociation constant (K_D) and the total number of binding sites (B_{max}) for each radioligand were estimated from saturation experiments. Results from competition experiments are expressed as pK_i values (mean ± s.e.mean of three determinations, unless stated otherwise). The Pearson product-moment correlation between pK_i values for the sites labelled by [^{3}H]-8-OH-DPAT in the presence of 1 μM WAY 100635 and for [^{3}H]-citalopram binding sites was calculated by means of the program SigmaStat (SPSS, Chicago, U.S.A.).

Drugs

[^{3}H]-8-OH-DPAT (TRK850, 160–240 Ci mmol^{-1}) was purchased from Amersham Pharmacia Biotech (Orsay, France), and [^{3}H]-citalopram (NET1039, 70–87 Ci mmol^{-1}) from NEN Life Science Products (Paris, France). (±)8-OH-DPAT hydrobromide, R(+)8-OH-DPAT hydrobromide, S(−)8-OH-DPAT hydrobromide, ritanserin, TFMPP hydrochloride, imipramine hydrochloride, indatraline hydrochloride, quipazine dimaleate, 1-naphtylpiperazine hydrochloride, dextromethorphan hydrobromide, and ketanserin tartrate, were purchased from RBI, and 5-HT creatinin sulphate from Sigma (Saint-Quentin Fallavier, France). RU 24969 hemisuccinate was purchased from Tocris (Biobloch, Illkirch, France). Risperidone was purchased from Janssen Biotech (Olen, Belgium). Paroxetine hydrochloride hemihydrate, WAY 100635 dihydrochloride and fluoxetine hydrochloride were synthesized at the Centre de Recherche Pierre Fabre. Citalopram was a gift from Lundbeck (Copenhagen, Denmark), and roxindole was a gift from Merck (Darmstadt, Germany).

Results

5-HT inhibited [^{3}H]-8-OH-DPAT binding (0.25 nM) in a biphasic manner in membrane homogenates from the raphe (pK_{i1}: 8.82 ± 0.01, pK_{i2}: 6.07 ± 0.05; $n = 4$) and from the hippocampus (pK_{i1}: 9.20 ± 0.06, pK_{i2}: 6.46 ± 0.52; $n = 3$). In the raphe, the low affinity site represented 36 ± 4% of the total population of binding sites, whereas in the hippocampus, the low affinity site represented only 5 ± 1% of the total population (Figure 1). Slope factors (equivalent to the Hill coefficient) for a one-site model were 0.26 ± 0.02 and 0.76 ± 0.05 in the raphe and hippocampus, respectively.

The selective 5-HT_{1A} antagonist, WAY 100635, inhibited [^{3}H]-8-OH-DPAT binding in the raphe in a biphasic manner (pK_{i1}: 8.65 ± 0.17, pK_{i2}: 4.26 ± 0.38, $n = 3$). The affinity of WAY 100635 for the second binding site, however, could not be determined accurately (the estimated K_i being close to the highest concentration tested) (Figure 2). The concentration of 1 μM of WAY 100635 was used subsequently to mask the 5-HT_{1A} binding sites.

In the presence of 1 μM WAY 100635 to mask the 5-HT_{1A} binding sites, 5-HT inhibited [^{3}H]-8-OH-DPAT binding in a monophasic manner (pK_i: 6.04 ± 0.07, $n = 3$) (Figure 3).

Results from saturation experiments with [^{3}H]-8-OH-DPAT and [^{3}H]-citalopram in the raphe area of the rat brain are reported in Table 1. Note that the B_{max} values for [^{3}H]-citalopram and for [^{3}H]-8-OH-DPAT in the presence of WAY 100635 are similar (47.7 and 47.6 fmol mg^{-1} wet weight, respectively).

The affinity of various compounds for sites labelled by 0.25 nM [^{3}H]-8-OH-DPAT in the presence of 1 μM WAY

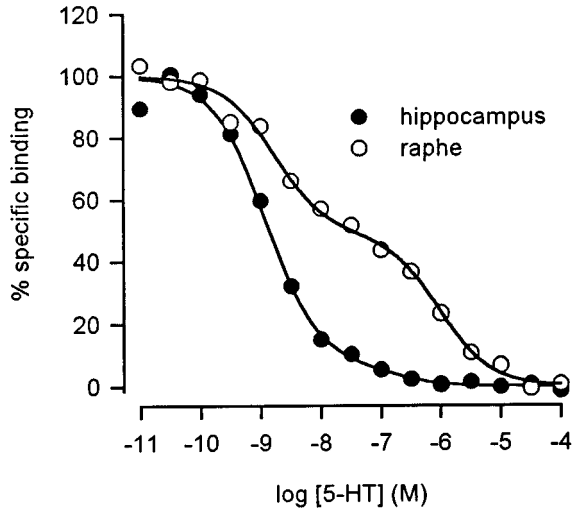

Figure 1 Inhibition of [^{3}H]-8-OH-DPAT binding by 5-HT in membrane homogenates from the raphe area and the hippocampus of the rat. Data shown are from representative experiments performed in duplicate (raphe) or triplicate (hippocampus). A two-site model fitted the data better than a one-site model in the raphe ($F_{(2,11)} = 92$; $P < 0.001$) and in the hippocampus ($F_{(2,11)} = 14$; $P = 0.001$).

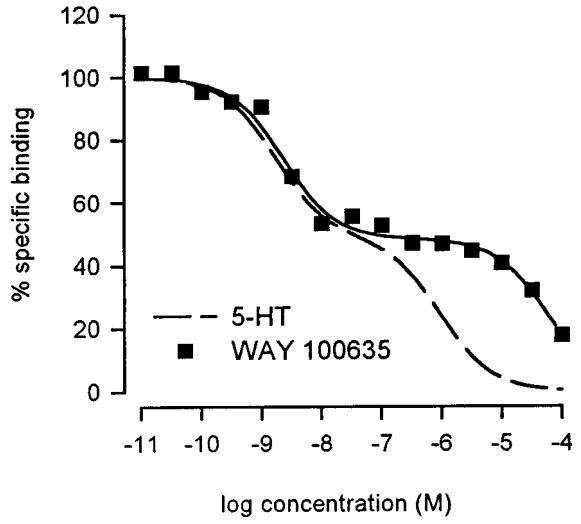

Figure 2 Inhibition of [^{3}H]-8-OH-DPAT binding by WAY 100635 in membrane homogenates from the raphe area of the rat. The dashed line depicts the inhibition curve of 5-HT shown in Figure 1. Data shown are from representative experiments performed in duplicate.

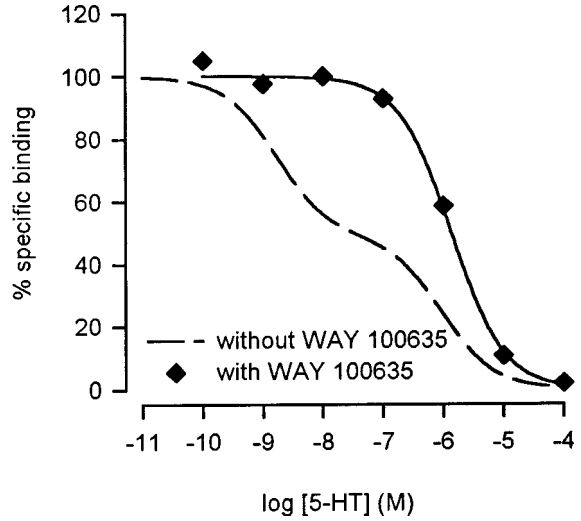

Figure 3 Inhibition of [^{3}H]-8-OH-DPAT binding in the presence of 1 μM WAY 100635 by 5-HT in membrane homogenates from the raphe area of the rat. The dashed line depicts the inhibition curve of 5-HT shown in Figure 1. Data shown are from representative experiments performed in duplicate.

Table 1 Equilibrium binding parameters of [^{3}H]-8-OH-DPAT alone or in the presence of 1 μM WAY 100635 and of [^{3}H]-citalopram in membranes from the raphe area of the rat brain

Ligand	K_D (nM)	B_{max} (fmol mg^{-1} wet weight)
[^{3}H]-8-OH-DPAT (0.25–8 nM)	3.72±0.28	12.3±0.2
[^{3}H]-8-OH-DPAT (1–480 nM) +WAY 100635 (1 μM)	44.6±8.4	47.7±8.6
[^{3}H]-citalopram	0.82±0.12	47.6±4.2

Binding parameters were determined from saturation isotherms as described in Methods. Data were analysed with the non-linear curve fitting program Kell, Radlig. Results are means±s.e.mean of 3–4 determinations.

100635 and for sites labelled by 0.5 nM [^{3}H]-citalopram are reported in Table 2. 5-HT, 8-OH-DPAT and its enantiomers, selective and non selective 5-HT uptake inhibitors, and several other serotonergic ligands possessing additional 5-HT uptake inhibiting properties, all inhibited the binding of [^{3}H]-8-OH-DPAT in the presence of 1 μM WAY 100635 and the binding of [^{3}H]-citalopram. For the compounds examined here, there was a significant correlation between their pK_i values at 5-HT uptake sites and at the sites labelled by [^{3}H]-8-OH-DPAT in the presence of 1 μM WAY 100635 ($r=0.80$, $P<0.001$, $n=17$) (Figure 4), suggesting these latter sites to be 5-HT uptake sites. The correlation, although high, was less than 1.0, and inspection of the standardized residuals showed R(+)8-OH-DPAT to be a significant outlier, because its residual (i.e., −2.18) was outside the interval containing 95% of the standardized residuals.

Because the concentration of 8-OH-DPAT used in the competition experiments (0.25 nM) was low compared with its K_D value for the non-5-HT_{1A} binding sites (44.6 nM), additional competition experiments with 8 nM [^{3}H]-8-OH-DPAT in the presence of 1 μM WAY 100635 were performed with some of the compounds. The results obtained were similar to those obtained with 0.25 nM [^{3}H]-8-OH-DPAT in the presence of WAY 100635 (pK_i values $n=2$: 5-HT 5.88; ritanserin 6.45; fluoxetine 6.82; citalopram 8.32; TFMPP 6.30).

Although R(+)8-OH-DPAT has moderate, stereoselective affinity for human cloned 5-HT_{1D} receptors, [^{3}H]-8-OH-DPAT binding in the presence of WAY 100635 was only weakly inhibited by the selective 5-HT_{1D} versus 5-HT_{1B} ligand, ketanserin (pK_i: 5.31, $n=2$).

Because 8-OH-DPAT has also weak affinity for α_1- and α_2-adrenoceptors and for dopamine D_2 receptors, selective antagonists at these receptors (prazosin, idazoxan, and raclopride, respectively) were tested. Their pK_i values for [^{3}H]-8-OH-DPAT in the presence of WAY 100635 and for [^{3}H]-citalopram sites were both less than 5. The pK_i of WAY 100635 for [^{3}H]-citalopram was also less than 5.

Discussion

The major finding of the present study is that, in the presence of the selective 5-HT_{1A} antagonist WAY 100635, the tritiated derivative of the prototypic 5-HT_{1A} agonist 8-OH-DPAT labelled non-5-HT_{1A} binding sites in the raphe area of the rat brain. Inhibition of [^{3}H]-8-OH-DPAT binding to these non-5-HT_{1A} binding sites with a series of compounds correlated well with their affinity for 5-HT uptake sites. Interestingly, however, R(+)8-OH-DPAT appeared to be an outlier, suggesting that 8-OH-DPAT binds to raphe sites other than 5-HT_{1A} binding sites and 5-HT uptake sites.

In addition to its 5-HT_{1A} agonist properties, 8-OH-DPAT has been reported to inhibit 5-HT uptake *in vitro* (Hamon *et al*., 1984) and *in vivo* (Assié & Koek, 1996). Labelling of 5-HT uptake sites by 8-OH-DPAT has been reported previously in the bovine raphe (Sprouse *et al*., 1993), consistent with the present data. The results of the present study indicate that [^{3}H]-8-OH-DPAT binds to 5-HT_{1A} and to non-5-HT_{1A} binding sites in the raphe area of the rat brain and in rat hippocampus. The proportion of non-5-HT_{1A} binding sites, however, is much higher in the raphe (36%) than in the hippocampus (5%). The potent and selective 5-HT_{1A} antagonist, WAY 100635 (Forster *et al*., 1995), also appears to bind to 5-HT_{1A} and to non-5-HT_{1A} binding sites in the raphe. The low affinity of WAY 100635 for non 5-HT_{1A} binding sites could not be characterized accurately, the K_i being close to the highest concentration

Table 2 Estimated affinity (pK_i) of various drugs for sites labelled by 0.25 nM [^{3}H]-8-OH-DPAT in the presence of 1 μM WAY 100635, and for sites labelled by 0.5 nM [^{3}H]-citalopram

	Compound	*[^{3}H]-8-OH-DPAT*	*[^{3}H]-citalopram*	*ratio**
1	5.HT	6.04±0.07	6.45±0.01	0.378
2	(±)8-OH-DPAT	7.32±0.15	6.52±0.10	5.85
3	R(+)8-OH-DPAT	7.61±0.09	6.52±0.15	13.26
4	S(−)8-OH-DPAT	6.03±0.03	6.56±0.18	0.341
5	paroxetine	8.38±0.10	10.21±0.05	0.014
6	indatraline	7.57±0.13	9.58±0.05	0.009
7	citalopram	8.26±0.19	9.07±0.05	0.130
8	fluoxetine	7.19±0.01	8.43±0.09	0.060
9	imipramine	6.78±0.11	7.96±0.03	0.063
10	dextromethorphan	6.96±0.14	7.63±0.10	0.203
11	1-naphtylpiperazine	6.56±0.12	6.61±0.13	0.898
12	TFMPP	6.36±0.08	6.31±0.08	1.13
13	ritanserin	6.60±0.13	6.30±0.04	1.82
14	risperidone	6.21±0.12	6.01±0.03	1.50
15	quipazine	7.15±0.16	7.63±0.06	0.294
16	RU24969	6.45±0.13	7.17±0.02	0.174
17	roxindole	8.39±0.01	9.18±0.13	0.173

Competition experiments were performed in membrane homogenates from the raphe area of the rat. Data were analysed with non-linear curve fitting program Kell, Radlig. Values are means±s.e.mean of three determinations each performed in duplicate ([^{3}H]-8-OH-DPAT) or triplicate ([^{3}H]-citalopram). *ratio of K_i values [^{3}H]-citalopram/[^{3}H]-8-OH-DPAT.

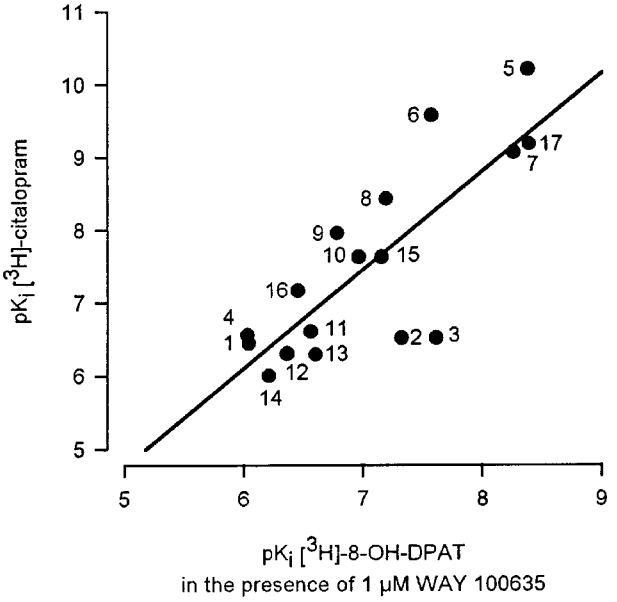

Figure 4 Correlation between affinity values of various compounds expressed as pK_i (see Table 2) for the sites labelled by [^{3}H]-8-OH-DPAT in the presence of 1 μM WAY100635 and for [^{3}H]-citalopram binding sites ($r=0.80$, $P<0.001$).

tested, but may correspond to the 5-HT uptake site. Because there was a more than 4 log unit separation between the affinities of WAY 100635 for the two sites, the concentration of 1 μM WAY 100635 was considered to be sufficient and selective to mask all 5-HT_{1A} receptors. In the presence of 1 μM WAY 100635, [^{3}H]-8-OH-DPAT appears to label 5-HT uptake sites. Indeed, in addition to 5-HT, 8-OH-DPAT and its enantiomers, selective and non selective 5-HT uptake inhibitors, as well as other serotonergic compounds possessing 5-HT uptake inhibiting properties, inhibited the non-5-HT_{1A} binding of [^{3}H]-8-OH-DPAT. Because the concentration of [^{3}H]-8-OH-DPAT used to label non-5-HT_{1A} binding sites (0.25 nM) was low compared with its K_D value at these sites (44.7 nM), competition experiments with some of the compounds were performed with 8 nM [^{3}H]-8-OH-DPAT and produced results similar to those obtained with 0.25 nM. The significant positive correlation between pKi values for [^{3}H]-citalopram binding and for [^{3}H]-8-OH-DPAT binding in the presence of WAY 100635 suggest that the two ligands may label the 5-HT uptake sites. This is further suggested, from saturation experiments, by the similar estimated B_{max} values for both ligands.

The results of the present study, however, suggest that, in addition to 5-HT uptake sites, other binding sites may be involved in the non-5-HT_{1A} binding of 8-OH-DPAT. Indeed, the ratio of K_i values at the site labelled by [^{3}H]-8-OH-DPAT in the presence of WAY 100635 and at the site labelled by [^{3}H]-citalopram varied markedly among the compounds tested (Table 2). Interestingly, the two isomers of 8-OH-DPAT appear to have different affinities for the sites labelled by [^{3}H]-8-OH-DPAT, but similar affinities for 5-HT uptake sites. R(+)8-OH-DPAT has about a 40 fold higher affinity than S(−)8-OH-DPAT for the former sites, suggesting that the (+) isomer may have some selectivity for non-5-HT_{1A}, non-5-HT uptake sites labelled by 8-OH-DPAT in the raphe.

Although the affinity of 8-OH-DPAT for the non-5-HT_{1A} binding sites is similar to its affinity for 5-HT_7 receptors (K_i: 35–52 nM) (Shen *et al.*, 1993; Ruat *et al.*, 1993), it is unlikely that these latter receptors are involved in the non-5-HT_{1A} binding of [^{3}H]-8-OH-DPAT reported here, because 5-HT has much higher affinity for 5-HT_7 receptors (0.6–1.5 nM) than for the non-5-HT_{1A} sites (941 nM). It would be interesting to know if the isomers of 8-OH-DPAT have some selectivity for 5-HT_7 receptors. To the best of our knowledge, however, these data are not available.

8-OH-DPAT has moderate affinity for cloned human 5-HT_{1D} receptors and lower affinity for 5-HT_{1B} receptors (Pauwels & Colpaert, 1996), and shows stereoselectivity at these sites, with R(+)8-OH-DPAT being 10 times more potent than S(−)8-OH-DPAT. The same authors (Pauwels *et al.*, 1995) have shown previously that ketanserin has about a 100 fold selectivity for human cloned 5-HT_{1D} versus 5-HT_{1B} receptors. In the present study, ketanserin had only very low affinity for the non 5-HT_{1A} binding sites of 8-OH-DPAT. Thus, it seems unlikely that 5-HT_{1D} receptors are involved in the non-5-HT_{1A} binding of 8-OH-DPAT examined here.

In addition to its affinity for the above mentioned 5-HT binding sites, 8-OH-DPAT has weak affinity for α_2-adrenoceptors (e.g., Brown *et al.*, 1990), for α_1-adrenoceptors, and for

dopamine D_2 receptors (e.g., Kleven *et al*., 1997). The non-5-HT_{1A} binding sites of [^{3}H]-8-OH-DPAT, however, do not appear to be α_1- or α_2-adrenoceptors, or dopamine D_2 receptors, because selective ligands at these receptors (prazosin, idazoxan, and raclopride, respectively), have no detectable affinity for the non-5-HT_{1A} binding sites.

Thus, in addition to 5-HT_{1A} receptors and 5-HT uptake sites, 8-OH-DPAT might recognise other, as yet unknown, serotonergic binding sites. Among the compounds tested here, R(+)8-OH-DPAT was found to have affinity for, and the highest selectivity at these additional, non-5-HT_{1A} binding sites. Thus, labelled R(+)8-OH-DPAT may be a useful tool to explore further the apparently novel serotonergic binding sites with which 8-OH-DPAT interacts in the raphe.

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