Characterization and distribution of putative 5-HT₇ receptors in guinea-pig brain

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1 In the presence of (–)-cyanopindolol (1.0 μ M) and sumatriptan (1.0 μ M), 0.5 nM [³H]-carboxamidotryptamine ([³H]-5-CT) labelled a single population of receptors in guinea-pig cerebral cortex membranes.

2 5-HT-displaceable binding was rapid, saturable and reversible. A high affinity binding site was characterized both by equilibrium saturation ($K_d = 0.76 \pm 0.28$ nM; $B_{max} = 68.1 \pm 26.7$ fmol mg^{–1} protein) and kinetic ($K_d = 0.18 \pm 0.05$ nM) analysis. The pharmacological profile of this site was similar to the profile obtained in transfected CHO-K1 cells expressing guinea-pig 5-HT₇ receptors.

3 Autoradiographic analysis revealed a discrete localization of binding sites in guinea-pig brain, with the highest density of sites in the medial thalamic nuclei and related limbic and cortical regions. Moderate levels of binding were detected in sensory relay nuclei, substantia nigra, hypothalamus, central grey and dorsal raphe nuclei. This distribution corresponded to that observed using *in situ* hybridization with [³⁵S]-UTP labelled riboprobes complementary to mRNA encoding the guinea-pig 5-HT₇ receptor.

4 In conclusion, under appropriate conditions, [³H]-5-CT labelled a single population of saturable binding sites that corresponded to an endogenous 5-HT₇ receptor in guinea-pig brain. The distribution of 5-HT₇ receptors in thalamocortical and limbic brain regions suggests a role for these receptors in sensory and affective behaviours.

Keywords: 5-Carboxamidotryptamine; 5-hydroxytryptamine; 5-HT receptors; guinea-pig thalamus; guinea-pig limbic system; 5-HT₇; autoradiography; *in situ* hybridization

Introduction

5-Hydroxytryptamine (5-HT) exerts a wide variety of behavioural and physiological effects through actions on multiple receptor subtypes. Pharmacological and molecular cloning approaches have identified at least fourteen distinct subtypes of mammalian 5-HT receptors, classified into seven families with unique structural, transductional and operational characteristics (Martin & Humphrey, 1994; Hoyer *et al.*, 1994). Endogenous functional equivalents have been identified for four major classes (5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄). Where functional correlates have not yet been identified (i.e. 5-HT₅, 5-HT₆, and 5-HT₇), the nomenclature recommendation has been to use the lowercase appellation (Hoyer *et al.*, 1994).

The 5-HT₇ receptor has been cloned from rat, mouse, guinea-pig and human cDNA (Plassat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Meyerhof *et al.*, 1993; Lovenberg *et al.*, 1993; Tsou *et al.*, 1994; Bard *et al.*, 1993). Despite a high degree of interspecies homology (95%), the receptor sequence exhibits low amino acid sequence homology (<40%) with other 5-HT receptors. The cDNA contains introns and predicts encoding of a seven-transmembrane receptor with a long carboxyl terminus. Cells transfected with the cDNA encoding the 5-HT₇ receptor express functional receptors coupled positively to adenylyl cyclase. The pharmacological profile of the 5-HT₇ receptor is unique but consistent across species (Eglen *et al.*, 1994b; Boess & Martin, 1994). 5-HT₇ receptors exhibit high affinity (pK_i 8.1–9.9) for 5-carboxamidotryptamine (5-CT), 5-HT, and 5-methoxytryptamine (5-MeOT), moderate affinity (pK_i 6.4–7.8) for (±)-2-dipropyl-amino-8-hydroxy-1,2,3,4-tetrahydronaphthalene (8-OH-DPAT), methysergide, ergotamine, and spiperone, and low affinity (pK_i <6.0) for pindolol, sumatriptan, and buspirone. The greatest abundance of 5-HT₇ mRNA is found in the brain, where it is localized to the thalamus, hypo-

thalamus, neocortex, olfactory tubercle, brainstem, and limbic regions (Plassat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Tsou *et al.*, 1994).

An important step for the classification of a cloned and expressed receptor is the demonstration of endogenous pharmacological correlates. Functional assays have revealed the presence of receptors that may correspond to the 5-HT₇ subtype in guinea-pig hippocampus (Shenker *et al.*, 1987; Tsou *et al.*, 1994; Alvarez *et al.*, unpublished) and several peripheral tissues including porcine vena cava (Sumner *et al.*, 1989), dog coronary artery (Cushing & Cohen, 1992), marmoset aorta (Dyer *et al.*, 1994) and guinea-pig ileum (Feniuk *et al.*, 1983; Eglen *et al.*, 1994a). To date, however, little is known about the binding characteristics or central nervous system distribution of endogenous 5-HT₇ receptors due, in part, to the lack of specific ligands.

The aim of the present study was to utilize [³H]-5-carboxamidotryptamine ([³H]-5-CT), to characterize, locate and quantify the density of endogenous 5-HT₇ receptors in guinea-pig brain. The binding profile of a pharmacologically isolated receptor was correlated with the cloned guinea-pig 5-HT₇ receptor. The distribution was examined by quantitative autoradiography and compared to that obtained using *in situ* hybridization of mRNA encoding this receptor.

A preliminary report of these data has been presented (Jakeman *et al.*, 1994a).

Methods

CHO-K1 cell binding studies

CHO-K1 cells were cotransfected with 4 mg pSW2-7c No. 3 (Tsou *et al.*, 1994) and 1 mg pSV2Neo in 100 mm dishes using the lipofectin method (Felgner *et al.*, 1987). Cells stably expressing the cloned guinea-pig 5-HT₇ receptor were grown in F-12 media supplemented with 10% foetal bovine serum (GIBCO-BRL) and harvested with 0.1% Na₂ EDTA diluted

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1:10 in phosphate buffered saline. Cells were homogenized (0.5 million cells ml⁻¹ of buffer) in Tris-EDTA buffer (composition, mM: Tris-base 50, Na₂ EDTA 0.5, MgSO₄ 10, CaCl₂ 2, pargyline 0.01, ascorbate 0.1%, pH 7.4 at 4°C). The cell homogenate was centrifuged at 45,000 g for 12 min, and the membrane pellet was washed, rehomogenized, and centrifuged twice.

For saturation experiments, cell membranes were incubated for 2 h at room temperature with 0.03–10 nM [³H]-5-CT in the above buffer. In competition studies, membranes were incubated with approximately 0.5 nM [³H]-5-CT for 2 h at room temperature in the presence or absence of competing drugs. Nonspecific binding was defined with 1.0 μM 5-HT. Bound and free radioactivity were separated by rapid filtration through 0.3% polyethylenimine (PEI) pretreated GF/B microplates and washed twice with ice-cold 50 mM Tris-HCl buffer (pH 7.4) on a Packard Filtermate cell harvester. Trapped radioactivity was counted in a Packard Top Count scintillation counter (Downers, Inc.).

Guinea-pig cortical membrane binding studies

Guinea-pig cerebral cortex tissue was dissected from whole guinea-pig brains (Rockland, Inc.), and homogenized (20 mg wet tissue ml⁻¹ buffer) as described above. The tissue pellet was rehomogenized and incubated at 37°C for 20 min. The tissue homogenate was then resuspended and rehomogenized twice. Membranes were incubated with [³H]-5-CT for 2 h in Tris-EDTA buffer (pH 7.4 at 21–23°C) containing 1.0 μM (–)-cyanopindolol and 1.0 μM sumatriptan, and filtered as described above.

For association studies, cortical membranes were incubated in 0.5 nM [³H]-5-CT for 0 to 3 h in the absence (total) or presence (non-specific) of 1.0 μM 5-HT, and the binding was then dissociated with 1.0 μM 5-HT for 0 to 2.5 h. Kinetic experiments were terminated by rapid filtration with 50 mM Tris-HCl buffer through 0.3% PEI-pretreated GF/B glass fibre filters. Bound radioactivity was determined using a Packard 2500R scintillation analyzer.

Saturation experiments in cortical membranes were performed as described for the cells. Competition studies were performed using 0.30–0.75 nM [³H]-5-CT. Filtermats were adhered to Multilex melt-on scintillator sheets and counted on a 1204 Betaplate TM BS Liquid Scintillation counter (Wallac Inc.).

Autoradiography

Six adult male Dunkin Hartley guinea-pigs (350–425 g) were killed by asphyxiation with CO₂ and the brains were removed and frozen on dry ice. Sequential sections of 20 μm thickness were cut at –20°C (2800E Jung Frigocut; Leica, Inc.), thaw-mounted onto gelatin-coated slides and stored for 1–7 days at –20°C. Sections were thawed and dried at room temperature, preincubated in 50 mM Tris buffer pH 7.4 for 30 min at room temperature, and then incubated for 2 h at room temperature in Tris-EDTA buffer containing 1.0 nM [³H]-5-CT in the presence of 3.0 μM (–)-cyanopindolol and 3.0 μM sumatriptan. Higher concentrations of [³H]-5-CT, (–)-cyanopindolol and sumatriptan were used in these experiments based on previous studies that have shown a 3–5 fold lower affinity of 5-HT ligands for receptors in tissue sections as compared with homogenate binding studies (Jakeman *et al.*, 1994b; Jakeman *et al.*, unpublished observations). Nonspecific binding was determined on adjacent sections by the addition of 1.0 μM 5-HT. After incubation, the slides were washed twice for 10 min in ice-cold 50 mM Tris (pH 7.4), dipped for 5 s in cold water, and dried quickly under a stream of cold air. Slides were apposed to autoradiographic film for 20 weeks and films developed in Kodak D-19.

In situ hybridization

The distribution of 5-HT₇ mRNA was determined in four separate experiments by *in situ* hybridization as described previously (Jakeman *et al.*, 1993; Tsou *et al.*, 1994). Briefly, [³⁵S]-UTP labelled cRNA probes were prepared from sense and antisense templates corresponding to the full open reading frame of the cloned guinea-pig 5-HT₇ receptor (Tsou *et al.*, 1994) using the Gemini II system (Promega Corp.). Sections were hybridized overnight at 55°C and washed for 1 h at 55°C in 0.1 × saline sodium citrate (SSC). Dried slides were apposed to autoradiographic film for 7–10 days.

Compounds

[³H]-5-CT (50.4 Ci mmol⁻¹) was purchased from Dupont/New England Nuclear (Boston, MA, U.S.A.). Amoxapine, atropine sulphate, bufotenine monooxalate, (+)-butaclamol, 5-CT, clozapine, cyproheptadine hydrochloride, epidine, haloperidol, histamine dihydrochloride, ketanserin tartrate, lisuride hydrogen maleate, (+)-lysergic acid diethylamide tartrate ((+)-LSD), mesulergine hydrochloride, metergoline, methiothepin mesylate, 5-MeOT, methylergonovine maleate, methysergide maleate, mianserin hydrochloride, naloxone hydrochloride, octoclotheptin maleate, 8-OH-DPAT, oxy-metazoline hydrochloride, pergolide methanesulphonate, pindobind_{1A}, (–)-pindolol, pirenperone, prochlorperazine dimaleate, ritanserin, 5-hydroxytryptamine hydrochloride (5-HT), spiperone hydrochloride, terguride hydrogen maleate, theophylline, and trifluoperazine dihydrochloride were purchased from Research Biochemicals International (Natick, MA, U.S.A.). 5-Benzyloxytryptamine (5-BeOT), dipropyl-5-carboxamidotryptamine (DP-5-CT), 5-hydroxy-N-ω-methyl-tryptamine (5-OH-NωMeT), 5-methoxy-N,N-dimethyltryptamine (5-MeDMT), 6-methoxytryptamine (6-MeOT) and tryptamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Endo-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride (BIMU-1), GR113808 ([1-[2-methylsulphonyl]amino]ethyl]-4-piperidinyl] methyl 1-methyl-1H-indole-3-carboxylate), DuP996 (3,3-bis(4-pyridinyl methyl)-1-phenylindolin-2-one), ondansetron, and Rzacopride were synthesized in the Institute of Organic Chemistry (Syntex Discovery Research). All compounds were dissolved to 10 mM in 50% dimethyl sulphoxide (DMSO) and 50% ethanol and stored at –20°C up to 2 months. Stock solutions were then serially diluted over the range of 1 pM to 100 μM in experimental buffer.

Data analysis

The analyses of saturation and kinetic binding data were performed using non-linear curve fitting programmes by In-Plot Scientific Graphics (GraphPad, Inc.) to yield K_d and B_{max} estimates. Kinetic data resulted in the observed association (K_{obs}) and dissociation rate constants (K₋₁) which were used to calculate K₊₁ and K_d estimates (Molinoff *et al.*, 1981). Hill coefficients and IC₅₀ values were obtained from the analysis of competition studies by use of a 4-parameter, logistic, iterative curve fitting programme. IC₅₀ values were then converted to K_i values by the Cheng-Prusoff correction (Cheng & Prusoff, 1973). Affinity estimates were presented as the negative log of the K_i (pK_i). The correlation plot was analysed by linear regression (InPlot Scientific Graphics). All values are reported as mean ± s.e.mean from 3–8 experiments as indicated.

Autoradiographs were analysed by digital image analysis with the MCID imaging system (Imaging Research, Inc.). Specific regions of interest were defined on cresyl violet stained histological sections using a standard rat brain atlas (Paxinos & Watson, 1986). For receptor binding autoradiographs, the optical density of each region was converted to fmol radioligand bound mg⁻¹ tissue equivalent (fmol mg⁻¹

t.e.) using ³H standards (Amersham Co.) and a calibration curve was generated for each film. *In situ* hybridization autoradiographs were analysed qualitatively with optical film density scaled from 0-++ for each brain region.

Results

Establishment of radioligand binding conditions

[³H]-5-CT, at 0.5 nM, was predicted to bind to 5-HT_{1A}, 5-HT_{1D}, and 5-HT₇ receptors in guinea-pig cerebral cortex (Zifa & Fillion, 1992; Boess & Martin, 1994). To determine the appropriate concentrations of ligands required to mask binding to 5-HT_{1A} and 5-HT_{1D} receptors, the competition of 0.3–0.7 nM [³H]-5-CT binding by (–)-cyanopindolol and sumatriptan was initially investigated. Both compounds recognized two distinct binding sites in guinea-pig cortical membranes. Thus, for (–)-cyanopindolol, a high affinity site accounted for 58% of the specific binding. The estimated affinity at this site (pK_i) was 7.0, which corresponded to its affinity at 5-HT_{1A} and 5-HT_{1B} receptors (Hoyer, 1991). A low affinity component was also observed, with an approximate pK_i of 5.8. Sumatriptan bound to approximately 20% of sites with high affinity ($pK_i = 6.8$), that corresponded to its affinity at 5-HT_{1D} receptors (Hoyer *et al.*, 1985; Boess & Martin, 1994). A low affinity component was also observed for sumatriptan with an approximate pK_i of 5.2. Therefore, in subsequent experiments, 1.0 μ M (–)-cyanopindolol and 1.0 μ M sumatriptan were included in the assay buffer to isolate operationally the (–)-cyanopindolol and sumatriptan insensitive components of specific [³H]-5-CT binding.

Figure 1 Kinetics of association (a) and dissociation (b) of [³H]-carboxamidotryptamine ([³H]-5-CT) binding in guinea-pig cerebral cortex membranes in the presence of 1.0 μ M (–)-cyanopindolol and 1.0 μ M sumatriptan. $K_{obs} = 0.033$; $K_{-1} = 5.99 \times 10^{-3}$; $K_d = 0.09$ nM. Similar results were obtained in 2 additional experiments ($n = 3$) as described in text.

In the presence of these ligands, the binding of 0.5 nM [³H]-5-CT to guinea-pig cerebral cortex membranes was specific, heat-sensitive, and proportional with tissue concentration over a wide range (5 to 40 mg of wet tissue ml⁻¹). Specific (i.e. 5-HT-displaceable) binding accounted for approximately 75% of total binding and less than 1% of the total free radioligand bound to filters. The specific binding was reduced to 17% and 10% following preincubation of membranes for 20 min at 60°C and 90°C, respectively.

Receptor characterization in guinea-pig cortical membranes and comparison with cloned guinea-pig 5-HT₇ receptor

The specific binding of 0.5 nM [³H]-5-CT in guinea-pig cortex membranes was best fit with monoexponential kinetic equations (Figure 1). Specific binding reached equilibrium within 90 min and remained stable for at least 3 h. Kinetic analysis yielded a K_{obs} value of 0.023 ± 0.003 min⁻¹. The binding was reversible by 1.0 μ M 5-HT with an estimated dissociation constant (K_{-1}) value of $6.3 \pm 0.8 \times 10^{-3}$ min⁻¹. Thus, the kinetic K_d derived from these studies was 0.18 ± 0.05 nM ($n = 3$).

[³H]-5-CT binding in guinea-pig cortex membranes was saturable and best described by a model for a single receptor population. Less than 10% of the free ligand bound over the concentration-range used (Figure 2). The mean estimated K_d and B_{max} values were 0.76 ± 0.28 nM and 68.5 ± 26.6 fmol mg⁻¹ protein, respectively ($n = 4$; Figure 2). The affinity for [³H]-5-CT was similar to that estimated in CHO-K1 cells

Figure 2 (a) Saturation analysis of [³H]-5-carboxamidotryptamine ([³H]-5-CT) binding to guinea-pig cerebral cortex membranes in the presence of 1.0 μ M (–)-cyanopindolol and 1.0 μ M sumatriptan. $K_d = 0.77$ nM; $B_{max} = 45.8$ fmol mg⁻¹ protein. Total (●), non-specific (○), and specific (◆) binding. Similar results were obtained in 3 additional experiments ($n = 4$) to yield $K_d = 0.76 \pm 0.28$ nM and $B_{max} = 68.5 \pm 36.6$ fmol mg⁻¹ protein. (b) Scatchard transformation of specific binding (●) from data in (a).

Figure 3 Displacement of 0.3–0.75 nM [³H]-5-carboxamidotryptamine ([³H]-5-CT) by selected compounds in guinea-pig cerebral cortex membranes (a) and membranes from CHO-K1 cells expressing the guinea-pig 5-HT₇ receptor (b). Displacement curves are representative of all compounds listed in Table 1. Representative compounds included 5-CT (○), 5-HT (■), methiothepin (◇), tryptamine (▲) and spiperone (△). Non-linear regression analysis yielded the following K_i values for each ligand in cerebral cortex (0.68, 1.05, 62, 65, 125 nM) and CHO-K1 cells (0.26, 0.40, 4.7, 21.6, 48.9 nM). All compounds displaced [³H]-5-CT to the non-specific level defined in the presence of 1.0 μ M 5-HT. Mean and s.e.mean of pK_i values for 3–8 determinations are listed in Table 1.

stably expressing the guinea-pig 5-HT₇ receptor (0.22 ± 0.02 nM). However, in the presence of masking compounds, the maximum binding capacity in the cortex was approximately one fifth that found in the transfected cells, i.e. 68.5 ± 26.6 fmol mg⁻¹ protein compared to 393 ± 48 fmol mg⁻¹ of protein.

Several ligands competed for [³H]-5-CT binding in a monophasic manner, exhibiting a wide range of affinities (Figure 3). All ligands in Table 1 displaced [³H]-5-CT to the nonspecific level defined by 1.0 μ M 5-HT. Indoles, such as 5-CT, 5-HT, 5-MeOT, and 5-OHMeT, showed the highest affinity with pK_i values ranging from 8.2 to 9.0. Of the ergots, the two ligands with highest affinity, lisuride and pergolide, yielded pK_i values of 7.73 and 7.75, respectively. Derivatives of spiperone showed moderate affinity, the greatest affinity being exhibited by pirenperone with a pK_i value of 7.28. Octoclotheptin and methiothepin exhibited affinity estimates of 7.49 and 7.34, respectively.

Several compounds failed to displace [³H]-5-CT binding ($pK_i < 5.0$) including the derivatives of pindolol, ondansetron, GR113808, BIMU-1, R-zacopride, atropine, histamine, naloxone, epidine, theophylline, γ -aminobutyric acid and DUP996.

A comparison of the affinities of ligands at [³H]-5-CT binding sites in guinea-pig cortex and transfected cells yielded a statistically significant correlation (Figure 4; $r^2 = 0.96$; $P < 0.01$). The rank order of ligand affinity in both assays was: 5-CT, 5-HT, 5-MeOT > DP-5-CT, lisuride, pergolide > (+)-LSD, methiothepin, metergoline, tryptamine > spiperone, ritanserin, ergotamine, clozapine >> (-)-pindolol and (-)-butaclamol. All of the compounds in Table 1 showed a slightly lower affinity in the cortex binding assay than in the cloned CHO-K1 cell membranes. However, the Hill slope values (n_H) for all compounds were not different from unity.

Receptor distribution in guinea-pig brain

The binding of [³H]-5-CT to guinea-pig brain sections was specific and heterogeneous (Figures 5 and 6, Table 2). The specific binding density following incubation with 1.0 nM [³H]-5-CT ranged from 0.96 to 19.33 fmol mg⁻¹, t.e. Specific binding displaced by 5-HT accounted for more than 70% of total binding in all regions that bound 4.0 fmol of [³H]-5-CT mg⁻¹, t.e. or more.

Figure 4 Correlation of the affinity of ligands at the [³H]-5-carboxamidotryptamine ([³H]-5-CT) binding site in guinea-pig cerebral cortex membranes and membranes from CHO-K1 cells expressing the 5-HT₇ receptor. Least squares linear regression equation $y = 0.96x - 0.34$; $r^2 = 0.96$; $P < 0.01$.

The highest receptor density was observed in the medial nuclei of the thalamus and associated limbic regions. The paraventricular nuclei of the thalamus (PVA, PV, PVP) displayed the greatest density of receptors (17–19 fmol mg⁻¹, t.e.). Other high density regions (>10 fmol mg⁻¹, t.e.) included related midline thalamic nuclei (IMD, CM, and IAM) and limbic areas including the basomedial, basolateral and medial amygdala, dentate gyrus (stratum moleculare), and entorhinal cortex.

Moderate binding densities (>8 fmol mg⁻¹, t.e.) were observed in superficial cortex (layers 2–4), hippocampal regions CA1–CA2 (stratum radiatum and stratum moleculare), substantia nigra, superior colliculus, and lateral septum. Slightly lower binding (>6 fmol mg⁻¹, t.e.) was detected in medial and lateral portions of the medial geniculate nucleus, the medial hypothalamic nuclei (VMH, DMH, MPA), ventral pallidum and globus pallidus, superior colliculus, midbrain central gray, the parabrachial nuclei and dorsal raphe nucleus.

Low binding densities (<5 fmol mg⁻¹, t.e.) were observed in several regions, including the lateral thalamic nuclei (LGN and LD), the deep layers of cortex, the medial septum, caudate putamen, midbrain interpeduncular nucleus, and several brainstem nuclei. Little to no specific binding was detected in the cerebellum.

5-HT₇ mRNA distribution in guinea-pig brain

The distribution of guinea-pig 5-HT₇ receptor mRNA was determined in separate animals using *in situ* hybridization (Figures 5 and 6, Table 2). The highest levels of expression were found in the medial thalamic nuclei (PVA, PV, PVP, CM, IAD) and hippocampal formation (DG>CA3>CA2>>CA1). Moderate expression was found throughout the superficial layers of cortex (2–4), medial geniculate nucleus, amygdala, and hypothalamus. Low hybridization was associated with most midbrain and hindbrain regions. The cerebellar granule cell layer exhibited very high levels of hybridization with both sense and antisense probes. Hybridization with sense strand probes in all other brain regions was not above background.

Discussion

Selective ligands for the newer members of the 5-HT receptor family, including the 5-HT₅, 5-HT₆ and 5-HT₇ subtypes, have not yet been identified. While there are reports of endogenous responses that may correspond to 5-HT₆ and 5-HT₇ receptors (Shenker *et al.*, 1987; Cushing & Cohen, 1992; Feniuk *et al.*, 1993; Shoefter & Waeber, 1994), endogenous correlates for

Table 1 Affinity of representative ligands competing for [³H]-5-carboxamidotryptamine ([³H]-5-CT) binding sites

Compound	CHO-K1 cells (1)		Guinea-pig cortex			
	pK _i		pK _i		n _H	
	mean	s.e.mean	mean	s.e.mean	mean	s.e.mean
5-CT	9.65 ± 0.09		9.01 ± 0.07		0.93 ± 0.04	
5-HT	9.60 ± 0.24		8.87 ± 0.06		1.01 ± 0.10	
5-MeOT	9.28 ± 0.07		8.80 ± 0.09		1.01 ± 0.12	
5-OH-ME-Nw tryptamine	8.95 ± 0.02		8.19 ± 0.06		0.95 ± 0.09	
DP-5-CT	8.27 ± 0.17		7.87 ± 0.02		0.96 ± 0.07	
5-MeOT N,N tryptamine	7.88 ± 0.05		6.97 ± 0.18		0.92 ± 0.04	
Tryptamine	7.72 ± 0.03		7.19 ± 0.10		0.94 ± 0.06	
Bufotenine	7.67 ± 0.01		7.02 ± 0.14		0.91 ± 0.03	
6-MeOT	7.44 ± 0.02		6.44 ± 0.03		0.86 ± 0.07	
8-OH-DPAT	7.39 ± 0.03		6.87 ± 0.09		1.01 ± 0.05	
5-Benzoxoytryptamine	6.59 ± 0.06		5.96 ± 0.16		1.05 ± 0.09	
Lisuride	8.53 ± 0.12		7.73 ± 0.07		0.92 ± 0.03	
Pergolide	8.53 ± 0.08		7.75 ± 0.09		0.87 ± 0.04	
Metergoline	8.16 ± 0.11		7.31 ± 0.08		1.02 ± 0.07	
Terguride	8.10 ± 0.10		7.38 ± 0.05		0.95 ± 0.05	
Methylergonovine	7.96 ± 0.04		7.28 ± 0.08		1.20 ± 0.12	
(+)-LSD	7.81 ± 0.03		7.51 ± 0.09		1.05 ± 0.04	
Mesulergine	7.81 ± 0.06		7.18 ± 0.09		0.96 ± 0.06	
Methysergide	7.65 ± 0.04		7.03 ± 0.07		0.96 ± 0.05	
Ergotamine	7.31 ± 0.01		6.86 ± 0.07		1.17 ± 0.06	
Pirenpirone	7.74 ± 0.04		7.28 ± 0.17		0.88 ± 0.04	
Spiperone	7.32 ± 0.05		6.67 ± 0.07		0.92 ± 0.07	
Ritanserin	7.34 ± 0.05		6.72 ± 0.11		1.05 ± 0.09	
Ketanserin	6.20 ± 0.06		5.54 ± 0.09		0.94 ± 0.13	
Haloperidol	5.48 ± 0.11		5.03 ± 0.12		1.00 ± 0.15	
Octoclotheptin	8.60 ± 0.20		7.49 ± 0.13		1.04 ± 0.08	
Methiothepin	8.43 ± 0.09		7.34 ± 0.08		1.06 ± 0.06	
Clozapine	7.32 ± 0.14		6.83 ± 0.12		0.83 ± 0.04	
Mianserin	6.97 ± 0.10		6.18 ± 0.07		1.05 ± 0.15	
Cyproheptadine	6.90 ± 0.03		6.30 ± 0.13		0.92 ± 0.04	
Amoxapine	6.73 ± 0.09		5.85 ± 0.05		0.90 ± 0.05	
Butaclamol	6.72 ± 0.01		6.06 ± 0.07		0.98 ± 0.04	
Trifluoroperazine	5.84 ± 0.04		<5			
Prochlorperazine	<5		<5			
Pindobind-5-HT _{1A}	5.72 ± 0.03		<5			
(-)-Pindolol	<4		<5			

Values represent 3–8 determinations. [³H]-5-CT (0.5 nM) binding in the presence of sumatriptan (1.0 μM) and (-)-cyanopindolol (1.0 μM). 5-HT (1.0 μM) was used to define non-specific binding. (1) Affinity values determined in CHO-K1 cells expressing guinea-pig 5-HT₇ receptor. n_H values in cloned cells were not different from unity (Alvarez *et al.*, unpublished).

Figure 5 Distribution of 5-HT₇ receptor binding and mRNA in guinea-pig coronal forebrain sections. (a–d) Histological sections stained with 0.25% Cresyl violet. (e–h) Autoradiographs of total binding from same sections following incubation in 1.0 nM [³H]-5-carboxamidotryptamine ([³H]-5-CT) in the presence of (–)-cyanopindolol and sumatriptan. (i–l) Autoradiographs from sections adjacent to those in h–n, with non-specific binding defined in the presence of 1.0 μM 5-HT. (m–p) Autoradiographs following *in situ* hybridization using [³⁵S]-UTP-labelled antisense (m, n, o) and sense (p) strand riboprobes. Darker areas correspond to higher mRNA levels. Section in panel m from a different experiment from n–p. Hybridization in ventromedial hypothalamus is dense just lateral to suprachiasmatic nuclei (arrowhead). Hybridization using sense strand probe was not above background except in hippocampal dentate gyrus (DG**). Abbreviations are defined in Table 2.

these receptor subtypes have not been clearly isolated. In the present study, a distinct binding site in guinea-pig cerebral cortex membranes was evaluated. This site has a pharmacological profile that corresponds closely to the cloned 5-HT₇ receptor and a distribution similar to the distribution of 5-HT₇ mRNA in guinea-pig brain.

[³H]-5-CT has been used previously to label 5-HT₇ receptors in transfected cells (Plassat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Bard *et al.*, 1993; Lovenberg *et al.*, 1993). However, this radioligand exhibits nanomolar affinity at 5-HT₁, 5-HT₂, 5-HT₅, 5-HT₇, and cloned 5-HT₄ receptors (Zifa & Fillion, 1992; Boess & Martin, 1994; Adham *et al.*, 1994). In contrast, [³H]-5-CT is more selective, since it binds with nanomolar affinity only to 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT₇ subtypes (Hoyer *et al.*, 1985; Heuring & Peroutka, 1987; Hoyer, 1991; Nowak *et al.*, 1993). In the present study, a single population of receptors was isolated from guinea-pig cerebral cortex membranes using [³H]-5-CT in the presence of (–)-cyanopindolol and sumatriptan to occupy 5-HT_{1A} and 5-HT_{1D} receptors. The specific binding under these conditions was saturable, reversible, and described by a single site receptor model in equilibrium, kinetic and competition analyses. All compounds displaced the radioligand to the non-specific level defined by 5-HT, indicating that no non-5-HT sites were labelled. However, it is likely that not all 5-HT₇ receptors were available for binding, as the masking compounds both have sufficient affinity for 5-HT₇ receptors to occupy a proportion of sites at 1.0 μM.

The affinities of several structurally diverse compounds that competed for [³H]-5-CT binding sites was compared to those obtained in CHO-K1 cells stably expressing the cloned guinea-pig 5-HT₇ receptor. The rank orders of affinity were highly correlated, suggesting pharmacological identity. To verify an absence of other 5-HT receptor subtypes in the cortex binding assay, several compounds that distinguish known 5-HT receptors were used. A lack of 5-HT₁ and 5-HT₂ receptors was suggested by the low affinity of 8-OH-DPAT (5-HT_{1A}), pindolol derivatives (5-HT_{1A}, 5-HT_{1B}) and ergotamine (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{5A,5B}). Subnanomolar concentrations of [³H]-5-CT were not expected to label 5-HT_{1E}, 5-HT_{1F}, 5-HT₂, 5-HT₃, 5-HT₄ or 5-HT₆ receptors (Zifa & Fillion, 1992; Boess & Martin, 1994). The absence of these receptors was further suggested by the poor activity of sumatriptan (5-HT_{1F}), ritanserin and mianserin (5-HT₂), ondansetron (5-HT₃) and GR113808 (5-HT₄).

Although the correlation between brain tissue and cloned receptor binding is very good, all the compounds tested were weaker in the cortical membranes. The reason for this is not clear. A possible factor may be that the assay relies on binding of an agonist radioligand. Large differences have been reported for agonist binding affinity (K_d) and functional potency (EC_{50}) in the cloned 5-HT₇ receptors (Bard *et al.*, 1993; Tsou *et al.*, 1994), suggesting that coupling efficiency of this receptor can greatly affect potency. In addition, some differences may exist in the relationship between the receptor and associated G-proteins in the overexpressed conditions as

Figure 6 Distribution of 5-HT₇ receptor binding and mRNA in guinea-pig coronal midbrain and hindbrain sections. Sections labelled as in Figure 5 (a–d) Cresyl violet; (e–h) total binding, autoradiography; (i–l) non-specific binding, autoradiography; (m–p) *in situ* hybridization using antisense (m, n, o) and sense (p) strand ³⁵S-labelled riboprobes. Hybridization using sense strand probe was not above background except in cerebellar granule cell layer (Cb**). Abbreviations are defined in Table 2.

opposed to the endogenous tissues. Such differences might affect the observed affinity of ligands competing with an agonist binding site. Further studies using a radiolabelled antagonist may address this issue directly.

A heterogeneous distribution of [³H]-5-CT binding sites was identified in guinea-pig brain sections with the highest binding densities in thalamo-cortical and limbic system areas. A qualitatively similar distribution was recently reported for 5-HT₇ receptors in rat and guinea-pig using [³H]-5-CT in the presence of 100 nM PAPP (4-[2-[4-[3-(trifluoromethyl)phenyl]-1-piperazinyl]ethyl]benzene amide) and 160 nM (–)-pindolol (Branchek *et al.*, 1993).

This distribution corresponded closely to the distribution of 5-HT₇ mRNA, although some differences were observed. For example, 5-HT₇ mRNA in hippocampus was highest in the dentate gyrus and CA3 region, while [³H]-5-CT binding was highest in the dentate gyrus and CA1-CA2 regions. These observations may suggest that 5-HT₇ receptors are located presynaptically on the Shaffer collaterals and associational projections of CA3 pyramidal neurones. In contrast, receptors on dentate granule cells may be located both pre- and postsynaptically. The moderate binding density in brain regions with undetected mRNA levels, including the substantia nigra, central grey, superior colliculus and trigeminal nucleus (sp5) may reflect a presynaptic location of 5-HT₇ receptors in these brain areas (Branchek *et al.*, 1993).

In many brain areas, the distribution of binding sites was complementary to that of other 5-HT receptors, particularly the 5-HT₁ subtypes (Waeber *et al.*, 1989; Miquel *et al.*, 1991; Pompeiano *et al.*, 1992; del Arco *et al.*, 1993; Bruinvels *et al.*, 1994). Overlapping 5-HT₇ and 5-HT₁ receptor distributions were found in lateral septum, entorhinal cortex, CA1-CA2

subfields of the hippocampus, amygdala, substantia nigra, superior colliculi, and hypothalamus, and claustrum (5-HT_{1E}, Barone *et al.*, 1993). The areas of similar distribution may reflect opposing regulation of adenyl cyclase activity by 5-HT.

Based on their distribution, functional implications of 5-HT₇ receptors may be suggested. Thus, binding in midline thalamic, limbic, and cortical structures suggest a role in affective behaviours (Paxinos, 1985; Turner & Herkenham, 1991). Moderate receptor densities in the medial geniculate nucleus, superior and inferior colliculi, central grey, and spinal trigeminal nuclei are also consistent with a role in the modulation of sensory information. Finally, receptors in substantia nigra and ventral pallidum would be consistent with possible actions of 5-HT₇ receptors in motor behaviours (Shen *et al.*, 1993; Branchek *et al.*, 1993).

Other roles for 5-HT₇ receptors activation can be inferred from the pharmacological binding profile. For example, 5-HT₇ receptors exhibit moderate to high affinity for clozapine, octoclothepein, pimozide, and risperidone. This has led to the hypothesis that activity at these receptors may contribute to the antipsychotic efficacy of these drugs (Roth *et al.*, 1994). Indeed, the low receptor density in striatum suggests that novel compounds acting selectively at the 5-HT₇ receptor subtype would have fewer extrapyramidal side effects.

The moderate affinity of 8-OH-DPAT at 5-HT₇ receptors is also important for proposing functional roles for these receptors. 8-OH-DPAT has been previously classified as a highly specific 5-HT_{1A} receptor agonist (Gozlan *et al.*, 1983; Boess & Martin, 1994). However, 8-OH-DPAT also has moderate affinity for the 5-HT₇ receptor. Thus, several behavioural responses previously ascribed to 5-HT_{1A} receptor activation may

also reflect involvement of 5-HT₇ receptors. For example, administration of 8-OH-DPAT can mimic 5-HT modulation of circadian rhythms in hypothalamic tissue slices. This effect has now been mimicked by cyclic AMP analogues and blocked by ritanserin, but not by pindolol, possibly implicating 5-HT₇ receptors in the modulation of circadian rhythms (Lovenberg *et al.*, 1993).

In summary, radioligand binding techniques have been used to identify a specific [³H]-5-CT binding site in guinea-pig brain with a pharmacological profile and distribution similar to the cloned 5-HT₇ receptor. The development of a radioligand binding assay may prove useful for characterization of 5-HT₇ receptors.

Table 2 Distribution of 5-HT₇ receptors in coronal guinea-pig brain sections

Region		Receptor autoradiography Specific bound (fmol mg ⁻¹ , t.e.)	s.e.mean	% spec. binding	In situ hybridization Optical density (see footnote)
Thalamus					
paraventricular n., anterior	PVA	19.33	±2.25	94%	++++
paraventricular n., poster.	PVP	17.77	±1.76	92%	+++
paraventricular n.	PV	17.28	±2.48	88%	+++
intermediodorsal n.	IMD	13.05	±1.48	87%	++
central medial n.	CM	10.67	±2.01	83%	+++
interanterodorsal n.	IAM	11.74	±1.82	87%	+++
medial geniculate n., ventral	MGV	8.16	±1.01	84%	++
medial geniculate n., medial	MGM	7.29	±0.88	84%	++
mediodorsal n.	MD	6.96	±1.52	80%	++
centrolateral n.	CL	5.99	±0.60	80%	++
lateral geniculate n.	LGN	4.88	±0.75	71%	+
laterodorsal n.	LD	1.70	±0.40	49%	+
Hippocampus					
dentate gyrus (mol. layer)	DG	14.77	±1.17	86%	++++**
CA1 (all layers)	CA1	9.64	±0.60	77%	±
CA2 (all layers)	CA2	9.48	±0.39	80%	++
CA3 (all layers)	CA3	5.77	±0.54	81%	+++
Cerebral cortex					
entorhinal cortex	Ent	11.79	±1.12	87%	++
parietal cortex, layers 2-4	Par	10.27	±1.19	89%	++
occipital cortex, layers 2-4	Oc	9.91	±1.42	84%	++
frontal cortex, layers 2-4	Fr	9.21	±0.97	88%	++
temporal cortex, layers 2-4	Te	9.09	±1.09	84%	++
cingulate cortex	Cg	7.29	±0.87	83%	++
perirhinal cortex	PRh	7.06	±0.91	85%	++
parietal cortex, layers 5-6	Par (5-6)	4.24	±0.59	76%	±
Amygdala					
medial amygdala n.	MeA	11.42	±0.87	86%	+
basomedial n.	BMA	10.72	±1.84	87%	++
basolateral n.	BLA	10.58	±1.63	89%	++
Septum and hypothalamus					
lateral septum	LS	8.68	±1.05	85%	+
ventromedial hypothal. n.	VMH	7.15	±0.96	86%	+++*
dorsomedial hypothal. n.	DMH	6.20	±0.85	76%	+
medial preoptic area	MPA	6.03	±1.04	83%	+
medial septum	MS	4.16	±0.99	75%	-
Basal ganglia					
claustrum	CI	9.17	±0.96	88%	+
ventral pallidum	VP	7.21	±1.17	84%	-
globus pallidus	GP	6.83	±1.04	81%	-
caudate-putamen	CPu	2.85	±0.44	68%	-
Midbrain					
substantia nigra, pars retic.	SNR	9.66	±1.04	87%	-
superior colliculus	SC	8.87	±0.76	84%	-
dorsal inferior colliculus	DCIC	6.76	±0.80	81%	+++
central grey	CG	6.31	±1.01	78%	+
interpeduncular n.	IPDN	4.04	±0.32	76%	-
zona incerta	ZI	3.12	±0.45	64%	+
Hindbrain					
dorsal raphe n.	DR	7.14	±0.33	79%	±
parabrachial n.	PB	6.07	±0.83	81%	±
locus coeruleus	LC	5.82	±0.78	77%	-
spinal trigeminal n.	Sp5	5.36	±0.63	81%	-
prepositus hypoglossal n.	PrH	4.38	±0.41	74%	-
n. solitary tract	Sol	4.23	±0.35	74%	±
hindbrain reticular form.	Ret	3.88	±0.49	72%	+
median raphe n.	MR	3.80	±0.40	73%	-
cerebellum	Cb	0.96	±0.21	41%	++++**

Receptor autoradiography values obtained by digital image analysis of [³H]-5-carboxamidotryptamine ([³H]-5-CT) binding for *n* = 6 brains. Qualitative analysis of ³⁵S-labelled riboprobe *in situ* hybridization (*n* = 4) expressed as relative optical density from autoradiographs. Key: +++++, very dark; ++++, dark; ++, moderate; +, light; ±, very light; -, none detected over sense control background.

*mRNA ventrolateral to suprachiasmatic nucleus; **dark hybridization in sense strand control sections.

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