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Functional characterization of the 5-HT terminal autoreceptor in the guinea-pig brain cortex

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1 In guinea-pig cerebral cortical slices *in vitro* we have shown that the rank order of potency of 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine and sumatriptan for inhibition of electrically stimulated [^{3}H]-5-HT release correlates well with published data on their 5-HT_{1D} receptor binding affinites.

2 Both the non-selective 5-HT_{1D} receptor antagonist, methiothepin and the selective 5-HT_{1D} receptor antagonist, N-[4-methoxy-3-(4-methyl-1-piperazinyl]phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazole-3-yl) [1,1-biphenyl]4-carboxamide (GR127935) increased stimulated [^{3}H]-5-HT release *per se* and also attenuated agonist-induced inhibition of [^{3}H]-5-HT release. GR127935 (10 nM-100 nM) produced a pA_2 of 9.0 against 5-HT, which is consistent with its 5-HT_{1D} receptor binding affinity.

3 From these findings we conclude that, in guinea-pig cerebral cortex, the 5-HT terminal autoreceptor is of the 5-HT_{1D} receptor subtype. However, three observations suggest the presence of multiple terminal autoreceptors: shallow inhibition curves to the agonists; a shallow Schild slope of GR127935 antagonism and differences in the maximal responses to 5-HT between whole cortex and frontal cortex.

Keywords: [^{3}H]-5-HT release; 5-HT_{1D} receptor; 5-HT autoreceptor; guinea-pig cerebral cortex

Introduction

Terminal 5-hydroxytryptamine (5-HT) autoreceptors in the rat are of the 5-HT_{1B} subtype (Engel *et al.*, 1986; Middlemiss & Hutson, 1990) but differ in pharmacological specificity from that of other species such as guinea-pig, rabbit, pig and human. Receptor agonist and antagonist potencies indicate that the 5-HT terminal autoreceptors in the latter species correlate with 5-HT_{1D} receptor binding (Fink *et al.*, 1988; Middlemiss, 1988; Hoyer & Middlemiss, 1989; Schlicker *et al.*, 1989; Starke *et al.*, 1989; Limberger *et al.*, 1991; Maura *et al.*, 1993).

Definitive pharmacological characterization of the 5-HT terminal autoreceptor has been difficult, due to the lack of selective ligands. However, with the identification of the selective 5-HT_{1D} receptor antagonist, GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazole-3yl)[1,1-biphenyl]-4-carboxamide, Skingle *et al.*, 1993) it is now possible to reinvestigate the hypothesis that the terminal autoreceptor is of the 5-HT_{1D} subtype.

Recent receptor cloning studies have revealed that there are at least two 5-HT_{1D} receptors: 5-$HT_{1D\alpha}$ (Hamblin & Metcalf, 1991; Weinshank *et al.*, 1992) and 5-$HT_{1D\beta}$ subtypes (Weinshank *et al.*, 1992). These findings have raised the question of which subtype, if either, or indeed both, correspond to terminal autoreceptors. There is limited evidence for the existence of more than one 5-HT terminal autoreceptor. Limberger and colleagues (1991) using the technique of *in vitro* [^{3}H]-5-HT release, suggested that both 5-HT_{1D} and 5-HT_{1B} are autoreceptors in rat brain. In addition Wilkinson & Middlemiss (1992) using the same technique, reported that pA_2 determinations for methiothepin varied depending on the agonist used. Recently we reported (Price *et al.*, 1993) that agonist inhibition curves to 5-HT, 5-CT and sumatriptan did not conform to a classical one site model, suggesting that there were multiple receptor subtypes.

5-HT fibres arise from two major nuclei in the brain: the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN). Some brain areas are preferentially innervated with fibres from one nucleus while others receive a mixed innervation (Kosofsky & Molliver, 1987). Therefore, there is a possibility that fibres arising from different raphe nuclei may contain different populations of autoreceptors. To investigate this possibility we compared 5-HT release from the whole cortex with that from the frontal cortex, an area which has been reported to be richly innervated by 5-HT fibres arising from the DRN.

Methods

Male guinea-pigs (400-500g) were killed by cervical dislocation, decapitated and the brains removed. The whole cortex or the frontal cortex were rapidly dissected and cross-chopped into 300 μm x 300 μm slices on a McIlwain chopper. The slices were incubated with 100 nM [^{3}H]-5-HT in the presence of pargyline (10 μM) at 37°C for 15 min. Slices were washed, suspended in 10 ml of Krebs solution (composition, mM: NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 10, L-ascorbate 0.06, Na_2EDTA 0.03) and 100 μl aliquots transferred to a Brandel superfusion 2000 apparatus. The slices were then superfused with oxygenated Krebs in the presence of the 5-HT uptake blocker, paroxetine (1 μM).

After 30 min of superfusion ($t=0$) samples were collected every 4 min for a duration of 80 min. Transmitter release was stimulated electrically at $t=-20$ min, $t=12$ min (S_1) and $t=56$ min (S_2) with 2 ms biphasic square wave pulses, 20 mA in amplitude, at a frequency of 1 Hz for 120 pulses. Agonists were superfused for 20 min at $t=44$ min and antagonists at $t=24$ min, and were present in the superfusion fluid for the remainder of the experiment.

At the end of the experiment the radioactivity in the slices and superfusate fractions were determined by scintillation spectrometry. Fractional release (FR) for each sample was calculated as the amount of radioactivity in a sample expressed as a fraction of the total radioactivity present, a sample being a 4 min period. Basal levels of [^{3}H]-5-HT release were calculated as the mean % FR (4 min)$^{-1}$ of the 2 samples either side of the S_1 and S_2 stimulations and were designated B_1 and B_2 respec-

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tively. Effects of drugs on basal levels were calculated as B_2/B_1 ratios while effects on stimulated release were calculated as $(S_2-B_2)/(S_1-B_1)$ ratios of FR. The results were then expressed as a % of control. Basal levels of release were also determined for the duration of each experiment. These were calculated as the sum of each % FR $(4\ min)^{-1}$ in the absence of electrical stimulation over the 80 min superfusion, divided by the number of 4 min fractions. Concentration-response curves were analysed with Grafit (Erithacus Software Ltd.), using a four parameter logistic fit.

5-Hydroxytryptamine creatinine sulphate (5-HT), pargyline and tetrodotoxin (TTX) were purchased from Sigma. [^{3}H]-5-HT (27.4 Ci $mmol^{-1}$) was purchased from New England Nuclear. Paroxetine HCl, 5-carboxamidotryptamine (5-CT), sumatriptan, ω-conotoxin MVII (CTX) and GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide) were synthesized by SmithKline Beecham.

Results

Whole brain cortex

Basal levels of [^{3}H]-5-HT release from slices of guinea-pig whole cortex were stable over the 80 min period, at $1.83\pm0.04\%$ ($n=48$) fractional release (FR) $(4\ min)^{-1}$. Electrical stimulation at 1 Hz increased the [^{3}H]-5-HT levels to $4.61\pm0.19\%$ FR $(4\ min)^{-1}$ ($n=30$) for S_1 and $4.39\pm0.20\%$ FR $(4\ min)^{-1}$ ($n=30$) for S_2, with a S_2/S_1 ratio of 1.09 ± 0.03 ($n=30$) after subtraction of basal values. More than 90% of this stimulated release was calcium-dependent and tetrodotoxin-sensitive, while 80% was ω-conotoxin (CTX)-sensitive. Less than 15% of the basal release was affected by any of these 3 treatments (Table 1).

In the guinea-pig whole cortex 5-HT, 5-CT and sumatriptan, when superfused during the second electrical stimulus, inhibited [^{3}H]-5-HT release without any effect on basal release. The rank order of potency of the 5-HT autoreceptor agonists was: 5-CT > 5-HT > sumatriptan (Figure 1, Table 2).The degree of maximum inhibition observed varied between agonists. 5-CT, 5-HT and sumatripan showed 98%, 83% and 66% maximum inhibition respectively. The concentration-response curves for both 5-CT and 5-HT had slopes less than one (Table 2).

Both methiothepin (10 nM – 10 μM) and GR127935 (10 nM – 10 μM) enhanced stimulated [^{3}H]-5-HT release *per se*, producing maximum increases of $140\pm9\%$ ($n=4$) and $124\pm13\%$ ($n=3$) of control respectively.

Methiothepin (10 nM) attenuated agonist-induced inhibition of [^{3}H]-5-HT release resulting in rightward shifts of the agonist curves and giving apparent pA_2 values of 8.34 ± 0.18 ($n=5$), 8.11 ± 0.17 ($n=3$) and 8.35 ± 0.09 ($n=2$) against 5-CT, 5-HT and sumatriptan respectively (Figure 1). The shifts of the agonist curves and the apparent pA_2 values were not significantly different from each other (unpaired *t* test).

Table 1 Effect of calcium ion depletion ($-Ca^{2+}$), tetrodotoxin (TTX) and conotoxin (CTX) on *in vitro* basal and stimulated [^{3}H]-5-HT release from the guinea-pig whole brain cortex

	% of control	
Treatment	*Basal*	*Stimulated*
Control	100±4%	100±3%
TTX (1 μM)	86±3%*	8±2%*
CTX (1 μM)	89±9%	21±7%*
$-Ca^{2+}$	99±12%	−6±10%*

Calcium was removed or TTX/CTX added at $t=24$ min, and were present in the superfusion fluid for the duration of the experiment. Results are expressed as % of control ± s.e.mean ($n=3-7$). *$P<0.05$ unpaired, *t* test.

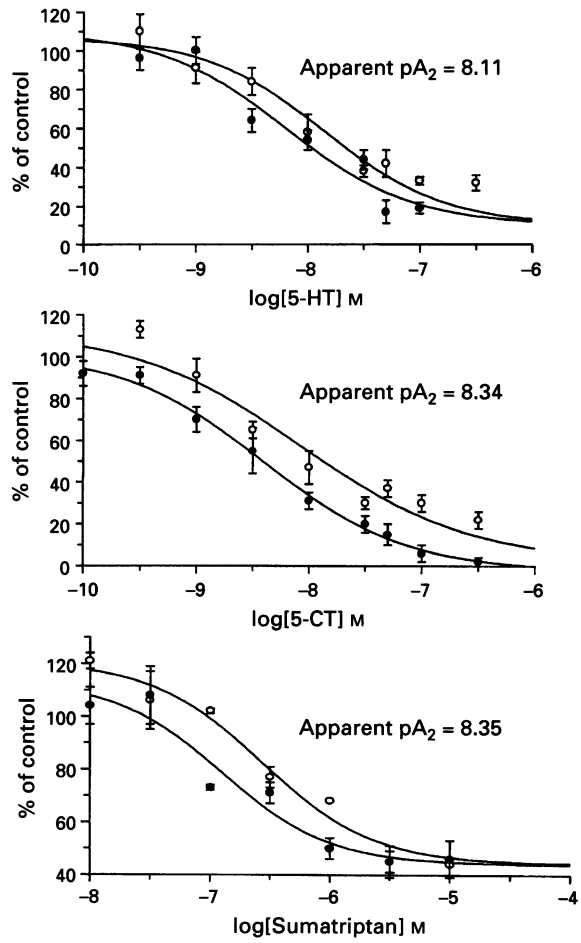

Figure 1 Inhibition of electrically stimulated [^{3}H]-5-HT release from guinea-pig whole brain cortical slices by 5-HT, 5-CT and sumatriptan, in the absence (●) and presence (○) of methiothepin (10 nM). Results are expressed as a % of control ± s.e. mean from 2–9 experiments.

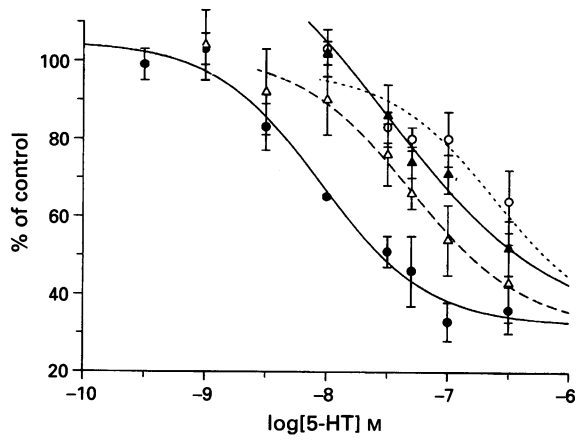

Figure 2 Inhibition of electrically stimulated [^{3}H]-5-HT release from guinea-pig whole brain cortical slices by (●) 5-HT ($n=15$) and attenuation of their inhibition with GR127935 at (△) 10 nM ($n=4$), (▲) 30 nM ($n=4$) and (○) 100 nM ($n=3$). Results are expressed as a % of control ± s.e. mean.

The selective 5-HT_{1D} receptor antagonist, GR127935 (10 nM – 100 nM), also attenuated the 5-HT-induced inhibition of [^{3}H]-5-HT release, producing parallel rightward shifts of the inhibition curve (Figure 2). Apparent pA_2 values were 8.61 ± 0.21 (n = 4), 8.44 ± 0.08 (n = 4) and 8.33 ± 0.14 (n = 3) at 10 nM, 30 nM and 100 nM respectively. Schild analysis of these data resulted in a pA_2 value of 9.03 with a slope of 0.67.

Frontal cortex

In the frontal cortex, both basal and control stimulated levels of release of [^{3}H]-5-HT were comparable to those seen in the whole cortex. Over an 80 min period the basal levels were 1.28 ± 0.04% FR (4 min)$^{-1}$ (n = 45). On electrical stimulation at 1 Hz the release increased to 2.76 ± 0.20% FR (4 min)$^{-1}$ (n = 16) for S_1 and 2.34 ± 0.24% FR (4 min)$^{-1}$ (n = 16) for S_2, with a S_2/S_1 ratio of 1.00 ± 0.04 (n = 16) after subtraction of basal values.

As in the whole cortex, methiothepin (1 μM) potentiated stimulation-evoked [^{3}H]-5-HT release (164 ± 12% (n = 15) of control). This effect was not significantly different from that observed in the whole cortex.

The receptor agonists studied in the frontal cortex revealed the same rank order of potency as that for the whole cortex (Table 2). However, some differences were apparent in the maximum inhibitions observed. In the frontal cortex 5-CT, 5-HT and sumatriptan gave E_{max} values of 86%, 53% and 72% respectively. The maximum effect seen with 5-HT was significantly less than that seen in the whole cortex ($P < 0.01$, unpaired t test), with a concurrent increase in the slope of the inhibition curve (see Figure 3). The concentration-response curves for 5-CT and sumatriptan had slopes less than one. A summary of the receptor agonist potencies in both regions of the guinea-pig brain is shown in Table 2, together with their 5-HT_{1D} binding affinities in guinea-pig cortex for comparison.

Discussion

We have demonstrated that agonist potencies at guinea-pig 5-HT terminal autoreceptors correlate well with their reported affinities for 5-HT_{1D} receptor binding, suggesting that the terminal autoreceptor is of the 5-HT_{1D} subtype. However, agonist potency alone is insufficient to characterize a receptor fully. Therefore, the nature of the autoreceptor was confirmed by demonstrating that GR127935, the selective 5-HT_{1D} receptor antagonist, attenuated 5-HT-induced inhibition of [^{3}H]-5-HT release with a potency consistent with its 5-HT_{1D} receptor affinity (Skingle *et al.*, 1993). Unfortunately, it is not possible to determine which of the 5-HT_{1D} receptor subtypes is the autoreceptor because GR127935 has similar affinity for both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors.

The anatagonist, ketanserin, has been reported to differentiate between 5-HT_{1D} receptor subtypes, displaying a hundred fold selectivity for 5-$HT_{1D\alpha}$ over the 5-$HT_{1D\beta}$ receptor subtype (Zgombick *et al.*, 1994). However, ketanserin is not an

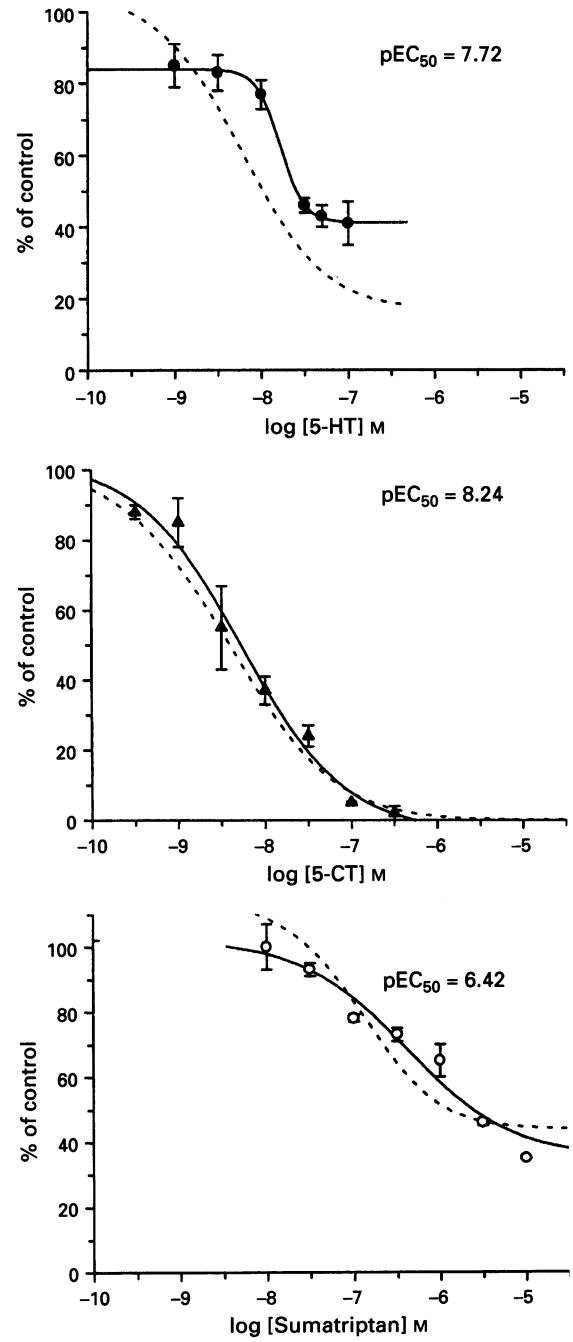

Figure 3 Inhibition of electrically stimulated [^{3}H]-5-HT release from guinea-pig frontal cortex slices with (●) 5-HT (n = 3), (▲) 5-CT (n = 3) and (○) sumatriptan (n = 3). The respective inhibitions present in the whole cortex for the 3 agonists are shown for comparison (dotted lines). Results are expressed as a % of control ± s.e. mean.

Table 2 Agonist potencies in inhibiting electrically stimulated [^{3}H]-5-HT release compared with 5-HT_{1D} receptor binding affinity, in the guinea-pig cortex

	[^{3}H]-5-HT release								5-HT_{1D} binding#
	Whole cortex				Frontal cortex				Whole cortex
Agonist	*pEC50*	*Slope*	*% E_{max}*	*n*	*pEC50*	*Slope*	*% E_{max}*	*n*	*pIC50*
5-HT	7.96 ± 0.16	0.61 ± 0.08	83 ± 6%	9	7.72 ± 0.10	2.99 ± 1.38	53 ± 6%*	3	8.06
5-CT	8.42 ± 0.14	0.67 ± 0.07	98 ± 1%	6	8.24 ± 0.18	0.79 ± 0.09	86 ± 7%	3	8.50
Sumatriptan	6.95 ± 0.20	1.10 ± 0.33	66 ± 10%	5	6.42 ± 0.24	0.73 ± 0.13	72 ± 9%	3	7.38

pEC_{50} values were calculated as 50% of the maximum response and analysed through Grafit, using a 4 parameter logistic fit. #5-HT_{1D} receptor binding data is taken from Beer *et al.* (1992). [^{3}H]-5-HT was used as the ligand and results are taken from a 2 site model (high affinity component quoted). *$P < 0.05$, unpaired t test c.f. pEC_{50} for 5-HT in whole cortex

ideal tool to use for characterization of the 5-HT terminal autoreceptor. In our hands this compound, and the closely related compound, ritanserin, display large, non-specific basal effects on [^{3}H]-5-HT release (unpublished observations). At concentrations that do not affect basal release of [^{3}H]-5-HT, ketanserin has been reported to be ineffective in attenuating 5-HT autoinhibition (Maura *et al.*, 1993). This implies that the 5-HT terminal autoreceptor is a 5-$HT_{1D\beta}$ receptor subtype. In support of this, the 5-HT terminal autoreceptor in rodents is accepted to be a 5-HT_{1B} receptor subtype (Engel *et al.*, 1986), which is the species homologue of the 5-$HT_{1D\beta}$ receptor (Adham *et al.*, 1991). Unfortunately, there are no 5-$HT_{1D\beta}$ selective ligands available and confirm these findings.

Regression analysis of the GR127935 attenuation of 5-HT inhibition of [^{3}H]-5-HT release resulted in a shallow Schild slope, indicative of action at more than one site. If this is the reason for the shallow Schild slope, it suggests that non-5-HT_{1D} receptors are also involved. Possible candidates are the recently identified subclasses 5-HT_{1F} (Amlaiky *et al.*, 1992; Adham *et al.*, 1993; Lovenberg *et al.*, 1993b), 5-HT_5 (Erlander *et al.*, 1993; Matthes *et al.*, 1993), 5-HT_6 (Monsma *et al.*, 1993) and 5-HT_7 (Lovenberg *et al.*, 1993a; To *et al.*, 1995). mRNA for the 5-HT_{1F}, 5-HT_{5B} and 5-HT_7 receptors have been detected in the raphe nuclei (Bruinvels *et al.*, 1994; Wisden *et al.*, 1993; Erlander *et al.*, 1993; To *et al.*, 1995), suggesting that they have autoreceptor function. In the guinea-pig whole cortex the slopes of the agonist curves for 5-HT and 5-CT were shallow, which may also indicate the existence of multiple autoreceptors. Sumatriptan gave a lower maximum response than either 5-HT or 5-CT, suggesting that it may be a partial agonist at the 5-HT terminal autoreceptor.

There may be an underlying anatomical reason for autoreceptor heterogeneity. 5-HT fibres originate from two main midbrain nuclei: the DRN and MRN and it is possible that these fibres may possess different terminal 5-HT autoreceptors. Some brain areas are preferentially innervated with fibres from one nucleus while others receive a mixed innervation. The whole cortex receives 5-hydroxytryptaminergic input from both the DRN and MRN, while the frontal cortex contains predominantly DRN projections (Kosofsky & Molliver, 1987).

There was one clear difference between the results obtained from the two cortical preparations: 5-HT gave a higher maximum and a more shallow concentration-response curve in the whole cortex than in the frontal cortex (the reason for the steep slope is unknown). In isolation, this observation implies that there are receptor differences between the preparations: a single autoreceptor in the frontal cortex and more than one subtype in other parts of the cortex. However, this hypothesis does not explain the observation that 5-CT and sumatriptan gave very similar concentration-response curves in both areas. Therefore, we cannot dismiss the possibility that different degrees of autoreceptor control (Blier *et al.*, 1990), receptor desensitization or partial agonist activity may also be contributing factors.

In summary, we have demonstrated that the pharmacology of the 5-HT terminal autoreceptor in the guinea-pig cortex is consistent with it being a 5-HT_{1D} receptor. We have also provided circumstantial evidence for the existence of multiple terminal autoreceptors. Definitive evidence may not be forthcoming until compounds are available that clearly differentiate between the 5-HT receptor subtypes that are candidate terminal autoreceptors.

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