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Glutamate release in human cerebral cortex and its modulation by 5-hydroxtryptamine acting at h 5-HT_{1D} receptors

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1 The release of glutamic acid and its modulation by 5-hydroxytryptamine (5-HT) in the human brain has been investigated in synaptosomal preparations from fresh neocortical samples obtained from patients undergoing neurosurgery to reach deeply sited tumours.

2 The Ca^{2+}-dependent K^+ (15 mM)-evoked overflow of glutamate was inhibited by 5-HT in a concentration-dependent manner (EC_{50} = 2.9 nM; maximal effect ≃50%). The inhibition caused by 5-HT was antagonized by the 5-HT_1/5-HT_2 receptor antagonist methiothepin. The 5-HT_{1B}/5-HT_{1D} receptor agonist sumatriptan mimicked 5-HT (EC_{50} = 6.4 nM; maximal effect ≃50%); the effect of sumatriptan was also methiothepin-sensitive. Selective 5-HT_{1A} receptor antagonists could not prevent the inhibition of glutamate release by 5-HT.

3 The 5-HT_{1B}/5-HT_{1D} receptor ligand GR 127935 and the 5-HT_{2C}/5-HT_{1B}/5-HT_{1D} receptor ligand metergoline were unable to prevent the 5-HT effect; instead they inhibited glutamate release, their effects being abolished by methiothepin. Some 5-HT_{1A} receptor antagonists also displayed intrinsic agonist activity.

4 The effect of sumatriptan was prevented by ketanserin, a drug known to display much higher affinity for recombinant h 5-HT_{1D} than for h 5-HT_{1B} receptors.

5 We propose that neocortical glutamatergic nerve terminals in human brain cortex possess release-inhibiting presynaptic heteroreceptors that appear to belong to the h 5-HT_{1D} subtype.

Keywords: Human cerebral cortex; glutamate release; 5-hydroxytryptamine-glutamate interaction; human native 5-hydroxytryptamine receptors; h 5-HT_{1D} receptor

Introduction

Pharmacological studies as well as molecular cloning of 5-HT receptors have revealed high receptor heterogeneity. Seven major types of the 5-hydroxytryptamine (5-HT) receptor have been identified and termed 5-HT_1 to 5-HT_7. Most of these receptors, particularly the 5-HT_1 type, are heterogeneous and exist as subtypes. Species homologues of the same receptor subtype may exist which, despite high structural homology, may display pronounced pharmacological differences (Hoyer & Middlemiss, 1989; Hoyer *et al*., 1994; Martin & Humphrey, 1994).

Among the functions that have been attributed to 5-HT receptors, several animal studies have shown that 5-HT receptor activation can mediate regulation of the release of various transmitters (references in Hoyer *et al*., 1994; Martin & Humphrey, 1994). Functional studies with native human brain 5-HT receptors have rarely been performed and deal almost exclusively with autoreceptors mediating feedback inhibition of 5-HT release (Schlicker *et al*., 1985; Galzin *et al*., 1992; Maura *et al*., 1993; Fink *et al*., 1995).

Animal studies suggest that 5-HT can interact with glutamate to inhibit excitatory transmission in the CNS. Data from electrophysiological (Aston-Jones *et al*., 1991; Sizer *et al*., 1992; Tanaka & North, 1993; Boeijinga & Boddeke, 1996), *in vitro* release (Raiteri *et al*., 1986; Maura *et al*., 1995; Maura & Raiteri, *et al*., 1996) and *in vivo* microdialysis (Srkalovic *et al*., 1994; Glass *et al*., 1995) studies have shown that multiple 5-HT receptors may be involved in these 5-HT glutamate interactions. Since excessive glutamate release has been implicated in a number of pathophysiological conditions, understanding how the release of this excitatory transmitter can be regulated in the human CNS may lead to novel therapeutic approaches.

In the present study, we investigated the effects of 5-HT on the release of glutamate from synaptosomes of human cerebral cortex. Activation of receptors of the human 5-HT_{1D} (h 5-HT_{1D}; former nomenclature: 5-$HT_{1D\alpha}$; see Hartig *et al*., 1996) subtype appears to inhibit potently depolarization-evoked glutamate release.

Methods

Characteristics of human specimens

Samples of human cerebral cortex were obtained from 9 female and 7 male patients (aged 19–70 years). The tissues had to be removed to reach deeply located tumours. Samples of frontal (5), temporal (8) and parietal (3) lobes were used. For details on premedication and anaesthesia see Maura *et al*. (1993). Immediately after removal, the tissue was placed in a physiological salt solution kept at 2–4°C.

Release experiments

Crude synaptosomes were prepared within 2 h. The tissue was homogenized in 40 vol of 0.32 M sucrose, buffered at pH 7.4 with phosphate. The homogenate was centrifuged (5 min, 1000 *g*, at 0–4°C) to remove nuclei and debris and synaptosomes were isolated from the supernatant by centrifugation at 12,000 *g* for 20 min. The synaptosomal pellet was then resuspended in a physiological medium having the following

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composition (mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 22 and glucose 11 (aerated with 95% O_2 and 5% CO_2); pH 7.2–7.4. Protein was determined according to Bradford (1976). Identical aliquots of the synaptosomal suspension (0.4–0.6 mg of protein) were distributed in a set of parallel superfusion chambers maintained at 37°C (Raiteri *et al*., 1974). Superfusion was then started with standard medium aerated with 95% O_2 and 5% CO_2 at a rate of 0.6 ml min^{-1} and continued for 48 min. At min 39 synaptosomes were depolarized by a 90 s pulse of 15 mM KCl replacing an equimolar concentration of NaCl. Fraction collection began at min 36, according to the following scheme: two 3 min samples (basal release) before and after one 6 min sample (basal release plus release evoked by high-K^+). Agonists were added concomitantly with high K^+; antagonists were present from 8 min before depolarization. When indicated, a calcium-free medium was introduced 18 min before depolarization.

Amino acid determination

The amount of endogenous glutamate released or remaining in the synaptosomes after superfusion was measured by h.p.l.c. The glutamate content of synaptosomes was measured in the supernatant obtained after homogenization (Ultra Turrax, max speed, 20 s) in ice-cold distilled water and centrifugation at 20,000 × *g* for 10 min. The analytical method (Tonnaer *et al*., 1983) involved precolumn derivatization with *o*-phthalaldehyde followed by separation on C_{18} reverse phase chromatography column (Chrompack, 10 cm × 4.6 mm, 3 μm) and three solvent discontinuous gradient, from 23% methanol in acetate buffer 0.1 M pH 6 to 46% methanol in acetate buffer 0.1 M pH 5.8, in 22 min, at a rate of 0.9 ml min^{-1}.

Calculation

The amount of endogenous glutamate released in each fraction collected was expressed as pmol mg^{-1} synaptosomal protein. The depolarization-evoked release (overflow) was estimated by subtracting the basal release from the total release in the 6 min fraction collected during and after the depolarization period. Depolarization-evoked overflow in the presence of drugs was calculated as percentage variation with respect to the control. EC_{50} values (half-maximum effective concentration) were derived from the concentration-response curves obtained by use of a four-parameter logistic function fitting routine (Sigma Plot software). Means ± s.e.mean of the given numbers (*n*) of experiments are presented throughout. Data presented in Table 1 and Figures 2 and 3 were compared by two-tailed Student's *t* test.

Materials

The following drugs were purchased: 5-hydroxytryptamine (5-HT) creatinine sulphate (Calbiochem, Los Angeles, CA, U.S.A.); phenylbiguanide and metergoline (RBI, Natick, MA, U.S.A.), spiperone (Sigma Chemical Co, St. Louis, MO, U.S.A.); ketanserin (Tocris Cookson, Bristol, U.K.). The following drugs were gifts: methiothepin (Hoffmann La Roche, Basel, Switzerland); sumatriptan and 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 (GR 127935; Glaxo Group Research, Greenford, U.K.); (**S**)-(+)N-tert-butyl-3-[4-(2-methoxyphenyl)piperazin-1-yl]-2-phenylpropionamide dihydrochloride ((+)-WAY 100135; Wyeth Research, Berkshire, U.K.).

Results

In the experiments presented throughout, the amino acid released in the 3 min fraction collected before the onset of K^+-stimulation (see Methods) amounted to 311 ± 41 pmol mg^{-1} protein (n = 16). The glutamate content of synaptosomes measured at the end of superfusion was 20 ± 1.9 nmol mg^{-1} protein (n = 4). The K^+-evoked overflow of glutamate was strongly dependent on the presence of Ca^{2+} ions in the superfusion medium: 15 mM KCl = 522 ± 46; 15 mM KCl (Ca^{2+}-free) = 72 ± 9 pmol mg^{-1} protein (n = 3). The basal release was not significantly Ca^{2+}-dependent (not shown).

The glutamate overflow elicited by 15 mM K^+ was inhibited by 5-HT in a concentration-dependent manner (EC_{50} = 2.9 nM; maximal inhibition ≃ 50%), when the indoleamine was added concomitantly with the depolarizing stimulus (Figure 1). Basal release was not affected by 5-HT (not shown). The inhibition of the overflow caused by 0.1 μM 5-HT was antagonized by 1 μM of the 5-HT$_1$/5-HT$_2$ receptor antagonist methiothepin (Figure 2), indicating involvement of 5-HT receptors. Spiperone, a drug able to block 5-HT$_2$ receptors, added at 1 μM, did not prevent the effect of 0.1 μM 5-HT (Table 1). The 5-HT$_3$ receptor agonist phenylbiguanide (1 μM) did not mimic 5-HT (not shown).

We next explored the possible involvement of a subtype of the 5-HT$_1$ receptor. The selective antagonist of the 5-HT$_{1A}$ subtype, (+)-WAY 100135 (Fletcher *et al*., 1993) and the non-selective 5-HT$_{1A}$ receptor blocker spiperone, both added at 1 μM, were unable to prevent the effect of 0.1 μM 5-HT on glutamate release (Table 1). Considering that 'classical' 5-HT$_{1B}$ receptors (present nomenclature: rat or r 5-HT$_{1B}$; Hartig *et al*., 1996) do not exist in the human brain (Hoyer *et al*., 1986), synaptosomes were exposed to the 5-HT$_{1D}$ receptor agonist sumatriptan (1 nM–10 μM). The drug mimicked 5-HT (Figure 1) and inhibited the K^+-evoked glutamate overflow (EC_{50} = 6.4 nM; maximal effect ≃ 50%) in a methiothepin-sensitive manner (Figure 2).

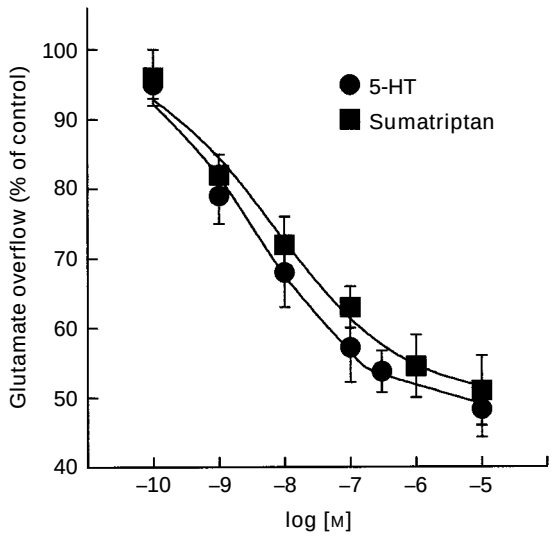

Figure 1 Effect of 5-HT receptor agonists on the overflow of endogenous glutamate evoked by depolarization of human cerebral cortical synaptosomes. Depolarization was performed by exposing synaptosomes to a 90 s pulse of 15 mM KCl during superfusion. Agonists were added concomitantly with high-K^+. Other experimental details as in Methods. Results are expressed as a percentage of the corresponding controls Data are means of 4 to 6 independent experiments; each experiment consisted of 3 replicate chambers for each condition. Vertical lines show s.e.mean.

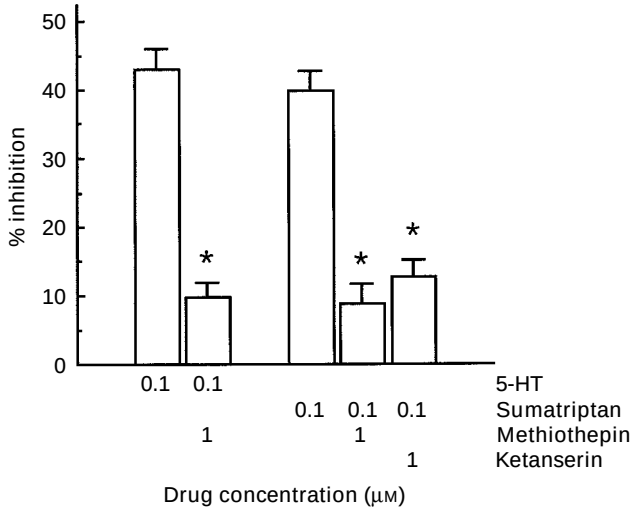

Figure 2 Antagonism by methiothepin or ketanserin of the inhibition by 5-HT or sumatriptan of the K^+-evoked glutamate overflow from human cerebrocortical synaptosomes. Data are expressed as % inhibition of the K^+-evoked overflow of glutamate. Agonists were added concomitantly with high-K^+; antagonists 8 min before. Data are means ± s.e.mean of 3 to 4 independent experiments, each consisting of three replicate chambers for each condition. $^*P<0.005$ when compared to the effect of the agonist alone.

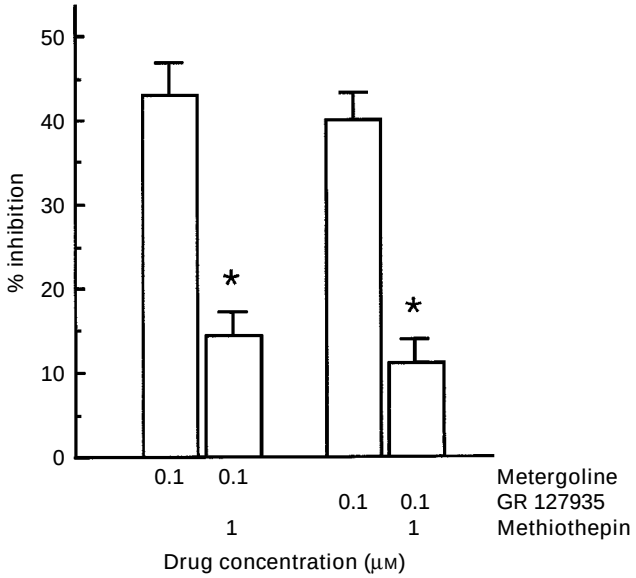

Figure 3 Antagonism by methiothepin of the inhibition by metergoline or GR 127935 of the K^+-evoked glutamate overflow from human cerebrocortical synaptosomes. Data are expressed as % inhibition of the K^+-evoked overflow of glutamate. Metergoline or GR 127935 were added concomitantly with high-K^+, methiothepin 8 min before. Data are means ± s.e.mean of 3 independent experiments, each consisting of 3 replicate chambers for each condition. $^*P<0.005$ when compared to the effect of the agonist alone.

Table 1 Effect of 5-HT receptor antagonists on the 5-HT inhibition of the depolarization-evoked release of endogenous glutamate from superfused human cerebrocortical synaptosomes

	% inhibition
5-HT (0.1 μM)	46.4 ± 3.8 (8)
5-HT (0.1 μM) + spiperone (1 μM)	44.3 ± 3.0 (4)
5-HT (0.1 μM) + (+)-WAY 100135 (1 μM)	46.3 ± 3.5 (4)
5-HT (0.1 μM) + metergoline (1 μM)	43.4 ± 4.0 (4)
5-HT (0.1 μM) + GR 127935 (1 μM)	47.0 ± 3.0 (4)

The effect was measured as % inhibition of depolarization-evoked glutamate release. The agonists were added concomitantly with 15 mM KCl and the antagonists were present starting 8 min before depolarization. Data are mean ± s.e.mean of *n* (number in parentheses) experiments in quadruplicate.

To confirm the involvement of 5-HT$_{1D}$ receptors, we tried to block the effect of 5-HT with GR 127935, a selective 5-HT$_{1D}$ receptor antagonist (Skingle *et al*., 1993; 1996), and with metergoline, an antagonist with selectivity for the 5-HT$_{2C}$/5-HT$_{1D}$ receptors (Heuring & Peroutka, 1987). However, as shown in Table 1, the inhibition of glutamate release by 0.1 μM 5-HT was not affected by either 1 μM GR 127935 or 1 μM metergoline.

It was recently found that GR 127935, as well as metergoline, can display agonistic properties at recombinant h 5-HT$_{1D}$ (Pauwels *et al*., 1996b; Watson *et al*., 1996; Zgombick *et al*., 1996). As illustrated in Figure 3, both drugs, added at 0.1 μM, strongly inhibited the K^+-evoked overflow of glutamate from human cortex synaptosomes; the effects of GR 127935 and metergoline were quantitatively similar to those of 5-HT and sumatriptan (cf with Figure 1); moreover, as with sumatriptan and 5-HT, the effects of GR 127935 and metergoline were prevented by methiothepin. Antagonists at 5-HT$_{1A}$ receptors have also been shown to behave as agonists at cloned h 5-HT$_{1D}$ receptors (Pauwels *et al*., 1996a). Interestingly, in experiments run in parallel with sumatriptan on the same synaptosomal preparations ($n=3$), (+)-WAY 100135 and spiperone inhibited the glutamate-evoked overflow. The inhibition caused by 1 μM (+)-WAY 100135 (49.3 ± 5.0%) or by 1 μM spiperone (46.1 ± 5.9%) did not differ significantly from that caused by sumatriptan (45.0 ± 3.7%) also added at 1 μM, its maximally effective concentration (see Figure 1).

Finally, the inhibition of glutamate release caused by 0.1 μM sumatriptan was largely antagonized by 1 μM ketanserin (Figure 2); besides being a classical 5-HT$_2$ antagonist, this drug has been shown to exhibit a much higher affinity for recombinant receptors of the h 5-HT$_{1D}$ subtype than for h 5-HT$_{1B}$ receptors (Zgombick *et al*., 1995) and to behave as an antagonist at peripheral native h 5-HT$_{1D}$ receptors (Kaumann *et al*., 1994).

Discussion

More than a decade ago it was found that the Ca^{2+}-dependent, depolarization-evoked release of glutamate from rat cerebellar synaptosomes could be potently inhibited through the activation of an unknown subtype of the 5-HT$_1$ receptor that we then suggested to be termed 5-HT$_{1D}$ (Raiteri *et al*., 1986). This receptor was subsequently shown to be activated with nanomolar affinity by sumatriptan (Maura & Raiteri, 1996). When the rat 5-HT$_{1D}$ (now termed r 5-HT$_{1D}$) receptor was cloned, it was proposed to represent the species homologue of the human 5-HT$_{1D}$ (now h 5-HT$_{1D}$) receptor (Hamblin *et al*., 1992; Hartig *et al*., 1996).

The aims of the present work were: to investigate if 5-HT can modulate the release of glutamic acid also in human brain; to establish if the modulation is a direct effect on glutamate-releasing axon terminals; to characterize pharmacologically the 5-HT receptors involved.

Firstly, it was found that the Ca^{2+}-dependent, depolarization-evoked overflow of glutamate from human neocortex synaptosomes was potently (EC_{50} = 2.9 nM) inhibited by 5-HT. The action of 5-HT was receptor-mediated because it was sensitive to methiothepin, a non-selective 5-HT$_1$/5-HT$_2$ receptor antagonist. It is likely that 5-HT acts directly on glutamate-releasing axon terminals, because the technique used to monitor release (a thin layer of synaptosomes immobilized on a microporous filter and up-down superfused) has often been shown to prevent indirect effects.

As the effect of 5-HT was not antagonized by spiperone (a 5-HT$_2$ receptor antagonist) and was not mimicked by phenylbiguanide (a 5-HT$_3$ receptor agonist), the involvement of a subtype of the 5-HT$_1$ receptor was investigated in detail. A role for 5-HT$_{1A}$ receptors can be ruled out by the inability of (+)-WAY 100135 and spiperone, two potent 5-HT$_{1A}$ receptor antagonists, to prevent the 5-HT inhibition of glutamate release. The finding that sumatriptan, a drug that binds with similar affinity to h 5-HT$_{1B}$ and h 5-HT$_{1D}$ receptors (Weinshank *et al*., 1992), inhibited glutamate release with affinity and efficacy almost identical to those of 5-HT implies the involvement of h 5-HT$_{1B}$/h 5-HT$_{1D}$ receptors. These receptors were originally found to display drug binding characteristics that seemed virtually indistinguishable (Weinshank *et al*., 1992). However, subsequently, the classical 5-HT$_2$ receptor antagonist ketanserin was proposed to permit discrimination between h 5-HT$_{1B}$ and h 5-HT$_{1D}$ receptors (Kaumann *et al*., 1994; Pauwels & Colpaert, 1995; Zgombick *et al*., 1995). More recently, the ability of two novel ligands to distinguish pharmacologically between the two cloned receptors has been communicated (Price *et al*., 1996).

We found that the inhibition of glutamate release caused by sumatriptan was strongly antagonized by ketanserin. Since ketanserin has marked selectivity (~100 fold) for the recombinant h 5-HT$_{1D}$ relative to the h 5-HT$_{1B}$ receptor (Zgombick *et al*., 1995), our result suggests that the receptors mediating inhibition of glutamate release in human brain may belong to the h 5-HT$_{1D}$ subtype. Conversely, the sumatriptan-sensitive human 5-HT autoreceptor that mediates inhibition of 5-HT release (Maura *et al*., 1993) is ketanserin-insensitive (Maura *et al*., 1993; Fink *et al*., 1995) and therefore it can be classified as h 5-HT$_{1B}$ subtype.

However, the sensitivity to ketanserin may not be the only criterion of discrimination between h 5-HT$_{1B}$ and h 5-HT$_{1D}$ receptors. In fact, metergoline and GR 127935, two high affinity ligands at 5-HT$_{1B}$/5-HT$_{1D}$ receptors (Hamblin & Metcalf, 1991; Bruinvels *et al*., 1992; Skingle *et al*., 1996) could not antagonize 5-HT but instead strongly inhibited the K^+-evoked overflow of glutamate (Figure 3). In sharp contrast, both metergoline (Galzin *et al*., 1992; Maura *et al*., 1993) and GR 127935 (unpublished result) blocked 5-HT at the autoreceptors regulating 5-HT release in the human neocortex, thus behaving as antagonists at native h 5-HT$_{1B}$ receptors.

The 5-HT$_{1A}$ receptor antagonists (+)-WAY 100135 and spiperone behaved quite similarly to metergoline and GR 127935. Although efficacy determinations were not carried out, metergoline, GR 127935, (+)-WAY 100135 and spiperone all appear to be as effective as 5-HT and sumatriptan at the heteroreceptor regulating glutamate release. The inability of these drugs, all added at 1 μM, to antagonize the effect of 0.1 μM 5-HT (Table 1) supports the view that they are agonists endowed with high efficacy. Antagonists at the 5-HT$_{1A}$ receptor have been shown to be ketanserin-sensitive agonists at cloned h 5-HT$_{1D}$ receptors and to display much higher affinities for h 5-HT$_{1D}$ receptors than for h 5-HT$_{1B}$ subtype (Pauwels *et al*., 1996a). It should be noted that, contradictory data have been obtained with GR 127935 at cloned h 5-HT$_{1B}$ and h 5-HT$_{1D}$ receptors, possibly due to the different expression system. The compound was an antagonist at cloned h 5-HT$_{1B}$ receptors, while showing intrinsic agonist activity at h 5-HT$_{1D}$ receptors (Pauwels & Palmier, 1995). However, such selectivity was not observed by Watson *et al*. (1996), since GR 127935 behaved as a partial agonist at both cloned receptors. As to metergoline, strong intrinsic agonist activity has been observed at h 5-HT$_{1D}$ receptors, but not at the h 5-HT$_{1B}$ subtype, in keeping with our data (Pauwels *et al*., 1996b).

The intrinsic agonist activity sometimes displayed in transfected cell line systems by otherwise pure receptor antagonists have been considered artifactual and due to excessive receptor expression (Hoyer & Boddeke, 1993). It is thought that, unless a large receptor reserve exists, these antagonists would be silent in native tissues. Whether our results with GR 127935 and metergoline at native human receptors and the data by Manuel *et al*. (1995), showing agonist activity of GR 127935 at ketanserin-sensitive receptors in the neonatal rat spinal cord, reflect a large receptor reserve is difficult to say. In the conditions of the present work both GR 127935 and metergoline behaved as 'quasi' full agonists at native h 5-HT$_{1D}$ receptors and may therefore inhibit glutamate release in the human brain. Since both metergoline (Galzin *et al*., 1992; Maura *et al*, 1993) and GR 127935 (unpublished observation) are antagonists at the h 5-HT$_{1B}$ autoreceptors in human neocortex, the two drugs appear to distinguish, similarly to ketanserin, between native h 5-HT$_{1B}$ and h 5-HT$_{1D}$ receptors. In particular: h 5-HT$_{1B}$ receptors are ketanserin-insensitive, but can be antagonized by metergoline and GR 127935; h 5-HT$_{1D}$ receptors are antagonized by ketanserin, but can be activated by metergoline and GR 127935.

The present results represent the first example of a functional h 5-HT$_{1D}$ receptor present in the adult human brain. Considering that the human terminal autoreceptor is h 5-HT$_{1B}$, the emerging pharmacological differences between h 5-HT$_{1B}$ and h 5-HT$_{1D}$ receptors may lead to the development of novel therapeutic agents. 5-HT inhibition of glutamatergic transmission may be involved in a variety of pathological conditions, including epilepsy (Favale *et al*., 1995; see Clough *et al*., 1996 and references therein). Selective agonists at h 5-HT$_{1D}$ receptors, able to reduce abnormally elevated glutamate release without inhibiting 5-HT release, may be effective anticonvulsants. In rat cerebellum, 5-HT was found to inhibit potently glutamatergic transmission through activation of 5-HT$_{1A}$ and r 5-HT$_{1D}$ receptors (Maura *et al*., 1995; Maura & Raiteri, 1996). Administration of the 5-HT$_{1A}$ agonist buspirone has recently been shown to ameliorate the symptomatology of patients with cerebellar ataxia (Lou *et al*., 1995; Trouillas *et al*., 1995; 1996). As r 5-HT$_{1D}$ and h 5-HT$_{1D}$ are species homologues, a selective h 5-HT$_{1D}$ agonist could be useful in cerebellar ataxias.

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