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5-HT_{1D} Receptors Regulate 5-HT Release in the Rat Raphe Nuclei

In Vivo Voltammetry and In Vitro Superfusion Studies

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The aim of the present study was to characterize the pharmacological profile of 5-hydroxytryptamine (5-HT) receptors modulating 5-HT release in the mesencephalic raphe region. In a first series of experiments, differential normal pulse voltammetry and nafion-coated electrodes were used to measure extracellular 5-HT in the dorsal raphe of anesthesized rats. The intravenous administration of the selective 5-HT_{1A} agonist 8-OH-DPAT (30 μg/kg) and the 5-HT_1 agonist TFMPP (0.5 mg/kg) reduced the 5-hydroxyindole signal by 23% and 18%, respectively. Pretreatment with the 5-HT_{1A} antagonist (+)WAY100135 (0.5 mg/kg IV) 30 minutes before the injection of the agonists, blocked the effect of 8-OH-DPAT but not that of TFMPP. The effect of TFMPP was blocked by (±)mianserin, a drug with high affinity for the rat 5-HT_{1D} receptor, suggesting a role of this receptor subtype in the modulation of 5-HT release at the cell body level of 5-HT neurons. This was confirmed by in vitro superfusion experiments using mesencephalic raphe slices. The prototypical 5-HT_1 agonist 5-carboxy-amiditryptamine (5-CT) and the 5-$HT_{1B/1D}$ agonist sumatriptan (1–1,000 nM) induced a concentration-dependent inhibition of the electrically evoked release of [^{3}H]5-HT from preloaded raphe slices. 8-OH-DPAT (100 nM) produced an inhibitory effect similar to that of sumatriptan (100 nM). The selective 5-HT_{1B} agonist CP 93129 (10–10,000 nM), had no effect in raphe slices, but it dose dependently inhibited [^{3}H]5-HT release from hippocampal slices where autoreceptors are of the 5-HT_{1B} subtype. The inhibitory effect of 5-CT was blocked by the 5-$HT_{1/2}$ antagonist methiothepin (1 μM), the 5-HT_{1A} antagonist S-UH-301 (1 μM), and the 5-$HT_{1B/1D}$ antagonist GR 127935 (1 μM). That of 8-OH-DPAT was blocked by S-UH-301 (1 μM) but not by GR 127935 (1 μM), and that of sumatriptan was blocked by GR 127935 (1 μM) but not by S-UH-301 (1 μM). These results show that, together with 5-HT_{1A} autoreceptors, 5-HT_{1D} receptors negatively modulate the somatodendritic release of 5-HT.
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Dorsal and median raphe nuclei give rise to most of the serotoninergic innervation of the forebrain (Anden et al. 1966; Dahlström and Fuxe 1964; Ungerstedt 1971). At somatodendritic and terminal levels autoreceptors control 5-HT release (Starke et al. 1989). However, while terminal autoreceptors control regional 5-HT release in projection areas in which they are located (Starke et al. 1989), somatodendritic 5-HT_{1A} autoreceptors, by reducing the firing activity of 5-HT neurons, may influence local 5-HT release not only within the raphe nuclei themselves (Blier et al. 1990; O'Connor and Kruk 1992) but also in the projection areas (Adell et al. 1993; Blier et al. 1990) of those nuclei. Physiologically, the activation of 5-HT_{1A} autoreceptors is determined by the extracellular concentration of 5-HT (Jacobs and Fornal 1991). Hence modulation of 5-HT availability at the somatodendritic level constitutes a major regulatory factor of the efficacy of 5-HT neurotransmission.

Several autoradiographic and binding studies have documented the presence of different 5-HT binding

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sites in the rat raphe nuclei (Herrick-Davis and Titeler 1988; Laporte et al. 1992; Waeber et al. 1988, 1989). However, the role of receptors other than 5-HT_{1A} in modulating 5-HT neurotransmission at the level of the cell body is not as well characterized as that of 5-HT_{1A} autoceptors. The lack of effect of 1-[3-(trifluoromethyl)-phenylpiperazine (TFMPP) and *m*-chlorophenylpiperazine (*m*CPP) on the firing activity of 5-HT neurons led Sprouse and Aghajanian (1987) to conclude that 5-HT_{1B} receptors were not present on the cell body of 5-HT neurons in the rat brain. More recently it has been demonstrated in vivo, that extracellular availability of 5-HT in the dorsal raphe nucleus of anesthetized rats can be modulated independently of 5-HT neuron firing frequency (Blier et al. 1990) and that 5-HT_{1D} receptors may be involved in this function (Piñeyro et al. 1993, 1994). Therefore, the aim of the present study was to further characterize the pharmacology of 5-HT receptors modulating 5-HT release in the midbrain raphe nuclei of the rat. Furthermore, it was deemed of interest to determine whether the putative 5-HT_{1D} receptors that modulate somatodendritic release of 5-HT were located in the mesencephalic region. An in vitro superfusion paradigm was used to assess the local effects of drugs. Superfusion experiments also allowed a detailed pharmacological characterization of the receptors involved in controlling 5-HT release in the midbrain raphe area, since drugs that cannot be used in vivo because they do not cross the blood-brain barrier could be used in vitro.

MATERIALS AND METHODS

Animals

One hour before starting in vivo voltammetry experiments, Sprague-Dawley rats (225–250 g) were injected with allopurinol (10 mg/kg IP) in order to avoid contribution of uric acid to the voltammetric signal (Blier et al. 1990). All the in vivo experiments were conducted under chloral hydrate anesthesia (initial dose: 400 mg/kg IP). For superfusion experiments rats were sacrificed by decapitation and their brains rapidly removed and dissected on an ice-cold plate. The midbrain raphe region was dissected as described by Kerwin and Pycock (1979) and slices of 0.4 mm thickness were prepared with a McIlwain chopper. Hippocampal slices of similar thickness were also prepared.

Voltammetric Experiments

Extracellular levels of indoleamines were determined by differential normal pulse voltammetry (DNPV) using multifiber pyrrolytic electrodes (20 fibers, 50 μm diameter, 500 μm length). Electrochemical measurements were performed using a Biopulse polarograph (Tacussel, France) with an auxiliary platinum electrode and a reference calomel electrode. The carbon-fiber electrodes were electrochemically pretreated in phosphate buffer saline by applying continuous direct current to generate potentials of +2.85 V, +2.4 V, and +1.6 V for 20 seconds each and were then nafion coated (+4 V, 5 sec). For in vitro and in vivo measurements the following parameters were used: ramp potential of 0 to 700 mV, scan rate of 3 mV/0.4 s, square wave pulse modulation of 50 mV amplitude, period of 90 ms, duration of 40 ms. The electrodes were calibrated in vitro in a phosphate buffer solution (PBS, pH 7.4) containing either 10 nM 5-HT, 10 μM 5-HIAA, or 10 μM uric acid. In vitro the 5-HT/5-HIAA selectivity ratio was 6,000 to 10,000 before and 5,000 to 9,000 after the experiments. With respect to 5-HT/uric acid selectivity the ratios before and after the experiment were 8,000 to 12,000 and 10,000 to 15,000, respectively. After calibration the electrodes were stabilized in PBS containing 10 nM 5-HT, and when the peak occurring at 300 mV was stable they were immediately implanted into the dorsal raphe (A = 0.43, L = 0 and V = 0.5, interaural) with a backward angle of 45°. Once the signal was stable in vivo (usually 1.5 hour after implantation), voltammograms were generated for 2.5 hours every 10 minutes. Drugs were administered in the first 30 minutes of this 2.5-hour period. The electrochemical signal was quantified by measuring its height. For each individual animal control values were the mean peak height occurring 30 minutes before drug injection, and subsequent changes were expressed as percentages of the pre-injection values. Following the in vivo experiments, there was an increase in the sensitivity of the electrodes for all compounds tested in vitro. This observation, together with the fact that the selectivity ratio was also maintained after the in vivo experiments, suggests that the nafion coating was not significantly disrupted during implantation into the dorsal raphe nucleus. Statistical analyses were performed by comparing the curves produced under various treatments by means of a two-factor analysis of variance (ANOVA).

Superfusion Experiments

Hippocampal and mesencephalic slices containing dorsal and median raphe nuclei were incubated for 30 minutes at 37°C in Krebs solution containing 20 or 100 nM [^{3}H]5-HT creatinine sulphate (specific activity 22.7 Ci/mmol, NEN Research Products, Mississauga, Canada), respectively, and bubbled with a mixture of 95% O_2/5% CO_2. The composition of the Krebs solution was 118 mM NaCl, 4.7 mM $CaCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 0.004 mM Na_2EDTA, and 0.11 mM ascorbic acid. At the end of the incubation period with [^{3}H]5-HT, one mesencephalic or two hippocampal slices were transferred into

each of the glass chambers and superfused continuously at a rate of 0.5 ml/minute with Krebs solution maintained at 37°C and saturated with the 95% O_2/5% CO_2 mixture. Nineteen consecutive 4-minute fractions were collected starting 60 minutes after the beginning of superfusion. The slices were stimulated twice 8 minutes (S_1) and 44 minutes (S_2) after the end of the washing period. The electrical field generated in the chambers between two platinum electrodes (2 cm apart) had a voltage drop of 5 V/cm. The stimulation parameters used were 360 pulses of 2-ms duration, 30 mA intensity, delivered for 2 minutes at 3 Hz. This stimulation frequency was chosen because it is within the range of the firing rate of 5-HT neurons recorded in freely moving cats (Jacobs 1985). The first stimulation period was always used as a control. The putative antagonists were added 20 minutes before S_1 and remained present thereafter, whereas the agonists were added 20 minutes before S_2 and also remained present until the end of the superfusion. At the end of the experiment slices were solubilized in 0.5 ml Soluene 350 (Packard Instruments, Downers Grove, IL, USA) and radioactivity in the slices and superfusate samples was determined by scintillation spectrometry. The amount of tritium released per 4-minute sample was expressed as a fraction of total tritium contained in the tissue at the start of the respective collection period. The overflow of tritium produced by the electrical stimulation was calculated as the total increase in radioactivity above the basal outflow of tritium determined in the sample immediately preceding the start of stimulation (Sp_1 and Sp_2). In order to assess the drug-induced changes of electrically evoked overlow of radioactivity, S_2/S_1 ratios were calculated, and Sp_2/Sp_1 ratios were examined to determine whether the drugs altered the basal outflow of radioactivity. IC_{50} values for the different agonists were determined by computer analysis (GRAPHPAD, Graphpad Software, San Diego, CA, USA) using concentration-effect curves based on three concentrations of the agonists. The amount of tritium released by electrical stimulation in brain slices under these conditions provides a reliable estimate of the release of tritiated 5-HT (Blier and Bouchard 1993; Moret and Briley 1990). Since 5,7-dihydroxytryptamine lesion abolished reuptake into different cortical subcellular fractions Sette et al. (1983) concluded that in terminal regions such as the frontal cortex [^{3}H]5-HT is captured selectively by 5-HT terminals. It is thus most unlikely that at the concentration used in the present study (100 nM), more than fourfold smaller than the reported K_D of the 5-HT transporter for 5-HT (Barker and Blakely 1995), [^{3}H]5-HT may be captured other than by 5-HT neurons in the dorsal raphe.

The n values refer to the number of superfusion chambers studied. Experiments in which an agonist was compared to the control situation or when the effect of the agonist alone was compared to that of the agonist in the presence of the antagonist, were always run in parallel. Since control results obtained from similar experiments performed on different days did not differ significantly from one another, they were pooled. All results are expressed as means ± SEM. Means were compared using the two-tailed Student's t test and curves by using a two-factor ANOVA. p Values smaller than .05 were considered significant.

Drugs

The following drugs were used: allopurinol purchased from Sigma Chemicals (St. Louis, MO, USA); (±)mianserin; 1-[3-(trifluoromethyl)phenyl]-piperazine (TFMPP), 5-carboxyamidotryptamine, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), (R)-5-fluoro-8-hydroxy-2-(dipropylamino)tetralin (S-UH-301) from Research Biochemicals Incorporated (Wayland, MA, USA); CP 93129 from Pfizer (Groton, CT, USA); GR 127935 and sumatriptan from Glaxo (Greenford, UK); methiothepin maleate from Hoffmann La Roche (Basel, Switzerland); (−)propranolol from Imperial Chemical Industries (Cheshire, UK); (+)WAY100135 from Wyeth Research (Berkshire, UK); NSD 1015 from Aldrich Chemicals (Milwaukee, WI, USA); and ketanserin from Janssen (Beerse, Belgium). Clorgyline was a kind gift from Dr. D. Murphy (NIH Clinical Center), and paroxetine was donated by SmithKline-Beecham (Harlow, UK).

RESULTS

In Vivo Studies

The traces shown in Figure 1 illustrate typical voltammograms obtained in vitro and in vivo. The electrochemical signal was detected in vivo at 286 ± 11 mV (n = 66 electrodes tested) within a range of 200 to 450 mV. The intravenous administration of the decarboxylase inhibitor NSD 1015 (100 mg/kg), which inhibits 5-HT and 5-HIAA synthesis, reduced the electrochemical signal by 92% ± 5% over a 2-hour period (n = 4). The monoamine oxidase inhibitor (MAOI) clorgyline (10 mg/kg IV) reduced the in vivo signal by 40% ± 6% in the same time (n = 3), indicating that the recorded peak corresponded not only to 5-HT but also to 5-HIAA.

The 5-HT$_1$ agonist TFMPP (0.5 mg/kg IV) reduced the 5-hydroxyindole signal by 18% ± 6% over a 2-hour period (Figs. 1A and 2A). 8-OH-DPAT (30 µg/kg IV) produced a 23% ± 5% decrease of the signal over the same time (Fig. 2B). The effect of TFMPP was blocked (±)mianserin but not by (+)WAY100135 (Fig. 2A). (+)WAY100135 blocked the effect of 8-OH-DPAT (Fig.

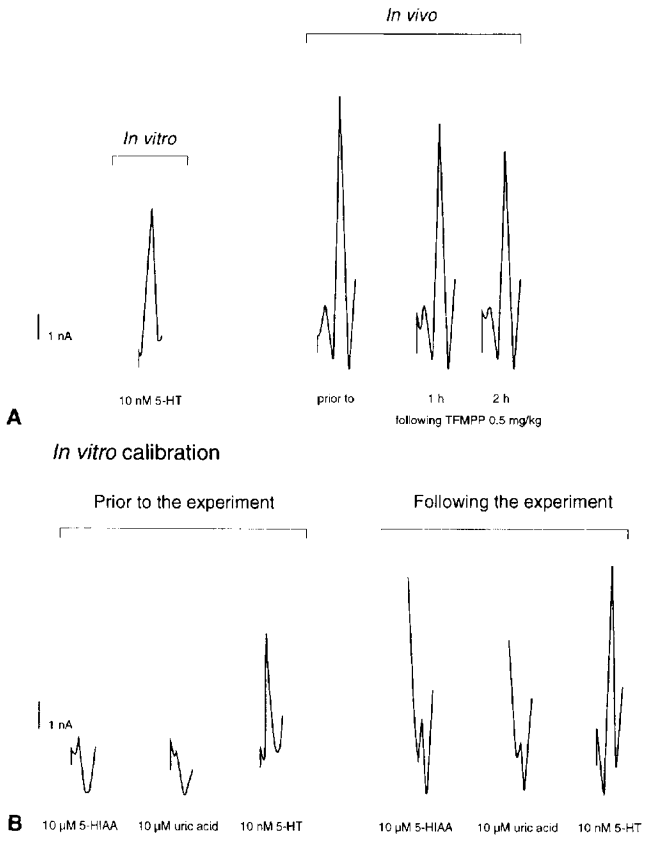

Figure 1. Individual voltammograms obtained with the same electrode. (**A**) voltammograms obtained in vitro and in vivo before and at various intervals following the administration of TFMPP (0.5 mg/kg) and (**B**) in vitro in phosphate buffer containing either 10 nM 5-HT, 100 mM 5-HIAA, or 100 nM uric acid before and after an in vivo experiment. In vivo voltammograms were obtained every 10 minutes. Those represented in the figure were obtained immediately before as well as 1 hour and 2 hours following the administration of TFMPP.

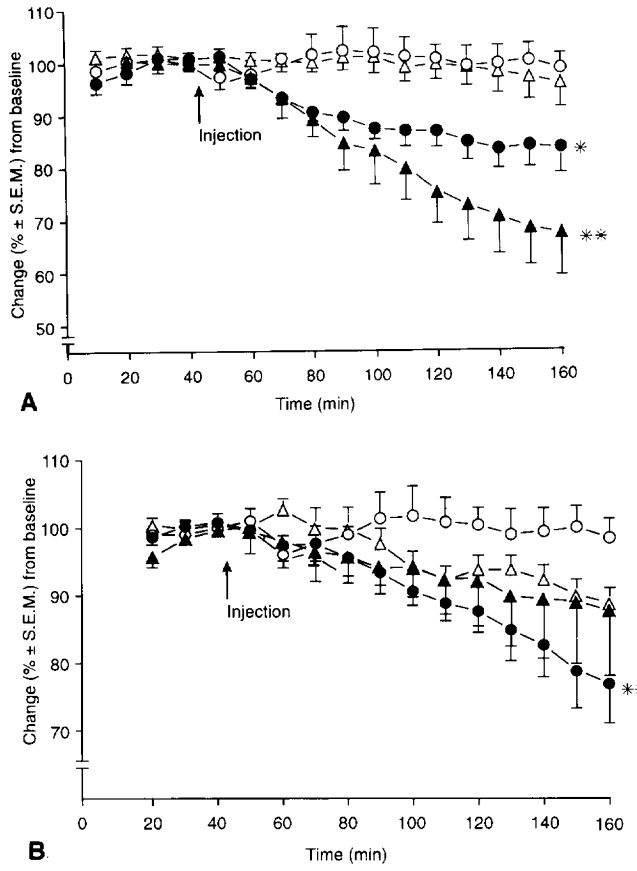

Figure 2. Modifications in the height of the 5-hydroxyindole peak recorded in the dorsal raphe nucleus following the intravenous administration of (**A**) TFMPP, 0.5 mg/kg (*solid circles*); (±)mianserin, 2 mg/kg, 30 minutes prior to TFMPP, 0.5 mg/kg (open triangles); (+)WAY100135, 0.5 mg/kg, 30 minutes prior to TFMPP, 0.5 mg/kg (*solid circles*); control (*open circles*); **(B)** 8-OH-DPAT, 30 μg/kg (*solid triangles*); (+) WAY100135, 0.5 mg/kg (open triangles); (+)WAY100135, 0.5 mg/kg, 30 minutes prior to 8-OH-DPAT, 30 μg/kg (*solid triangles*); control (open circles). Each value is expressed as a percentage of baseline ± SEM, the mean of the voltammograms of the 30-minute period before administration of the agonist being taken as 100%. $n = 5$; $p < .01$; ** $p < .001$ using two-factor ANOVA to compare with control curve.

2B). The coadministration of the 5-HT_{1A} antagonist (+)WAY100135 and TFMPP produced greater reduction in baseline values than that produced by TFMPP alone (Fig. 2A), the latter phenomenon being probably due to an intrinsic effect of (+)WAY100135 because by itself this drug reduced the voltammetric signal by 8% ± 5% (Fig. 2B).

In Vitro Studies

The electrically evoked release of [^{3}H]5-HT from raphe slices was almost entirely calcium dependent, as demonstrated by its near-complete suppression by the removal of calcium from the perfusion medium (S_2/S_1 ratios: 1.0 ± 0.1; $n = 10$; and 0.1 ± 0.1, $n = 4$, control and calcium-free superfusion medium, respectively; $p <$ .001). On the other hand, spontaneous outflow of tritium remained unchanged following calcium omission (Sp_2/Sp_1 ratios: 0.76 ± 0.02, $n = 10$, and 0.77 ± 0.06, $n = 4$ in slices superfused with normal and calcium-free Krebs, respectively). The differential effect observed on evoked and spontaneous tritium outflow following calcium omission is probably due to the different release mechanisms involved in each case. Indeed, it has been recently suggested that 5-HT may be released via two different mechanisms: (1) calcium-dependent, depolarization-induced vesicular release, and (2) cytoplasmic release, which occurs through the 5-HT carrier, by exchange diffusion and is driven by sodium gradient (Azmitia and Whitaker-Azmitia 1995).

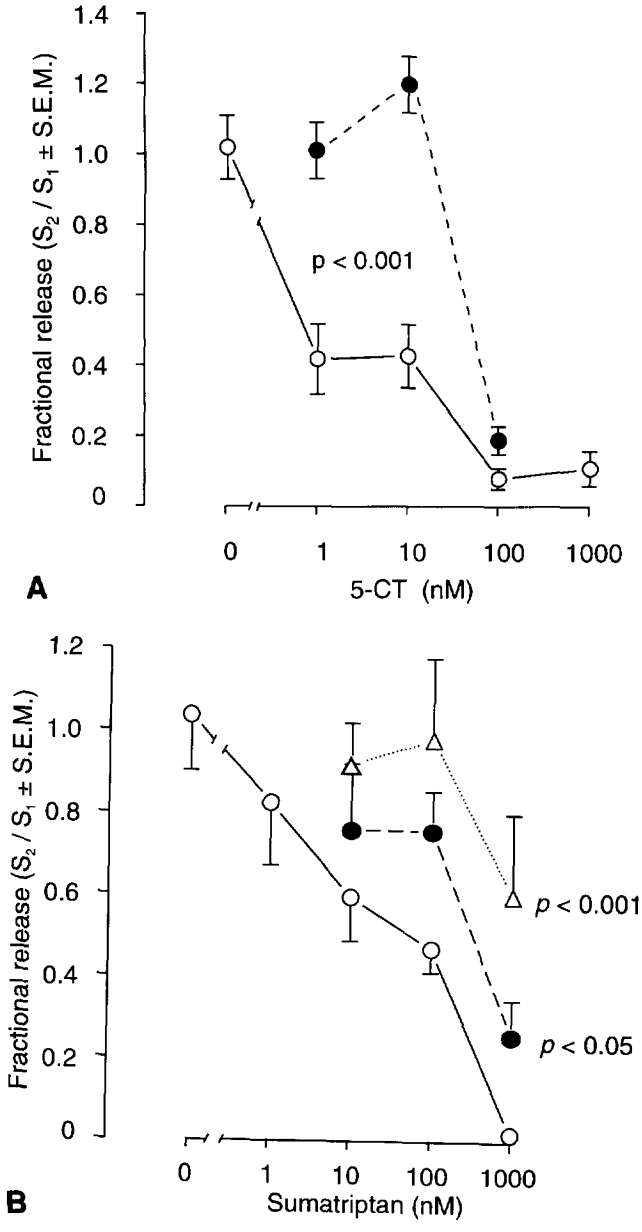

Figure 3. Concentration-effect curves of (**A**) 5-CT introduced 8 minutes before S_2 on the release of tritium elicited by the electrical stimulation of mesencephalic raphe slices preloaded with [^{3}H]5-HT (*open circle*). The *solid circles* show the curve for 5-CT in the presence of 1 μM methiothepin introduced 20 minutes before S_1; (**B**) sumatriptan introduced 8 minutes before S_2 (*open circles*). The filled circles show the curve for sumatriptan in the presence of 1 μM methiothepin and in the presence of GR 127935 (*open triangles*) introduced 20 minutes before S_1. Each point represents the mean ± SEM of 5 to 10 slices obtained from at least 2 to 5 rats. Curves were compared using a two-factor ANOVA.

Effect of 5-HT$_1$ Receptor Agonists on [^{3}H]5-HT Release. The 5-HT$_1$ agonist 5-CT and 5-HT$_{1D/1B}$ agonist sumatriptan (Hoyer 1991), introduced 8 minutes before S_2, produced a concentration-dependent inhibition of electrically evoked tritium release from mesencephalic slices (Figs. 3A and 3B). Respective IC$_{50}$ values of 5-CT and sumatriptan, calculated from the dose-response curves, were of 8 nM and 87 nM, indicating that 5-CT is more potent than sumatriptan ($p < .001$, when both curves were compared by ANOVA). Indeed, while a 100-nM concentration of 5-CT produced a near-complete inhibition of [^{3}H]5-HT release (92%), 100 nM of sumatriptan induced a submaximal inhibition (50%). At the same concentration the selective 5-HT$_{1A}$ agonist 8-OH-DPAT also induced a 50% reduction in evoked [^{3}H]5-HT release from mesencephalic slices (Fig. 4). Respective S_2/S_1 ratios for control slices and slices incubated with 100 nM 8-OH-DPAT were 1.0 ± 0.1 (n = 10) and 0.5 ± 0.1 (n = 5; $p < .01$). Except for 5-CT, which at the highest concentration used (1 μM) slightly but significantly reduced the Sp_2/Sp_1 ratio (control Sp_2/Sp_1 ratio: 0.76 ± 0.02, n = 10; 1 μM 5-CT Sp_2/Sp_1 ratio: 0.66 ± 0.02, n = 10; $p < .01$), basal tritium outflow remained unchanged throughout this series of experiments.

Unlike the 5-HT$_{1A}$ agonist 8-OH-DPAT and the 5-HT$_{1B/1D}$ agonist sumatriptan, the selective 5-HT$_{1B}$ agonist CP 93129 (Koe et al. 1992) did not inhibit 5-HT release from mesencephalic slices. Interestingly, the same doses that were ineffective at the cell body level in mesencephalic slices significantly reduced electrically evoked release of tritium in hippocampal slices (Fig. 5), where terminal autoreceptors are known to be of the 5-HT$_{1B}$ subtype (Maura et al. 1986, 1987). Concentrations of CP93129 higher than 100 nM increased spontaneous tritium outflow in hippocampus (Sp_2/Sp_1 ratio for 1 μM: 1.8 ± 0.1, n = 5, $p < .001$ compared to control) and dorsal raphe (respective Sp_2/Sp_1 ratios for 1 μM and 10 μM: 1.0 ± 0.1 and 0.9 ± 0.1, n = 5, $p <$ .001 compared to control).

Surprisingly, in a broad concentration range (100 nM–10 μM), TFMPP did not inhibit the electrically evoked release of [^{3}H]5-HT from mesencephalic slices. However, at the highest concentrations assessed (10 μM–1 mM), it produced a dose-dependent increase in spontaneous tritium outflow, suggesting that it could be acting as a displacing agent of the intraneuronal stores of tritium. To test this possibility the effect of TFMPP was assessed in the presence of the selective 5-HT reuptake inhibitor (SSRI) paroxetine (1 μM) in the superfusion medium. Paroxetine, however, did not prevent the effect of TFMPP on spontaneous outflow but, in fact increased the Sp_2/Sp_1 ratio when the drug combination was used. These results are presented in Table 1.

Effects of 5-HT Antagonists on the Response to 5-HT$_1$ Agonists in the Mesencephalic Slices. Figure 3A shows the concentration-response curve for 5-CT in the absence and presence of methiothepin (1 μM). In the pres-

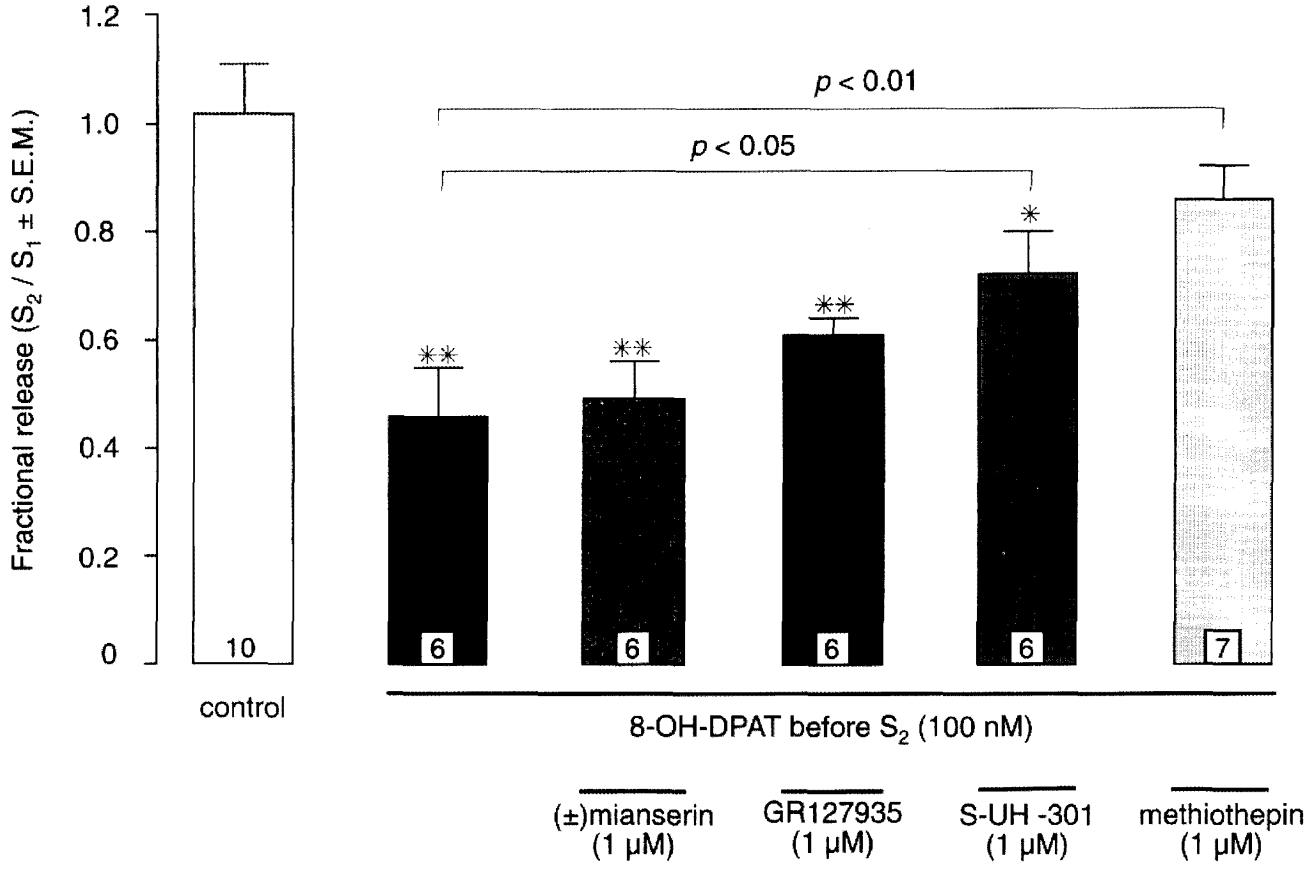

Figure 4. Effect of different 5-HT_1 antagonists on the inhibition of electrically evoked release of tritium produced by 8-OH-DPAT in mesencephalic raphe slices preloaded with [^{3}H]5-HT. The antagonists were introduced 20 minutes before S_1 and remained present in the superfusate until the end of the experiment. 8-OH-DPAT was introduced 20 minutes before S_2 and also remained present until the end of the experiment. Values are expressed as means ± SEM for which the number of experiments per group is given at the bottom of each column. Slices were obtained from three to five rats. The means were compared using a nonpaired Student's t test.

ence of methiothepin there was a significant reduction in the inhibition of the evoked [^{3}H]5-HT overflow ($p < .001$, comparing both curves by two-way ANOVA), with an eightfold increase in IC_{50} values (IC_{50} for 5-CT: 8-nM; IC_{50} for 5-CT + methiothepin, 1 μM: 63 nM). The effect of 5-CT (100 nM) was also blocked by the 5-HT_{1A} antagonists S-UH-301 (Fig. 6A; Björk et al. 1991) and (+)WAY100135 (Fletcher et al. 1993; Routledge et al. 1993). Observed S_2/S_1 ratios were 0.08 ± 0.03 for 5-CT (100 nM, $n = 10$) and 0.74 ± 0.06 for 5-CT (100 nM) plus (+)WAY100135 (1 μM; $n = 3$), respectively ($p < .001$). The 5-$HT_{1B/1D}$ antagonist GR 127935 (Skingle et al. 1993) and (±)mianserin, a drug with high affinity for the rat 5-HT_{1D} receptor subtype (Hamblin et al. 1992a) also blocked the effect of 5-CT (Fig. 6A).

The effect of 8-OH-DPAT (100 nM) was blocked by methiothepin and S-UH-301, but neither by the 5-$HT_{1B/1D}$ receptor antagonist GR 127935 nor by (±)mianserin (Fig. 4). The latter observations thus confirm that at the concentration of 100 nM, inhibition of stimulated [^{3}H]5-HT release by 8-OH-DPAT is a 5-HT_{1A}-mediated effect.

The concentration-effect curve of sumatriptan was shifted to the right by (±)mianserin ($p < .05$) and GR 127935 ($p < .001$; Figure 3B). The calculated IC_{50} values for sumatriptan, sumatriptan + (±)mianserin (1 μM), and sumatriptan + GR 127935 (1 μM) were 85, 227, and 467 nM, respectively. Methiothepin, but not the 5-HT_{1A} antagonist S-UH-301, blocked the effect of 100 nM sumatriptan (Fig. 6B). This observation indicates that the inhibitory effect of sumatriptan on electrically evoked release of [^{3}H]5-HT is not a 5-HT_{1A}-mediated response. The 5-$HT_{1A/1B}$ antagonist (−)propranolol, at a concentration of 1 μM, did not block the effect of sumatriptan (100 nM) either (respective S_2/S_1 ratios for sumatriptan and sumatriptan (±)propranolol were 0.4 ± 0.1 and 0.6 ± 0.1 ($n = 9$, $p = .6$). However, at a higher concentration of (−)propranolol (3 μM) the effect of sumatriptan was completely abolished (respective S_2/S_1 ratios for sumatriptan and sumatriptan

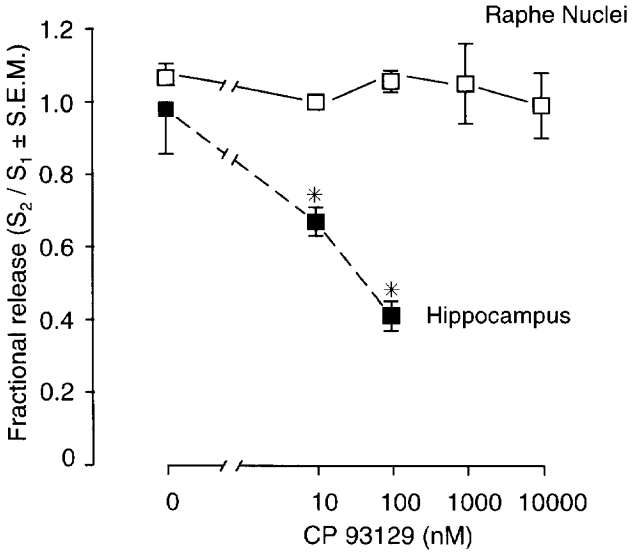

Figure 5. Concentration-effect curve of CP 93129 introduced 8 minutes before S_2 on the release of tritium elicited by the electrical stimulation of mesencephalic raphe slices (*open squares*) or hippocampal slices (*solid squares*) preloaded with [^{3}H]5-HT. Each point represents the mean ± SEM of 5 to 10 slices obtained from at least 2 to 5 rats. * $p < .001$ when compared to the control value using a nonpaired Student's t test.

(−)propranolol were 0.4 ± 0.1 and 1.1 ± 0.2 (n = 6, $p < .05$).

The effects of the antagonists themselves on evoked and basal tritium release, were assessed by comparing S_1 and Sp_1 values obtained in experiments in which the antagonist were introduced 20 minutes before S_1, to those obtained in control experiments. Methiothepin, GR 127935, and ketanserin significantly increased the amount of [^{3}H]5-HT released by S_1 as well as spontaneous tritium outflow (Sp_1). Presently ketanserin is the only drug that has been shown to distinguish between 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptor subtypes (Doménech et

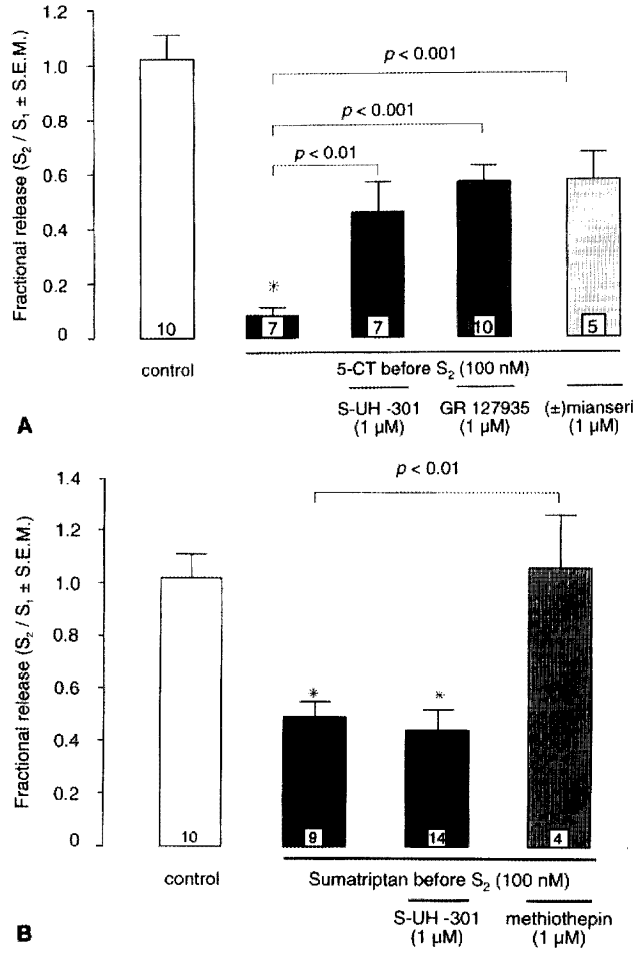

Figure 6. Effect of different 5-HT antagonists on the inhibition of electrically evoked release of tritium produced by (**A**) 5-CT or (**B**) sumatriptan in mesencephalic raphe slices preloaded with [^{3}H]5-HT. The antagonists were introduced 20 minutes before S_1 and remained present in the superfusate until the end of the experiment. 5-CT or sumatriptan were introduced 20 minutes before S_2 and also remained present until the end of the experiment. Values are expressed as means ± SEM for which the number of experiments per group is given at the bottom of each column. Slices were obtained from three to five rats. The means were compared using a nonpaired Student's t test.

Table 1. Effect of TFMPP on Spontaneous and Evoked Release of [^{3}H]5-HT from Mesencephalic Slices

		S_2/S_1	Sp_2/Sp_1
Control		1 ± 0.1	0.76 ± 0.02
TFMPP	0.1 μM	1.0 ± 0.1	0.77 ± 0.03
	1 μM	0.9 ± 0.1	0.73 ± 0.08
	3 μM	0.9 ± 0.1	0.80 ± 0.03
	10 μM	1.0 ± 0.1	1.07 ± 0.01**
	100 μM	–	2.81 ± 0.31**
	1000 μM	–	9.63 ± 1.97
Paroxetine	(1 μM)		
+ TFMPP	1 μM	1.2 ± 0.1	1.00 ± 0.06**
	10 μM	3.1 ± 0.6*	2.5 ± 0.1**

* $p < .05$ (two-tailed Student's t test); ** $p < .001$ (two-tailed Student's t test); n = 4 to 10 slices obtained from 2 to 5 rats.

Table 2. Effects of Putative 5-HT$_{1D}$ Antagonists on Spontaneous Release of [^{3}H]5-HT from Mesencephalic Slices

	Sp_1	S_1
Control	1.50 ± 0.07	1.8 ± 0.2
Methiothepin (1 μM)	2.00 ± 0.09*	6.8 ± 0.9**
GR 127935 (1 μM)	1.73 ± 0.08	4.5 ± 0.2**
Ketanserin (0.3 μM)	3.31 ± 0.15**	4.4 ± 0.5**
Ketanserin (1 μM)	4.31 ± 0.25**	5.8 ± 0.5**
Ketanserin (1 μM) + Paroxetine (1 μM)	6.30 ± 0.20**	

* $p < .01$ (two-tailed Student's t test); ** $p < .001$ (two-tailed Student's t test); n = 10 to 30 slices obtained from 5 to 15 rats.

al. 1994). Its effect on the inhibition of [^{3}H]5-HT release produced by sumatriptan could not be assessed because it induced a two- to threefold increase in spontaneous tritium overflow. This increase in outflow was not blocked by 1 μM of the SSRI paroxetine, introduced 20 minutes before S_1 (Table 2).

DISCUSSION

In the present study in vivo voltammetry and in vitro superfusion paradigms were used to demonstrate that functional 5-HT$_{1D}$ receptors modulate 5-HT release within the mesencephalic (dorsal and median) raphe nuclei of the rat brain. On the one hand, voltammetry results showed that 5-HT$_1$ receptors other than 5-HT$_{1A}$, and possibly 5-HT$_{1D}$, play a functional role in modulating in vivo the release of 5-HT in the dorsal raphe nucleus of the rat. On the other hand, the in vitro superfusion paradigm was used to complete the pharmacological characterization of the 5-HT receptor subtypes regulating 5-HT release in the mesencephalic raphe nuclei. Moreover, since somatodendritic sites are severed from their afferents in mesencephalic slices, this paradigm also allowed us to confirm that 5-HT release at the cell body level is modulated locally by 5-HT$_{1D}$ receptors. It was not possible, however, to determine whether 5-HT$_{1D}$ receptors in the rat mesencephalon are located on 5-HT neurons themselves or on interneurons, or whether they control somatodendritic or axon collateral release of 5-HT. However, given the small number of axon terminals in the dorsal raphe nucleus (Descarries et al. 1982) and the presence of abundant vesicles containing 5-HT in the dendrites of 5-HT neurons (Chazal and Ralston 1987), somatodendritic release might well be the predominant source of extracellular 5-HT in the raphe nuclei.

The electrochemical results obtained in vitro are consistent with the notion that electrically pretreated carbon-fiber and nafion-coated electrodes do not detect 5-HIAA (Fig. 1A). In the present study a 5,000- to 10,000-fold selectivity for 5-HT compared to 5-HIAA was achieved. For 5-HT/uric acid a 8,000- to 15,000-fold selectivity factor was also attained (Fig. 1A). In vivo the administration of the xanthine oxidase inhibitor allopurinol (10 mg/kg IP), which brings uric acid to undetectable levels in the brain (Cespuglio et al. 1986) further ensured the lack of contribution of uric acid to the electrochemical signal. Though the in vitro selectivity of the electrodes for 5-HT was still present after the in vivo experiment (see Materials and Methods section and Fig. 1A), during the time they were implanted into the dorsal raphe nucleus these electrodes detected 5-HIAA as well as 5-HT. The shape of the voltammograms obtained from the dorsal raphe in the present study is comparable to that monitored in vivo with DPV and uncoated carbon-fiber electrodes (Crespi 1990). However, the intravenous administration of the MAOI clorgyline produced a much smaller decrease of the voltammetric signal in the present study (40%) than in the latter one (70%). It is noteworthy, however, that the decrease in the voltammetric signal produced by blocking the production of 5-HIAA with clorgyline was smaller than that produced by the decarboxylase inhibitor NSD 1015, which blocks 5-hydroxytryptophan conversion into 5-HT. Moreover, the concentration of extracellular 5-HT in the dorsal raphe calculated from our experiments was of 12 nM, a value that is in perfect agreement with that of 10 nM obtained in vivo from the rat dorsal raphe by Crespi et al. (1988) using single-fiber nafion-coated electrodes. These observations thus indicate that in spite of the fact that in vivo the electrodes detected not only 5-HT but also 5-HIAA, the concentration of 5-HT was not overestimated as would have been the case if 5-HIAA had largely contributed to the signal. On the other hand, results from microdialysis studies suggest that the extracellular somatodendritic concentration of 5-HT would be lower (3–5 nM; Adell et al. 1993; Bel and Artigas 1992). However, a concentration gradient could account for this discrepancy because in the latter studies the dialysis probe was placed in the immediate vicinity (0.5 mm lateral from bregma) of the raphe nuclei while in the present one the tips of the electrodes were positioned directly into the dorsal raphe. In contrast with previous results, nafion-coated electrodes used in the present study detected 5-HIAA as well as 5-HT, which is presumably due to the fact that it is more difficult to coat entirely with nafion multifiber electrodes like the ones used in this study than single-fiber electrodes like those used by Crespi et al. (1988).

At the doses used the nonselective 5-HT$_1$ agonist TFMPP (Schoeffter and Hoyer 1989) and the selective 5-HT$_{1A}$ agonist 8-OH-DPAT produced similar attenuation in the 5-hydroxindole peak recorded from the dorsal raphe nucleus (Fig. 2). The inhibitory effects observed in the present study are consistent with those previously reported by Blier et al. (1990) following systemic administration of 8-OH-DPAT (30 μg/kg IV) and RU 24969 (10 mg/kg IP). In the Blier et al. (1990) study, however, the reduction in the voltammetric signal 2 hours after the injection of both agonists was of about 50%. Given that nafion-coated electrodes were used in the present study but not in the former, a reduced contribution of 5-HIAA to the voltammetric peak could explain the smaller effect observed. The fact that the 5-HT$_{1A}$ antagonist (+)WAY100135 (Fletcher et al. 1993) blocked the effect of 8-OH-DPAT, but not that of TFMPP, indicates that only 8-OH-DPAT-induced decrease in dorsal raphe extracellular indoles is mediated via 5-HT$_{1A}$ receptors. (+)WAY100135 produced an apparent potentiation of the effect of TFMPP (Fig. 2A). This could be due to an intrinsic effect of (+)WAY100135

because by itself (+)WAY100135 produced a small reduction in the voltammetric signal (8%, NS; Fig. 2B). Two mechanisms could explain the intrinsic activity of (+)WAY100135 observed in the present study: (1) a direct partial agonistic effect at 5-HT$_{1A}$ receptors (Escandon et al. 1994); or (2) an antagonistic activity at α_1-adrenergic receptors (Lanfumey et al. 1993). It is worth noting that at the dose used (0.5 mg/kg IV) in the present study, (+)WAY100135 has been shown to decrease 5-HT neuron firing activity by 20% (Fletcher et al. 1993), in keeping with its putative 5-HT$_{1A}$ partial agonistic action.

(±)Mianserin is a nonselective 5-HT antagonist, its highest affinity being for 5-HT$_{2A}$ (8 nM; Hoyer 1991) and rat 5-HT$_{1D}$ binding sites (30 nM; Hamblin et al. 1992a). This drug readily distinguishes 5-HT$_{1D}$ and 5-HT$_{1B}$ sites in rat cell lines as it binds to the former with an affinity more than 10,000-fold higher than for the latter (Hamblin et al. 1992a). Because TFMPP is an agonist selective for 5-HT$_1$ receptors (Shoeffter and Hoyer 1989), the reduction induced by this drug on extracellular 5-hydroxyindole concentration in the dorsal raphe cannot be ascribed to the stimulation of 5-HT$_{2A}$ receptors, even if its effect is blocked by (±) mianserin. The ineffectiveness of (+)WAY100135 to block the effect of TFMPP ruled out a role for 5-HT$_{1A}$ receptors, and the ability of (±)mianserin to suppress the TFMPP-induced decrease in the voltammetric signal suggests that 5-HT$_{1D}$, rather than 5-HT$_{1B}$, receptors mediate the effect of systemic administration of TFMPP. To test this contention the pharmacological profile of the receptors that regulate somatodendritic 5-HT release was studied in vitro.

The following evidence confirms the presence of 5-HT$_{1A}$ receptors that negatively control 5-HT release within the rat mesencephalic raphe nuclei: (1) the inhibitory effect of the 5-HT$_1$ agonist 5-CT on tritium-evoked release was blocked by 5-HT$_{1A}$ receptor antagonists UH-301 (Fig. 6A) and (+)WAY100135 (Björk et al. 1991; Fletcher et al. 1993; Routledge et al. 1993); and (2) the 5-HT$_{1A}$ agonist 8-OH-DPAT inhibited tritium release (Fig. 4).These observations are consistent with the suppressant effect of microiontophoretic application of 8-OH-DPAT on the firing activity of 5-HT neurons in the dorsal raphe (Blier et al. 1987). 8-OH-DPAT also has affinity for 5-HT$_{1D}$ receptors (Schoeffter and Hoyer 1990). However, at the concentration used (100 nM) these receptors were not activated because the 5-H$_{1B/1D}$ antagonist GR 127935 did not block the effect of 8-OH-DPAT (Fig. 4).

Sumatriptan, which binds with nanomolar affinity to 5-HT$_{1D}$ sites (Hoyer 1991), also inhibited [^{3}H]5-HT release from slices containing the mesencephalic raphe nuclei (Fig. 3B), and this effect was blocked by GR 127935 (Fig. 3B). However, sumatriptan has only a 10-fold selectivity in discriminating 5-HT$_{1D}$ from 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors (Hoyer 1991). A role for 5-HT$_{1A}$ receptors in this inhibitory effect of sumatriptan may be ruled out since the 5-HT$_{1A}$ antagonist S-UH-301 (Björk et al. 1991) did not alter the effect of sumatriptan (Fig. 6A). Evidence supporting the involvement of 5-HT$_{1D}$, and not that of 5-HT$_{1B}$, receptors in the inhibitory effect of sumatriptan is twofold: (1) (±)mianserin, which does not significantly bind to 5-HT$_{1B}$ receptors (Hamblin et al. 1992a), blocked this effect of sumatriptan (Fig. 3B), (2) though the selective 5-HT$_{1B}$ agonist CP 93129 (Koe et al. 1992) inhibited 5-HT release from hippocampal slices where 5-HT release is controlled by 5-HT$_{1B}$ receptors (Maura et al. 1986, 1987), this drug was devoid of effect on the evoked release of [^{3}H]5-HT from mesencephalic slices (Table 1). At some of the concentrations used (1 and 10 μM), CP 93129 produced an increase in basal outflow of tritium from mesencephalic slices. It is unlikely, however, that the effect of CP 93129 on basal outflow could account for its lack of effect on electrically evoked release of [^{3}H]5-HT because at the concentration of 100 nM, which did not modify basal outflow (Table 1), it did not affect the evoked release of tritium. The fact that (−)propranolol (1 μM) did not block the effect of sumatriptan (100 nM) further supports the notion that 5-HT$_{1D}$, not 5-HT$_{1B}$, receptors negatively regulate 5-HT release in the raphe nuclei. The effectiveness of higher concentrations (3 μM) to block the effect of sumatriptan (100 nM) could be due to a loss of selectivity. Indeed, (−)propranolol binds to rat 5-HT$_{1D}$ receptors with a K_D of 1,800 nM (Hamblin et al. 1992a). Because methiothepin blocked the effect of sumatriptan (Fig. 6B) and 5-HT$_{1E}$ receptors have only modest affinity for this drug (McAllister et al. 1992; Zgombick et al. 1992), it is improbable that the latter receptors account for the results obtained with sumatriptan. Sumatriptan also binds to 5-HT$_{1F}$ sites; in contrast 5-CT binds only with low affinity to these sites (Glennon and Dukat 1995). It is therefore difficult to reconcile a role of 5-HT$_{1F}$ receptors with the inhibitory effect of 5-CT, which, on the other hand, is blocked by the 5-HT$_{1D/1B}$ antagonist GR 127935 (Fig. 6A).

The increase in [^{3}H]5-HT release observed with methiothepin and GR 127935 (Table 2) is consistent with previous findings (Moret and Briley 1993; Starkey and Skingle 1994; Wilkinson and Middlemiss 1992) and has been attributed to the blockade of autoreceptor activation caused by released 5-HT. Interestingly, the 5-HT$_{1A}$ antagonist S-UH-301 (Björk et al. 1991) did not produce an increase in tritium-evoked release, although other 5-HT$_{1A}$ antagonists such as (+)WAY100135 have been shown to do so (Starkey and Skingle 1994). This could be explained by the lower affinity of S-UH-301 for the 5-HT$_{1A}$ receptor. Indeed, respective IC$_{50}$ values for S-UH-301 and (+)WAY100135 in displacing [^{3}H]8-OH-DPAT from 5-HT$_{1A}$ receptors are 157 and 34 nM (Cliffe et al. 1993; Cornfield et al. 1991).

The present results are in agreement with those of Starkey and Skingle (1994) showing the presence of functional 5-HT_{1D} receptors in the dorsal raphe of the guinea pig. Molecular biology techniques have revealed that there exist two 5-HT_{1D} receptor subtypes: 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ (Hamblin and Metcalf; Hamblin et al. 1992a, 1992b; Weinshank et al. 1992). The pharmacological profiles of these two receptors are very similar (Hartig et al. 1992), ketanserin being the only compound tested thus far able to differentiate them (Doménech et al. 1994). Because of these similarities, Starkey and Skingle (1994) were unable to conclude whether one or the two 5-HT_{1D} receptor subtypes control 5-HT release at the somatodendritic level. The effect of ketanserin on sumatriptan-induced inhibition of evoked [^{3}H]5-HT release could not be assessed in the present study because at the concentrations tested (0.3 and 1 μM) ketanserin doubled to tripled spontaneous 5-HT outflow, an effect that could not be blocked by the SSRI paroxetine (Table 2). However, since rat 5-HT_{1D} and 5-HT_{1B} receptors are species homologues of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors, respectively (Hartig et al. 1992), it is possible, that in those species where both receptors exist, including humans (Weinshank et al. 1992), it is the 5-$HT_{1D\alpha}$ subtype that would control somatodendritic release of 5-HT.

Consistent with the present findings, Davidson and Stamford (1994) have recently reported that 5-HT_1 receptors, other than 5-HT_{1A} autoreceptors, regulate 5-HT release in the rat dorsal raphe nucleus. The drugs used in the latter study, unlike the present one, did not allow the authors to conclude which 5-HT_1 receptor subtype was involved. We have recently provided evidence that 5-HT_{1A} and 5-HT_{1D} receptors are not functionally redundant because 5-HT_{1A} receptors control 5-HT release in the cell body area by regulating 5-HT neuron-firing activity, whereas 5-HT_{1D} receptors control release in a firing-independent manner (Piñeyro et al. 1993, 1994). Furthermore, Craven et al. (1994) have recently found that GR 127935 does not attenuate 5-HT induced inhibition of firing of 5-HT neurons recorded from guinea pig midbrain slices.

TFMPP inhibited 5-HT release in vivo but not in vitro (Fig. 2A and Table 2). This is not surprising considering that similar results have been obtained when the effect of RU 24969 was assessed in the rat brain. In vivo RU 24969 decreased 5-HT release in frontal cortex (Blier et al. 1990; Crespi et al. 1990) but in vitro it increased 5-HT release from hippocampal slices (Auerbach et al. 1990). The enhancing effect of RU 24969 and TFMPP on 5-HT release in vitro were abolished by the SSRI fluoxetine. It was concluded from this set of results that at low concentrations both agonists stimulate the terminal autoreceptor and inhibit 5-HT release, whereas at higher concentrations, they interact with the 5-HT carrier (Auerbach et al. 1990). In the present study, the effect of TFMPP on the evoked release of [^{3}H]5-HT was tested over a large range of concentrations, and no significant inhibition was observed. The possibility of an interaction with the 5-HT carrier was also explored; paroxetine did not block the effect of TFMPP on the Sp_2/Sp_1 ratio; in fact, it potentiated the releasing effect of TFMPP. Another explanation for these results could be derived from the observation of Sinton and Fallon (1988) that TFMPP decreases 5-HT neuron-firing activity in the dorsal raphe and stimulates that of 5-HT neurons in the median raphe. Because mesencephalic slices used in the present study contained both nuclei, it is therefore possible that the effects on both nuclei cancel each other out. Alternatively, it should be considered that TFMPP-induced changes in the in vivo voltammograms may have reflected a decrease not only in 5-HT but also in 5-HT metabolism. In fact, systemic administration of RU 24969 has been found to decrease 5-HT release and 5-HT metabolism, monitored in frontal cortex with nafion-coated and uncoated electrodes, respectively (Crespi et al. 1990).

In conclusion, the results obtained in the present study demonstrate the existence of functional 5-HT_{1D} receptors that control the release of 5-HT from mesencephalic raphe nuclei in the rat brain.

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