Drug-Induced Regulation of [¹²⁵I]Iodocyanopindolol-labeled 5-Hydroxytryptamine_{1B} Receptor Binding Sites in the Central Nervous System

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Little is known about the regulation of 5-hydroxytryptamine_{1B} (5-HT_{1B}) receptors, a putative terminal autoreceptor in the central nervous system. We studied the regional responses of [¹²⁵I]iodocyanopindolol ([¹²⁵I]ICYP)-labeled central 5-HT_{1B} sites to chronic treatment with 5-HT agonists and antagonists at a dose of 10 mg/kg/d IP for 30 consecutive days in the rat. In controls, there were 3.4-fold regional differences in B_{max}, with a rank order of brainstem > hippocampus > cortex, striatum > spinal cord, and K_ds were slightly lower in striatum and spinal cord. RU 24969 significantly reduced B_{max} 23 to 63% in cortex, hippocampus, striatum, brainstem, and spinal cord without a change in K_d except for a 1.7-fold increase in cortex and spinal cord. The

putative 5-HT_{1B} agonist (m-trifluoromethyl-phenylpiperazine (TFMPP), but not [1-(3-chlorophenyl)piperazine] (m-CPP) or the 5-HT_{1B} antagonists pindolol or quipazine, reduced the B_{max} of cortical 5-HT_{1B} sites (–16%). Chronic treatment with the 5-HT antagonists methysergide, pindolol, propranolol, ritanserin, metergoline, or methiothepin did not significantly affect striatal B_{max} or K_d compared to respective vehicles. The data demonstrate significant changes in maximum number of 5-HT_{1B} receptors in response to chronic agonist but not antagonist treatments at the dose studied. [*Neuropsychopharmacology* 10:259–264, 1994]

KEY WORDS: Serotonin_{1B} receptor; [¹²⁵I]ICYP; RU 24969; m-CPP; Tolerance; 5-HT_{1B} sites

Recently, the demonstration of 5-HT_{1B} sites in human brain utilizing molecular genetic techniques (Jin et al. 1992) has renewed interest in this site for which much data has been accumulated in rodents. The 5-HT_{1B} receptor, the purported 5-HT terminal autoreceptor (Engel et al. 1983; Middlemiss 1984), is enriched in striatum (caudate-putamen) (Pazos and Palacios 1985), a

basal ganglia region of importance to certain human dyskinesias or movement disorders. 5-HT_{1B} sites may regulate γ -aminobutyric acid (GABA) release in striatum, blocking the GABA_B synaptic potential in mid-brain dopamine neurons (Johnson et al. 1992), and cerebellar Purkinje cells (Maroteaux et al. 1992). The 5-HT_{1B} receptor is coupled to the inhibition of adenylyl cyclase by the inhibiting G-protein G_i (Bouhelal et al. 1988; Pleus and Bylund 1992). A marker behavioral correlate of 5-HT_{1B} receptor activation has been proposed as stimulatory effects on locomotion based on RU 24969 (Green et al. 1984; Oberlander et al. 1986; Pranzatelli et al. 1987).

Evidence for differential regulation of 5-HT receptor subtypes in the mammalian CNS is accumulating. There have been few studies of the regulation of 5-HT_{1B} receptors. Behavioral tolerance has been

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demonstrated in rodents following repeated administration with RU 24969, *m*-CPP, or CGS 12066B (Sills et al. 1985; De Souza et al. 1986; Oberlander et al. 1987; Frances and Monier 1991). Information on chronic effects of 5-HT_{1B} antagonists is not available. Desensitization and down-regulation by 5-HT has been demonstrated in nonneural cell lines in vitro (Pleus and Bylund 1992) but not in brain or spinal cord.

As part of a systematic effort to characterize drug-induced regulation of 5-HT receptor subtypes, we studied the effects of chronic treatment with 5-HT agonists and antagonists on central 5-HT_{1B} binding sites in the rat. RU 24969, TFMPP, and *m*-CPP were chosen as 5-HT_{1B} agonists, and pindolol and quipazine as 5-HT_{1B} antagonists (Glennon 1987; Middlemiss 1985), drugs for which behavioral data are available. However, TFMPP and *m*-CPP have activity at 5-HT_{1C} sites and RU 24969, TFMPP, *m*-CPP and quipazine also bind to 5-HT₂ sites (Hoyer et al. 1985). [¹²⁵I]ICYP has been used to selectively label 5-HT_{1B} sites in the presence of isoproterenol to suppress binding to β -adrenoceptors (Offord et al. 1988; Ariani et al. 1989; Pleus and Bylund 1992).

METHODS

Animals

Male 200 to 250 g Sprague-Dawley rats (Charles River, Wilmington, MA) were housed three to a cage with access to food and water under constant temperature (23°C) and a 12-hour light/12-hour dark cycle.

Drugs

Drug solutions were prepared immediately before use and sonicated to ensure that they were dissolved. For injections, ($\pm$)DOI[1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane] HCl (Research Biochemicals, Inc., Natick MA; RBI), TFMPP (*m*-trifluoromethyl-phenylpiperazine) HCl (RBI), *m*-CPP [1-(3-chlorophenyl)piperazine] HCl (Sigma Chemical Co., St. Louis MO), and methysergide maleate (Sandoz, East Hanover, NJ) were dissolved in 0.9% saline, quipazine maleate (RBI) in 0.9% saline acidified with 1 drop/ml of 1 N HCl, ($\pm$)pindolol (Sigma) and ($-$)propranolol (Wyeth-Ayerst Research, Inc., Princeton, NJ) in 0.9% saline containing 10% ascorbic acid, and metergoline (Farmitalia Carlo Erba, Milan, Italy), methiothepin (Hoffman La Roche, Nutley, NJ), ritanserin (Janssen Pharmaceutica, Beerse, Belgium) in 100% propylene glycol. For receptor binding studies, the following drugs were dissolved in assay buffer: 5-HT creatinine sulfate (Sigma), ($\pm$)isoproterenol HCl (Sigma), RU 24969 (Roussel-UCLAF, Romainville, France). [¹²⁵I]ICYP (specific activity 2200 Ci/mmol) was purchased from Dupont-New England Nuclear

(Wilmington, DE). Other reagents were obtained from Sigma.

Drug Injections

Rats were injected IP once daily for 30 consecutive days with 10 mg/kg of a single drug or respective vehicle (saline, 10% ascorbic acid, saline acidified with HCl, or 100% propylene glycol). The use of a 10 mg/kg dose was somewhat arbitrary in terms of 5-HT_{1B} receptor affinities but was selected to allow comparison with behavioral studies (Green et al. 1984; Sills et al. 1985; De Souza et al. 1986; Frances and Monier 1991) and with regulation studies of other 5-HT receptors (Larsson et al. 1990; Pranzatelli 1991; Murthy and Pranzatelli 1992; Pranzatelli et al. 1993).

Dissections

Rat brains were dissected 24 hours after the last drug injection on a glass petri dish over ice into cortex, hippocampus, striatum, brainstem (inclusive of midbrain, pons, and medulla), diencephalon (thalamus and hypothalamus), cerebellum. Whole spinal cord was also removed. Tissues were frozen on dry ice and then stored at -70°C until use.

[¹²⁵I]ICYP Binding

In the following description, all concentrations are stated as final concentrations. Frozen tissues were homogenized in 200 volumes of 50 mmol/L Tris HCl-base buffer (pH 7.4 at 37°C) using a Brinkmann Polytron (setting 5 for 10 seconds), preincubated for 15 minutes at 37°C, and centrifuged at $49,000 \times g$ for 20 minutes. The striatal pellet, due to the small size of the region and amount of sample available for binding assays, was resuspended in 100 volumes of assay buffer (Tris HCl-base, pH 7.4 at 37°C, 0.1% ascorbic acid w/v, 4 mmol/L MgCl₂, and 10 $\mu\text{mol/L}$ pargyline), but all other pellets were resuspended in 50 volumes. After reviewing several different methods of performing [¹²⁵I]ICYP binding (Offord et al. 1988; Ariani et al. 1989; Matsumoto et al. 1992; Pleus and Bylund 1992) that differ in concentrations of isotope or isoproterenol used, and based on preliminary experiments, our radioligand binding assays consisted of 200 μl tissue homogenate, which was added last to 16×100 mm borosilicate tubes, 12.5 μl of 10 $\mu\text{mol/L}$ 5-HT or buffer blank, 12.5 μl of 30 $\mu\text{mol/L}$ isoproterenol, and 25 μl of [¹²⁵I]ICYP. Our Scatchard plots were linear using 12 isotope concentrations (0.02 to 1.0 nmol/L) in brainstem, therefore, in subsequent experiments we used six isotope concentrations (0.02 to 0.6 nmol/L) to allow control and drug-treated samples to be run at the same time

Figure 1. Regional saturation analyses of [¹²⁵I]ICYP-labeled 5-HT_{1B} sites in (A) brainstem, (B) striatum, (C) hippocampus, (D) cortex, and (E) spinal cord of controls. The data are means of five separate experiments performed in duplicate. For Scatchard plots (inserts), units are pmol/g for BOUND and pmol/g/nmol/L for B/F (Bound/Free).

under the same conditions. Scatchard studies in brainstem performed in the absence and presence of isoproterenol revealed that 84% of [¹²⁵I]ICYP-specific binding was to 5-HT_{1B} binding sites. Assays were pipetted on ice in a fume hood with the hood light turned off. A 15-minute incubation in a covered shaking water bath at 37°C was terminated by filtration through a Brandel M48-R Harvester on Schleicher and Schuell No. 32 glass fiber filters (Keene, NH) with two 5 ml washes of ice-cold Tris HCl-base buffer (pH 7.7 at 25°C). Radioactivity was counted in a Beckman gamma counter at 77% counting efficiency. Specific binding was defined as the difference between radioligand binding in the presence and absence of unlabeled 5-HT. B_{max} , K_d , IC_{50} , K_i , and n_H were calculated using the computer-assisted EBDA program (McPherson 1983).

Competition experiments were performed in striata from rats treated chronically with 5-HT agonists,

Figure 2. Regional effects on B_{max} of chronic treatment with 10 mg/kg RU 24969. Data are means $\pm$ SEM of five separate experiments performed in duplicate. Regions are cortex (CTX), hippocampus (HIPP), striatum (STR), brainstem (BS), and spinal cord (SC). There were statistically significant main effects on B_{max} ($F = 36.64$, $p = .0001$) for CNS region ($F = 56.40$, $p = .0001$), chronic drug treatment ($F = 84.24$, $p = .0001$), and the interaction of region and drug treatment ($F = 3.55$, $p = .02$). There were significant differences in K_d (nmol/L) ($F = 9.03$, $p = .0001$) for drug treatment ($F = 21.15$, $p = .0001$) only in cortex (saline 0.19 ± 0.01 , RU 24969 0.33 ± 0.04) and spinal cord (saline 0.14 ± 0.02 , RU 24969 0.23 ± 0.04). *.01 $\leq p < 0.05$; **.001 $\leq p < .01$; ***.0001 $\leq p < .001$, t -test.

antagonists, or vehicle for 30 consecutive days. Unlabeled 5-HT or RU 24969 was allowed to compete with [¹²⁵I]ICYP. Twenty-one concentrations of competing drug from 10^{-11} to 10^{-4} M were used. 5-HT affinity was calculated by the computer assisted EBDA program according to the Cheng-Prusoff equation (1973):

$$K_i = IC_{50}/1 + \frac{[I]}{K_d},$$

where $K_d = 0.12$ nmol/L and $[I] = 0.04$ nmol/L. IC_{50} conversion to K_i is accurate only when n_H equals unity.

Statistics

Data were analyzed by analysis of variance (ANOVA) using general linear models (PROC GLM) of SAS statistical software (SAS 1988). Preplanned post hoc comparisons of least square means were made using t -tests (PDIF).

Figure 3. Effect of chronic treatment with 5-HT_{1B} receptor agonists and antagonists on the density of cortical 5-HT_{1B} binding sites. Drugs are 0.9% saline (SAL), RU 24969 (RU), *m*-CPP (M-CP), TFMPP (TFM), quipazine (QP), DOI, and pindolol (PIND). Data are means $\pm$ SEM of five separate experiments performed in duplicate. Chronic drug treatment significantly altered $B_{\max}$ ($F = 4.93$; $p = .002$, ANOVA). K_d was significantly altered only by RU 24969 ($F = 8.97$, $p = .0001$, ANOVA). Corresponding values for K_d (nmol/L) were 0.19 ± 0.01 (vehicle) and 0.33 ± 0.04 (RU 24969). * $.01 \leq p < .05$; ** $.001 \leq p < .01$; *** $.0001 \leq p < .001$, *t*-test.

RESULTS

Regional Comparison of 5-HT_{1B} Binding Sites

Saturation analyses are shown in Figure 1. Scatchard plots were linear and fit a single-site model. There were significant regional differences in specific binding to 5-HT_{1B} sites ($F = 55.7$, $p = .0001$, ANOVA). Specific binding was approximately 84 to 72 percent of total binding at low to high isotope concentrations, respectively, in striatum, brainstem, hippocampus, and cortex. In spinal cord, it was lower: 70 to 44 percent of total isotope binding, respectively ($p = .0001$, *t*-test).

There were large regional differences in the maximum density of 5-HT_{1B} binding sites in vehicle-injected rats ($F = 55.31$, $p = .0001$). The rank order of $B_{\max}$ was brainstem > hippocampus > cortex, striatum > spinal cord. There were small regional differences in K_d (nmol/L) in saline-injected controls: ($F = 6.53$, $p = .002$) with the rank order hippocampus, brainstem, cortex > spinal cord, striatum: hippocampus, 0.21 ± 0.02 ; brainstem, 0.19 ± 0.01 ; cortex, 0.19 ± 0.01 ; spinal cord, 0.14 ± 0.02 ; and striatum, 0.12 ± 0.02 .

Chronic Agonist Injections

Regional studies were performed on brain and spinal cord from rats treated chronically with RU 24969 (Fig. 2). Significant reductions in $B_{\max}$ were found in brain-

Table 1. Effect of Chronic Treatment with 5-HT Antagonists on Striatal 5-HT_{1B} Receptors

Vehicle or Drug	<i>n</i>	$B_{\max}$ (pmol/g)	K_d (nmol/L)
Saline	7	6.0 ± 0.4	0.12 ± 0.01
Methysergide	8	6.4 ± 0.8	0.13 ± 0.02
Propranolol	7	6.9 ± 0.9	0.11 ± 0.01
Ascorbic acid	8	6.7 ± 0.7	0.11 ± 0.01
Pindolol	5	6.9 ± 0.9	0.11 ± 0.01
Propylene glycol	5	4.9 ± 0.7	0.14 ± 0.02
Ritanserin	5	4.3 ± 0.9	0.13 ± 0.02
Metergoline	5	4.9 ± 0.6	0.14 ± 0.01
Methiothepin	4	5.1 ± 1.0	0.13 ± 0.02

Data are mean $\pm$ SEM of separate experiments performed in duplicate. $B_{\max}$, K_d and n_H values were determined using EBDA. All values for n_H were not significantly different than 1.0.

stem (−46%), spinal cord (−63%), striatum (−35%), cortex (−24%) and hippocampus (−23%). There was a 1.7-fold increase in K_d cortex ($p = .0001$) and spinal cord ($p = .007$, *t*-test).

In a comparison of 5-HT agonists in cortex (Fig. 3), RU 24969 (−24%) and TFMPP (−16%) reduced $B_{\max}$ significantly. DOI, a 5-HT_{2/1C} agonist, and *m*-CPP had no statistically significant effects on binding parameters.

Chronic Antagonist Injections

In cortex, quipazine and pindolol had no statistically significant effects on binding parameters. In striatum (Table 1), propranolol, pindolol, ritanserin, metergoline, and methiothepin also did not have significant effects compared to their respective vehicles.

Competition Studies

In naive rat striatum, the K_i for RU 24969 was 1.9 ± 0.9 nmol/L, and the value for n_H was 0.70 ± 0.04 . The K_i for 5-HT in saline-treated controls was 9.7 ± 0.2 nmol/L, and the value for n_H was 0.76 ± 0.03 . Striatal receptor occupancy was approximately 19% as determined by EBDA in competition experiments.

Chronic treatment with 5-HT agonists or antagonists had no significant effect on the K_i of 5-HT in striatum. The values for K_i were 7.3 ± 0.5 nmol/L in the chronic RU 24969-treated group, 10 ± 1 nmol/L in the *m*-CPP-treated group, and 10 ± 1 in the pindolol-treated group.

DISCUSSION

The main finding of this study was that chronic treatment with the 5-HT_{1B} agonist RU 24969 significantly down-regulated 5-HT_{1B} sites in multiple brain regions

and spinal cord and that there were significant regional differences in the magnitude of down-regulation. A pattern of 5-HT agonist-induced receptor recognition site down-regulation detectable by regional binding assays has now been shown for 5-HT_{1C} (Pranzatelli et al. 1993), 5-HT₂ (Pranzatelli 1991), but not 5-HT_{1A} sites (Larsson et al. 1990). The behavioral correlate of 5-HT_{1B} receptor down-regulation would appear to be tolerance to the stimulatory effect of RU 24969 on locomotor activity (Oberlander et al. 1987). Daily injection of mice for 3 days with 5 mg/kg RU 24969 reduced the behavioral effect of RU 24969 and of other 5-HT_{1B} agonists (Frances and Monier 1991). Even a single dose of RU 24969 in rats decreased subsequent RU 24969-evoked locomotion (Oberlander et al. 1987).

5-HT antagonist-induced upregulation of 5-HT_{1B} sites was not found at the doses studied. Earlier studies using nonselective 5-HT antagonists had predicted upregulation of 5-HT₁ receptors, but so far, no 5-HT₁ subtype has exhibited upregulation after chronic 5-HT antagonist treatment at a 10 mg/kg dose (Pranzatelli 1991; Pranzatelli et al. 1993). Unlike 5-HT_{1C} and 5-HT₂ sites, 5-HT_{1B} antagonists also do not induce down-regulation of 5-HT_{1B} sites. It can be argued, however, that the 5-HT_{1B} antagonists we studied do not have high enough affinity at 5-HT_{1B} sites and may give rise to false negatives at the dose studied. The K_i values for metergoline, methiothepin, propranolol, quipazine, and pindolol at rat cortical 5-HT_{1B} sites labeled with [¹²⁵I]CYP are 17, 43, 46, 223, and 240 nmol/L, respectively (Offord et al. 1988; Hoyer et al. 1985). The affinity of metergoline and methiothepin should be sufficient to demonstrate trophic effects with chronic administration at the dose used. Studies using higher doses will be necessary to explore this issue further. Evidence for metergoline being a partial agonist rather than an antagonist on 5-HT_{1D β} receptors has been presented recently (Miller et al. 1992).

There are differences in the B_{max} rank order of 5-HT_{1B} sites we report and that suggested by autoradiographic studies. Several explanations are possible. The differences in B_{max} for the regions in question (striatum, cortex, hippocampus, brainstem) are not great given the SEMs. The data are expressed as pmol/g rather than the fmol/mg protein used in most autoradiography papers. The data are saturation data, whereas most autoradiographic determinations have been at a single isotope concentration. It is difficult to make exact comparisons between homogenate binding and autoradiographic data. Our striatal binding was performed at 100 volumes of tissue, whereas other regions were done using 50 volumes. This higher volume for striatum was necessary because of small region size and the tissue requirements for the binding assays. The brainstem and hippocampus do contain areas high in 5-HT_{1B} sites, such as substantia nigra and subicu-

lum, respectively. We do note that Blurton and Wood (1986), in homogenate binding assays using [³H]5-HT at a single concentration, found the following regional distribution: frontal cortex 3.08 ± 0.25 , hippocampus 2.98 ± 0.20 , striatum 3.53 ± 0.20 , midbrain 3.79 ± 0.57 , pons-medulla 2.04 ± 0.05 . Their midbrain had a value higher than striatum, however, the inclusion of pons-medulla in our brainstem piece would be expected to lower the B_{max} according to their data. Their studies were not saturation studies, however.

The lack of effect of chronic treatment with *m*-CPP compared to TFMPP and RU 24969 on 5-HT_{1B} sites may relate to the 4.3-fold higher affinity of TFMPP and 25- to 100-fold higher affinity of RU 24969 in the rat (Sills et al. 1984; Hoyer 1988; Offord et al. 1988; Matsumoto et al. 1992), although there may be other pharmacokinetic differences between drugs. Our binding data suggest that, at a dose of 10 mg/kg, *m*-CPP does not act at 5-HT_{1B} receptors in the rat, whereas this dose in the same rats does down-regulate 5-HT_{1C} sites in spinal cord (Pranzatelli et al. 1993) but not 5-HT_{1A} sites in brainstem (Murthy and Pranzatelli 1992). It has been suggested that although indole 5-HT_{1B} agonists stimulate hyperlocomotion by stimulating presynaptic 5-HT_{1B} sites, phenylpiperazine 5-HT_{1B} agonists exert the opposite behavioral effect by acting postsynaptically (Oberlander et al. 1987). However, pharmacological studies of the hypoactivity induced by *m*-CPP and TFMPP have associated it with 5-HT_{1C} receptors instead of 5-HT_{1B} receptors (Kennett and Curzon 1988; Lucki et al. 1989).

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