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Mianserin-Induced 5-HT_2 Receptor Downregulation Results in Anxiolytic Effects in the Elevated Plus-Maze Test

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ABSTRACT

Benjamin, D., E.I. Saiff, T. Nevins, and H. Lal: Mianserin-induced 5-HT_2 receptor downregulation results in anxiolytic effects in the elevated plus-maze test. Drug Dev. Res. **26**:287–297, 1992.

Several agents that downregulate 5-HT_2 receptors produce anxiolytic effects in humans, but the role of 5-HT_2 receptor downregulation has been difficult to assess because of their other actions. To test the effects of pharmacological downregulation of 5-HT_2 receptors on exploratory behavior in the mouse, mianserin, a drug known to downregulate 5-HT_2 receptors after a single dose, was administered 30 min, 48 hr, or 18 days prior to testing in the elevated plus-maze. Following testing in the elevated maze, the head-shake response to 4-iodo-R-(−)-2,5-dimethoxyphenylisopropylamine (DOI), a selective 5-HT_2/5-HT_{1C} agonist was assessed, and in a separate group of animals 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, β_1,β_2, and 5-HT_2 agonist and antagonist binding was quantified autoradiographically. Mianserin pretreatment resulted in a significant dose-related anxiolytic effect in the elevated plus maze evidenced by increases in the percentage of entries to, and time spent on the open arms. Head-shakes induced by DOI were also dose-dependently decreased as a result of mianserin pretreatment. At this time, the binding of the 5-HT_2 receptor antagonist, 7-amino-8 [^{125}I]ketanserin was decreased by 50%. Binding of DOI to 5-HT_2 receptors was decreased by 46%, and to 5-HT_{1C} receptors was decreased by 53%, but no other changes were found in any of the other receptor types examined. These findings demonstrate that the 5-HT_2 receptor plays at least a permissive role in anxiety-like behaviors, since an intact 5-HT_2 system is necessary for the full expression of the anxiety-like response, but the role of

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5-HT_{1C} receptor downregulation in the effects of mianserin cannot be ruled out at this time. © 1992 Wiley-Liss, Inc.

Key words: serotonin, anxiety, 5-HT_{1C} receptors, autoradiography

INTRODUCTION

After acute administration, established antidepressant drugs produce anxiogenic effects [Linnoila et al., 1987] or no specific effect [Chopin and Briley, 1987] but have been found to produce anxiolytic effects (increments in novelty supressed feeding) following repeated administration [Bodnoff et al., 1989]. Many antidepressants downregulate either 5-HT_2 receptor numbers or responsiveness following chronic administration [Peroutka and Snyder 1980; Paul et al., 1988]. The anxiolytic effects of 5-HT_2 antagonists have been demonstrated in clinical studies [Ceulemans et al., 1985; Reyntjens et al., 1986] and in animal models [Bennett et al., 1989; Stutzman et al., 1991]. Agonists at the 5-HT_{1A} receptor, which exhibit clinically significant anxiolytic activity, potently antagonize 5-HT_2 receptor mediated effects [Arnt and Hytell, 1989]. Taken together, these studies suggest that decrements in the responsivity of the 5-HT_2 receptor system underlie anxiolytic effects.

The present study addresses this issue by assessing anxiolytic effects of pretreatment with mianserin, an atypical antidepressant drug that has been shown to downregulate 5-HT_2 receptors after a single dose [Blackshear and Sanders-Bush, 1982]. It was hypothesized that downregulation of 5-HT_2 receptors would result in an anxiolytic profile in the elevated plus-maze test. After testing in the elevated plus-maze, functional and physical downregulation of 5-HT_2 receptors was verified. As a preliminary evaluation of its specificity, the effect of mianserin pretreatment on 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, β_1,β_2, and 5-HT_2 agonist and antagonist binding were also determined.

MATERIALS AND METHODS

Animals

Male NIH Swiss mice (Harlan Sprague-Dawley, Frederick, MD) 1–3 months old were housed 10 per cage on cedar bedding. Rodent chow (Purina) and tapwater were constantly available. The mice were 18 g on arrival, and were tested when they had attained a weight of 26 ± 2 g. House lights were on a 12 hr light/dark cycle, and the vivarium rooms were maintained at a temperature of 23° ± 3°C. Animals were always tested during the light cycle between 0900 and 1600 h.

Elevated Plus-Maze

Test apparatus. The plus-maze was constructed from acrylic and consists of two open arms, 30 × 5 cm, and two enclosed arms, 30 (l) × 5 (w) × 15 (h) cm. The arms extend from a central platform 5 × 5 cm. The open arms, the central platform, and the floor of the closed arms are made of black, opaque acrylic. The walls of the close arms are made from clear acrylic, 1 cm thick. The apparatus was mounted on an acrylic base, raising it to a height of 38.5 cm above the floor. Three stopwatches were used: one to measure the total test period, one to measure the time spent on the open arms, and one to measure the time spent on the closed arms.

Drug administration. Mice were injected intraperitoneally (ip) with drugs dissolved in 0.9% saline solution, at a volume of 10 ml/kg body weight. The pretest injection period was 30 min. Mice were tail-marked immediately after injection and returned to the home cage until just before the test; at this time they were taken to the test room and allowed 5 min to habituate to the new location.

Test procedure. Mice were removed from the home cage and taken to the test room in a 10 × 12-inch transport cage. In the test cubicle, the transport cage was placed on the floor and the mouse allowed a 5-min pretest habituation period. After the 5-min pretest habituation period, the test animal was lifted by the tail and placed on the plus-maze, facing the center platform. Each arm entry and the time spent on each arm were recorded. Mice were taken to have entered an arm when all four paws crossed the entrance. The stopwatch for the arm the animal was on was stopped when the front paws crossed into the center zone. The test period was 5 min. Between tests, the maze was wiped clean with a water-dampened sponge. Mice were tested only once, and 10–12 mice were in each test group. The percentage of time spent on the open arm was calculated by dividing the time spent on the open arm by the sum of time spent on the closed arm and time spent on the open arm. The resulting proportion was multiplied by 100 to yield the percentage.

DOI-Induced Head Shakes

Head-shakes observed following DOI injection are mediated by 5-HT_2 receptors and reflect 5-HT_2 receptor function [Arnt and Hytell, 1989; Niemegeers et al., 1983]. A 1-mg/kg ip dose of DOI was administered 2 hr after testing in the plus-maze. Immediately following injection, mice were placed in Plexiglas observation cages and the number of head shakes observed for 20 min in drug-treated mice was compared with the number of head-shakes observed in controls. Receptor binding studies were not performed on tissue from mice that had received DOI.

Quantitative Autoradiography Protocols

Tissue section preparation for autoradiography. Immediately following sacrifice by decapitation, brains were rapidly removed and frozen using powdered dry ice. Brains were then stored at −80°C until sectioning. Coronal tissue sections (20-μm thickness) were cut at four neuroanatomical levels, through the raphe nuclei, through the amygdala, through the septal nuclei, and most anteriorly, through the nucleus accumbens and prefrontal cortex [Paxinos and Watson, 1982].

A Reichert-Jung Frigocut 2100 cryostat was used to cut tissue sections, with the temperature maintained at −21°C. Consecutive superimposable sections were used to determine total and nonspecific binding. Sections were cut and thaw-mounted on microscope slides subbed with a gelatin (1%, w/v) and chrome alum (chromium potassium permanganate; 0.1% w/v) solution. Sections were collected such that 14 slides, the number necessary for all the different binding assays conducted, were taken from each brain, and each slide had one section from each of the four areas. Following thaw-mounting, the sections were stored at 4–5°C. Every binding assay was concluded by dipping sections once in deionized water to remove excess buffer salts, and the slides were allowed to dry overnight in a stream of cool air.

5-HT_{1A}. A Tris–HCl, 50 nM buffer, pH 7.4, was used. Tissue sections were first washed in ice-cold buffer for 15 min. Next, sections were incubated for 30 min in the presence of 2 nM tritiated 8-hydroxy-2-n-(dipropylamino)tetralin ([^{3}H]-8-OH-DPAT, New England Nuclear (Boston, MA), specific activity 127 Ci/mM) at 23°C. Adjacent sections were incubated with [^{3}H]-8-OH-DPAT and 10 mM unlabeled 8-OH-DPAT, to define nonspecific binding. A third group of adjacent sections was sometimes incubated with (−)-pindolol (1 μM), to determine whether [^{3}H]-8-OH-DPAT was binding to 5-HT reuptake sites; if this was so, (−)-pindolol, which has no significant affinity for 5-HT reuptake sites would yield a different pattern of nonspecific binding. This was found not to be the case; therefore, most of the assays reported here used 8-OH-DPAT as the agent to define nonspecific binding. Following incubation sections were washed twice, for 15 min, in ice-cold buffer. This protocol was adapted from Pazos and Palacios [1985; for review, see Pazos et al., 1988].

5-HT_{1B}. A 50-mM Tris–HCl buffer, pH 7.4, with 150 mM NaCl and 7 mM isoproterenol was used. 140 pM [^{125}I]cyanopindolol (2,200 Ci/mM on arrival, New England Nuclear, Boston, MA), was used as the radioligand. Nonspecific binding was defined using 5-HT (10 mM) or CGS 12066B (1 μM). Tissue was washed twice, for 15 min in ice-cold buffer before a 90-min incubation at 23°C. Following incubation, sections were washed twice in ice-cold buffer, for 15 min each wash. This protocol was adapted from Offord et al. 1988.

5-HT_{1C}. [^{125}I]-(−)-DOI (2,000 Ci/mM at reference date; New England Nuclear) at a concentration of 200 pM, in the presence of spiperone, 100 nM, (to cover 5-HT_2 receptors) was used to label the 5-HT_{1C} receptors. Tris–HCl, 50 mM, pH 7.4, containing 0.4 mM $CaCl_2$, 0.1% ascorbate, and 0.1% bovine serum albumin was the buffer used. Nonspecific binding was defined as binding that was not displaced by DOI (1 mM). Sections were washed twice, for 15 min each wash, with ice-cold buffer prior to incubation, and then incubated for 1 hr at room temperature. Following incubation, sections were washed twice for 15 min each wash with ice-cold buffer. The 5-HT_{1C} agonist binding procedure was adapted from McKenna et al. [1987].

5-HT_2 antagonist binding. 7-Amino-8-[^{125}I]ketanserin (1,969 Ci/mM at reference date, Amersham, Arlington Heights, IL), at a concentration of 200 pM was used to label amine release sites and 5-HT_2 receptors. Tris–HCl buffer was used, at a concentration of 50 mM, with a pH of 7.4. Nonspecific binding was defined using tetrabenazine (1 mM), prazosin 1 (mM), and pirenpirone, methysergide, or ritanserin (1 mM) to cover amine release sites, α_1 receptors, and 5-HT_2 receptors, respectively. Tissue sections were incubated for 60 min at room temperature. Incubation was followed by two 10-min washes in ice-cold buffer. This procedure was adapted from Schotte and Leysen [1989].

5-HT_2 agonist binding. [^{125}I]-(−)-DOI (2,000 Ci/mM at reference date; New England Nuclear) at a concentration of 200 pM was used to label the high agonist-affinity 5-HT_2 receptors in a Tris–HCl, 50 mM, pH 7.4, buffer containing 0.4 mM $CaCl_2$, 0.1% ascorbate, and 0.1% bovine serum albumin. Nonspecific binding was defined as that not displaced by DOI (1 mM). Sections were washed twice with ice-cold buffer prior to incubation and then incubated for 1 hr at room temperature. Following incubation, sections were washed twice for 15 min with ice-cold buffer for 15 min each wash. The 5-HT_2 agonist binding procedure was adapted from McKenna et al. [1987].

β-Adrenoceptors. β-Receptor binding was measured using essentially the same protocol as for 5-HT_{1B} receptors, except that 5-HT, 10 mM was added to buffers for both specific and nonspecific binding to cover the 5-HT_{1B} sites, and nonspecific binding was defined using 10 mM isoproterenol. In addition, ICI 118,551 (50 nM) was used in other tissue sections to cover β_2 receptors, thus allowing separate analysis of β_1 and β_2 receptors.

Autoradiography protocols. After drying, sections were apposed to [^{3}H]Hyperfilm (Amersham). X-ray film cassettes were used for the exposure, and were loaded in total darkness. The cassettes remained in total darkness until development. Tritium or ^{125}I-standards (Amersham), calibrated against brain tissue, were exposed in every cartridge, so that a scale could be constructed to relate optical density in exposed film to nCi/mg tissue. Using this scale, the amount of drug bound at specific areas was calculated, using the specific activity of the ligand (a conversion factor was used to give the specific activity for the date of exposure, for both the radioligand and the standards). Film was exposed, and the period of exposure varied from 8 hr to 3 weeks. Exposure periods were determined empirically; sections were exposed until the darkest areas of the darkest specific binding image were just short of opaque. This was considered the optimal image density because it allowed for maximal sensitivity in the lightest regions, while the darkest areas would still fall on the linear portion of the calibration curve. Exposure periods varied as a function of the specific activity of the ligand and the density of binding sites.

Image analysis. Autoradiograms were analyzed using a Cohu video camera (purchased from Imaging Research Inc, Hamilton, Canada) and a Macintosh IIcx (Macintosh, Cupertino, CA), running the Image 1.10 system (W. Rosband, Research Services, NIMH) for image analysis. Optical densities were digitized, and nonspecific binding images were subtracted from total binding images to yield specific binding images. Gray levels for total, specific, and nonspecific binding were calibrated against Amersham tritium or ^{125}I standards, so that binding at any area of the section could be expressed in pmol/g tissue.

Drugs and Chemicals

Serotonin hydrochloride, 8-OH-DPAT, CGS 12066B, mCPP, (−)-pindolol, and TFMPP were obtained from Research Biochemicals Inc. (Natick, MA) as were DOI, mianserin, and prazosin. Pirenpirone and ritanserin were obtained from Janssen Biologicals (Beerse, Belgium). Tetrabenazine was a gift from Hoffmann-La Roche (Nutley, NJ), and ICI 118,551 was a gift from Imperial Chemical Industries (London). All other chemicals were of the highest grade commercially available.

RESULTS

Effects of a single dose of mianserin administered 30 min, 48 hr, or 18 days prior to testing in the elevated plus-maze. Mianserin, a drug with the well-characterized ability to downregulate 5-HT_2 receptors after a single dose, was tested for its ability to induce increases in percent open arm entries and time in the elevated plus-maze. Mice were injected 48 hr prior to testing, this allowed sufficient time for the elimination of the mianserin [Sanders-Bush et al., 1987] so that residual drug would not remain during testing.

Mianserin, administered 30 min prior to testing in the elevated plus-maze, produced variable results that did not differ significantly from control values (Fig. 1). When administered 48 hr prior to testing in the plus-maze, mianserin induced significant, dose-related increases in the percentage of open arm entires and time spent on the open arm. The effect of mianserin administered 18 days prior to testing on the percent open arm entries, and time was still significant but substantially less than the effect at 48 hr post-treatment (Fig. 1). Mianserin pretreatment produced a modest increase in the total number of entries, at doses of 2.5–10 mg/kg, but not at 20 mg/kg (Table 1).

Effect of mianserin administered 48 hr prior to testing on the number of head-shakes observed after DOI injection. Head-shakes observed after DOI injection were attenuated when mianserin was administered 48 hr prior to testing. This effect was dose related, reaching significance at 5 mg/kg; the maximal attenuation of head-shakes was observed at 10 mg/kg (Fig. 2).

Effect of mianserin pretreatment on 5-HT_2 receptor antagonist binding. Mianserin produced highly significant decreases in the density of autoradiographically measured cortical 5-HT_2 receptors when administered at a dose of 10 mg/kg, 48 hr prior to sacrifice. This decrease in the specific binding of 7-amino-8-[^{125}I]ketanserin, a 5-HT_2 antagonist, was significant ($P < 0.05$) at the 10-mg/kg dose of mianserin (Fig. 3).

Effect of mianserin pretreatment on 5-HT_2 and 5-HT_{1C} agonist binding. Following pretreatment with mianserin (10 mg/kg ip, 48 hr prior), the specific binding of the 5-HT_2/5-HT_{1C} agonist, [^{125}I]-DOI to 5-HT_2 receptors in cortex and to 5-HT_{1C} receptors in the choroid plexus of the lateral ventricles was significantly decreased (Fig. 4).

Effect of mianserin on 5-HT_{1A}, 5-HT_{1B}, β_1, and β_2 receptor binding. No change was detected in the binding of any of the other four receptors examined 48 hr after mianserin (10 mg/kg). It should be noted that the tissue sections were from mice that had been tested in the elevated maze, in which mianserin produced an anxiolytic response, adjacent to those in which we had had detected a decrease in 5-HT_2 receptor binding.

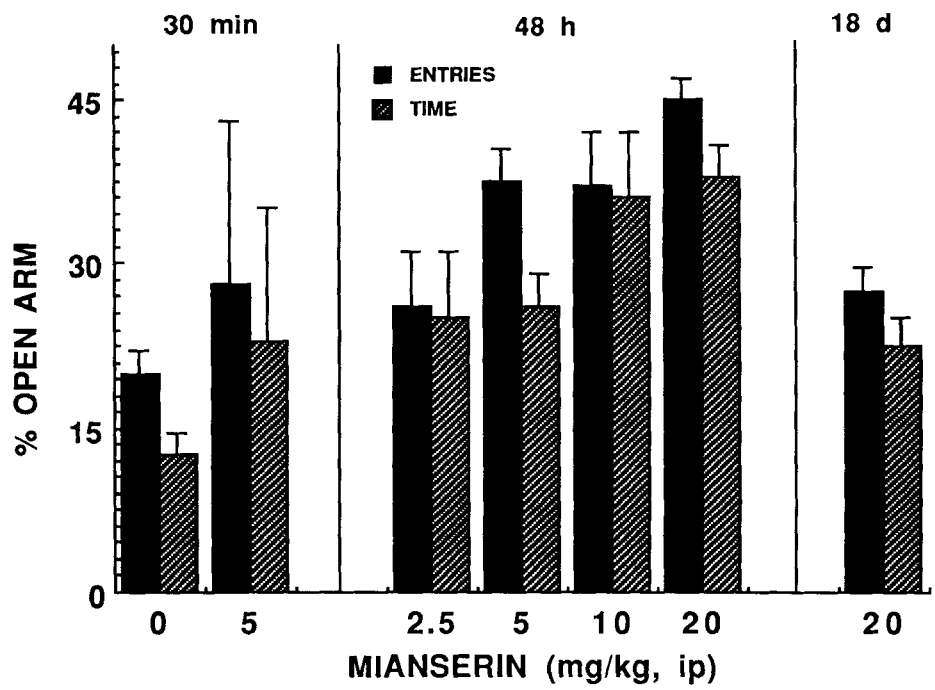


Fig. 1. Variable results were observed when mianserin was administered 30 min prior to testing. Forty-eight hr following injection, the percentage of open arm entries and time were significantly greater than vehicle, following a dose of 2.5 mg/kg, and the percentage of open arm entries was maximal following a dose of 20 mg/kg (overall effect $P < 0.001$, ANOVA; $P < 0.05$ for individual comparisons). The effect of mianserin on percentage of open arm time was maximal at 10 mg/kg. The effect of mianserin administered 18 days prior to testing on percentage of open arm entries and time was still significant at this time ($P < 0.05$, independent t-test), but significantly less than the effect at 48 hr post-treatment. Twelve mice were tested at each dose and time.

TABLE 1. Effect of Mianserin (48-hr pretreatment) on the Total Number of Entries Made by Mice in the Plus-Maze

Mianserin (mg/kg, ip)	Entries	
	Mean	±SEM[a]
0	11.7	0.74
2.5	15.6	1.78
5.0	16.3	1.33
10.0	17.1	1.50
20.0	12.6	1.06

[a]Twelve mice were tested at each dose.

DISCUSSION

Mianserin produces a rapid downregulation of 5-HT_2 sites after a single dose [Blackshear and Sanders-Bush, 1982; Sanders-Bush et al., 1987], so this drug was tested for its ability to produce anxiolytic effects in the elevated plus-maze. The elevated plus-maze para-

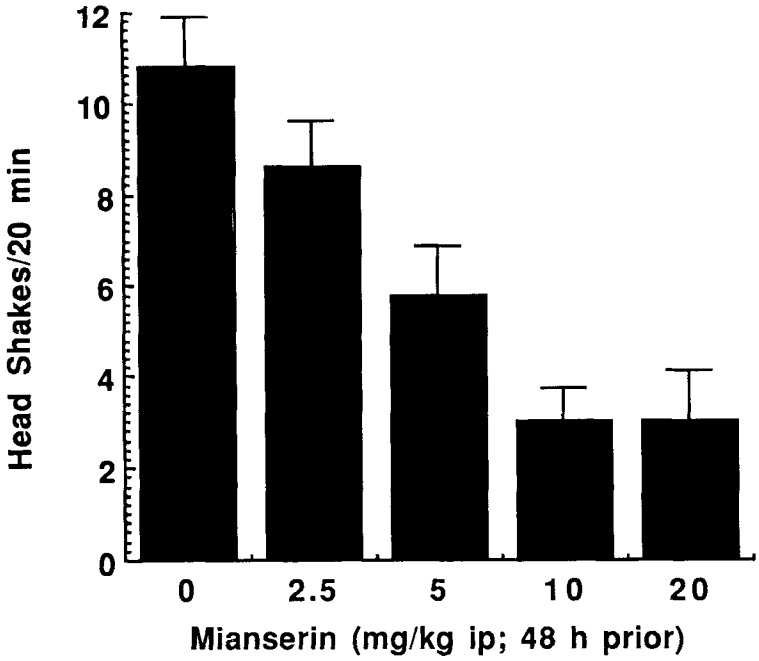
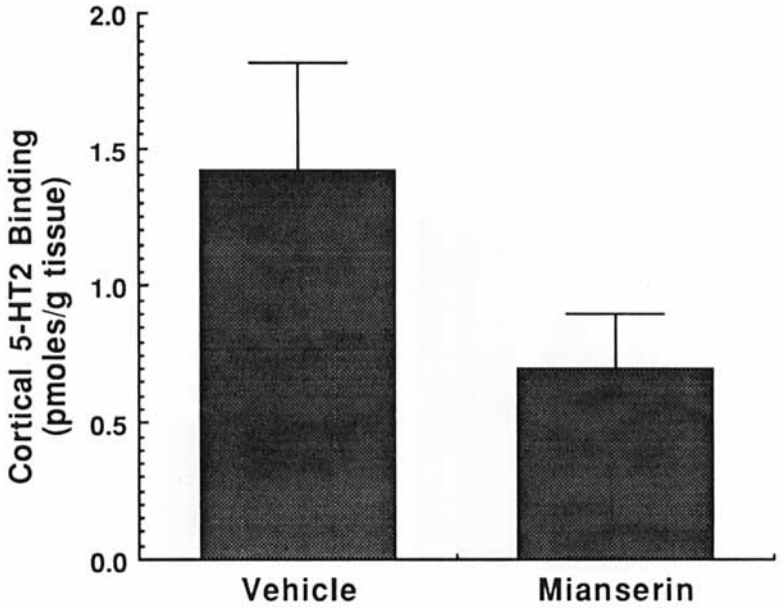


Fig. 2. Effects of a single dose of mianserin on headshakes induced by DOI (1 mg/kg) 48 hr later. Suppression reached significance at a dose of 5 mg/kg; the effect was maximal at 10 mg/kg (individual comparisons, $P < 0.05$, following significant one-way ANOVA). Five mice were tested at each dose.

digm was chosen as the anxiety analogue for this study because it has been validated pharmacologically, physiologically, and behaviorally [Benjamin et al., 1990a,b; Lister, 1987; Pellow et al., 1985] and because it identifies all known anxiolytics. Furthermore, the plus-maze paradigm is particularly well suited to ex vivo experiments such as the present study, because mice are only tested once. Mianserin did not produce consistent anxiolytic effects after acute administration. Administration of mianserin 48 hr prior to testing in the plus-maze resulted in significant, dose-related increases in the percentage of open arm entries and time spent on the open arm. Furthermore, the anxiolytic effect of mianserin pretreatment had decreased, although not to control values 18 days after a single 20-mg/kg dose.

Following a single dose, mianserin has a half-life of 1–3 hr in brain, so it would not be present in any significant quantity 48 hr later [Sanders-Bush et al., 1987]. Mianserin treatment, 48 hr prior to testing resulted in the attenuation in mice of head-shakes consequent to DOI administration. This effect was dose related, reaching significance at 5 mg/kg, and was maximal at 10 mg/kg. The anxiolytic effect of mianserin in the plus-maze and its attenuation of head-shakes was accompanied by a significant reduction in 5-HT_2 receptor density. 5-HT_{1C} receptor numbers were significantly reduced as well, this effect of mianserin was marked; a single 10-mg/kg dose of mianserin reduced 5-HT_{1C} binding to an undetectable level in the cortex. Since gepirone and its metabolite 1-PP decreased the numbers of 5-HT_2 receptors and did not downregulate 5-HT_{1C} receptors following chronic administration [Benjamin et al., 1990b; manuscript in preparation], but still produced an anxiolytic effect, the anxiolytic effect produced by mianserin can be attributed to its effects on 5-HT_2 receptors. Mianserin pretreatment did not affect 5-HT_{1A}, 5-HT_{1B}, β_1, or β_2 receptors, which further suggests that the anxiolytic effect of mianserin is mediated specifically by the 5-HT_2 receptor. It should be



added, however, that β and 5-HT_{1B} receptor densities were measured using antagonists, which would probably not detect different agonist affinity states, and that the behavioral effect observed might be mediated by a change in a receptor that we did not examine; although this cannot be disproved, the evidence strongly suggests that the anxiolytic effects are mediated by the 5-HT_2, and possibly the 5-HT_{1C} receptor.

These results suggest that the 5-HT_2 receptor has an inhibitory influence on the percentage open arm entries in the plus-maze and that 5-HT_2 receptor downregulation (i.e., decreases from control values) has an anxiolytic influence on plus-maze behavior. The latter explanation is preferred, because mianserin had an anxiolytic effect in the rat plus maze, whereas DOI alone did not produce any consistent effect on plus-maze behavior but could antagonize the anxiolytic effects produced by ritanserin [Tomkins et al., 1990]. Downregulation of 5-HT_2 receptors consistently results in an anxiolytic-like effect in the elevated plus-maze. Furthermore, it appears that the 5-HT_2 receptor plays a permissive role in anxiety-like behaviors, since an intact 5-HT_2 system is necessary for the full expression of the anxiety-like response.

It has been demonstrated repeatedly that mianserin has clinically significant anxiolytic effects at least equal to those of the benzodiazepines [Murphy 1978; Bjertnaes et al., 1982; Kahn et al., 1986; Khan et al., 1983; Granier et al., 1985; Sorensen et al., 1985], although the anxiolytic effects of mianserin have only been reported once prior to this study [Bodnoff et al., 1989] in animals. A possible explanation is that, prior to 1989, mianserin was only tested acutely, where it did not appear to produce any systematic effect (Fig. 1) [Chopin and Briley, 1987]. Deakin [1988] briefly reviewed the clinical evidence suggesting that anxiety disorder and nonpsychotic depression were not readily distinguishable and may actually be different individual expressions of a common neurochemical etiology. Both depression and anxiety disorder respond to antidepressants, all of which downregulate 5-HT_2 receptors. This line of

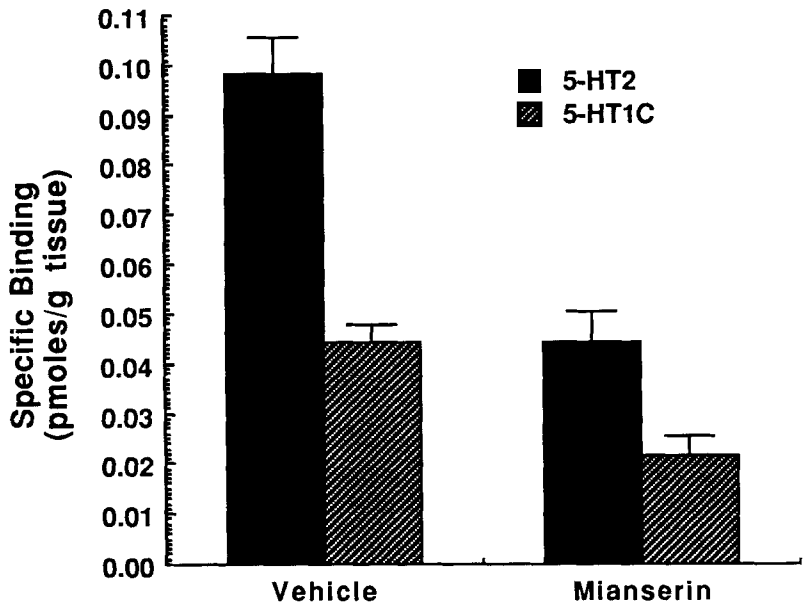


Fig. 4. Effects of a single dose of mianserin (10 mg/kg ip, 48 hr before sacrifice) on binding of [^{125}I]-DOI to 5-HT$_2$ receptors in cortex layer 4 and 5-HT$_{1C}$ receptors in the choroid plexus of the lateral ventricles. Pretreatment with mianserin resulted in highly significant decreases in specific [^{125}I]-DOI binding (P <0.001 in cortex, P <0.002 in choroid plexus, independent t-test, n = 5 per group).

reasoning led to the hypothesis that supersensitivity of the 5-HT$_2$ receptor system causes anxiety disorder and nonpsychotic depression. This hypothesis is also supported by the recent report by Arora and Meltzer [1989] of increased 5-HT$_2$ receptor binding in the blood platelets of depressed patients.

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