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ORIGINAL INVESTIGATION

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The role of 5-HT receptor subtypes in the anxiolytic effects of selective serotonin reuptake inhibitors in the rat ultrasonic vocalization test

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Abstract We evaluated whether the anxiolytic effects of selective serotonin reuptake inhibitors (SSRIs) in the rat ultrasonic vocalization (USV) test are preferentially mediated by (indirect) activation of 5-HT_{1A}, 5-$HT_{1B/1D}$, 5-HT_{2A}, 5-HT_3 or 5-HT_4 receptors. The SSRIs, paroxetine (ED_{50} in mg/kg, IP: 6.9), citalopram (6.5), fluvoxamine (11.7) and fluoxetine (> 30), dose dependently reduced shock-induced USV. The effects of paroxetine (3.0 mg/kg, IP) were not blocked by the selective 5-HT_{1A} receptor antagonist, WAY-100635 (3.0 mg/kg, IP), the 5-$HT_{1B/1D}$ receptor antagonist, GR 127935 (30 mg/kg, IP), the nonselective 5-HT_{2A} receptor antagonists, ritanserin (3.0 mg/kg, IP) and ketanserin (1.0 mg/kg, IP), the 5-HT_3 receptor antagonist, ondansetron (0.1 mg/kg, IP), or the 5-HT_4 receptor antagonist, GR 125487D (3.0 mg/kg, SC). In contrast, the selective 5-HT_{2A} receptor antagonist, MDL 100,907 (0.1 mg/kg, IP), completely prevented the paroxetine-induced reduction of USV. Under similar conditions, WAY-100635 blocked the anxiolytic-like effects of the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT [(±)-8-hydroxy-2-(di-*n*-propylamino)tetralin, 1.0 mg/kg, IP], and ritanserin, ketanserin, and MDL 100,907 blocked the anxiolytic-like effects of the mixed 5-$HT_{2A/2C}$ receptor agonist, DOI [1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, 3.0 mg/kg, IP]. WAY-100635 (1.0 mg/kg, IP) in combination with ritanserin (3.0 mg/kg, IP), but not ondansetron (0.1 mg/kg, IP), GR 125487D (3.0 mg/kg, SC), or GR 127935 (30 mg/kg, IP), attenuated the USV reducing effects of paroxetine. Although the results suggest that selective stimulation of 5-HT_{1A} and 5-HT_{2A} receptors produces a decrease of USV, we postulate that only 5-HT_{2A} receptors play a pivotal role in the effects of SSRIs in this model of anxiety.

Key words 5-HT_{1A} receptors · 5-HT_{2A} receptors · Anxiety · Paroxetine

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Introduction

Although it is now well established that selective serotonin (5-HT) reuptake inhibitors (SSRIs) possess antidepressive and anxiolytic properties, their underlying neuropharmacological mechanism of action is still not understood (for discussion, see De Vry 1996). Owing to their 5-HT reuptake-inhibiting effects, SSRIs increase synaptic levels of 5-HT (e.g., Bel and Artigas 1993; Kreiss and Lucki 1995), leading to increased activation of a variety of 5-HT receptor subtypes. However, it is not yet known which 5-HT receptor subtypes mediate the therapeutic effects of SSRIs and whether different 5-HT receptor subtypes are involved in their antidepressive and anxiolytic effects. In addition to their well characterized effects on 5-HT reuptake, some SSRIs inhibit the reuptake of other neurotransmitters, such as dopamine and noradrenaline (e.g., Gobert et al. 1996; Stanford 1996), and it is unclear whether these properties contribute to the therapeutic efficacy of SSRIs.

The recent availability of 5-HT receptor agonists and antagonists with a high degree of selectivity for particular 5-HT receptor subtypes has made it possible to characterize the 5-HT receptor subtypes involved in the effects of SSRIs in animal models of depression and anxiety (e.g., De Vry et al. 1995; Sanchez and Meier 1997, for review, see De Vry 1996). Thus, it has been suggested that the 5-HT_{1A} receptor subtype is critically

involved in the antidepressive properties of SSRIs (Detke et al. 1995; De Vry 1996), although a contributing role of other 5-HT receptor subtypes cannot be excluded.

The exact role of 5-HT receptor subtypes in the anxiolytic effects of SSRIs is also unclear (Murphy and Pigott 1990). The results of preclinical studies suggest that stimulation of 5-HT_{1A}, 5-$HT_{1B/1D}$ and 5-$HT_{2A/2C}$ receptors results in anxiolysis. Thus, in the adult rat ultrasonic vocalization (USV) test of conditioned anxiety, the 5-HT_{1A} receptor agonists, 8-OH-DPAT [(±)-8-hydroxy-2-(di-*n*-propylamino)tetralin], ipsapirone and buspirone, the 5-$HT_{1B/1D}$ receptor agonist, TFMPP [1-(3-trifluoromethylphenyl)piperazine], and the 5-$HT_{2A/2C}$ receptor agonist, DOI [1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane], were found to reduce USV, whereas the 5-HT_3 receptor agonist, mCPBG (*m*-chlorophenylbiguanadine), was inactive. Selective blockade of 5-HT receptors with either the 5-HT_{1A} receptor antagonist, WAY-100635, the 5-$HT_{2A/2C}$ receptor antagonist, ritanserin, the 5-HT_3 receptor antagonists, ondansetron and ICS 205-930, or the 5-HT_4 receptor antagonist, SB 204070, did not affect USV (Winslow and Insel 1991a,b; De Vry et al. 1993,1995; Sanchez 1993; Molewijk et al. 1995; Maurel Remy et al. 1996; Schreiber et al. 1996). Although such studies may indicate that particular 5-HT receptor subtypes are possibly involved in the mechanism of action of SSRIs, they do not provide direct evidence for such a role. The very few studies that have used a more direct approach, which assessed whether the anxiolytic effect of a SSRI is affected by co-treatment with a 5-HT receptor subtype-selective compound (either agonist or antagonist), have produced less consistent results. Thus, antagonism as well as potentiation of the anxiolytic effects of SSRIs following treatment with 5-HT_{1A} receptor antagonists has been reported, and ritanserin was found not to antagonize the effects of an SSRI (Njung'e and Handley 1991; Ichimaru et al. 1995; McNicoll et al. 1995). However, it should be realized that these studies generally used 5-HT receptor antagonists with a limited degree of selectivity. A detailed, comparative evaluation of the role of 5-HT receptors in the anxiolytic effects of SSRIs, using a broad range of selective 5-HT receptor ligands has not been reported.

In this study, we evaluated the contribution of particular 5-HT receptor subtypes to the anxiolytic actions of SSRIs in a more systematic manner. We chose the USV test of conditioned anxiety because it reliably detects the anxiolytic properties of a variety of SSRIs, including fluoxetine, paroxetine, sertraline, fluvoxamine and citalopram (Winslow and Insel 1991a; however, see also Bartoszyk et al. 1997; De Vry et al. 1993; Molewijk et al. 1995; Sanchez and Meier 1997). We performed antagonism experiments to determine the role of particular 5-HT receptor subtypes in the effects of an SSRI, by combining antagonists selective for particular 5-HT receptor subtypes with a fixed dose of paroxetine. The antagonists used were the 5-HT_{1A} receptor antagonist, WAY-100635 (Fletcher et al. 1996), the 5-$HT_{1B/1D}$ receptor antagonist, GR 127935 (Skingle et al. 1993), the 5-HT_{2A} receptor antagonists, ritanserin, ketanserin and MDL 100,907 (Sorensen et al. 1993; Marwood 1994; Kehne et al. 1996), the 5-HT_3 receptor antagonist, ondansetron, and the 5-HT_4 receptor antagonist, GR 125487D (Gale et al. 1994). The doses of the antagonists were selected as being those doses that blocked the anxiolytic effects induced by an agonist selective for that receptor. Finally, in order to evaluate whether combined activation of particular 5-HT receptor subtypes underlies the anxiolytic effects of an SSRI, we compared the effectiveness of combined treatment with the 5-HT_{1A} receptor antagonist, WAY-100635, and GR 127935, ritanserin, ondansetron, or GR 125487D with the effectiveness of either treatment administered alone.

Materials and methods

Animals

Male Wistar rats (Harlan-Winkelmann, Borchen, Germany), weighing 180–200 g at the beginning of the experiment, were housed in groups of four in standard Makrolon cages (45 × 30 × 30 cm) with free access to food and water. Animals were kept under a 12-h light-dark cycle (lights on from 7:00 a.m.). Temperature and humidity were held constant at 21 ± 1°C and 60 ± 5%, respectively. All experiments were carried out in conformity with the ethical rules of the German Law on the Protection of Animals.

Materials and procedures

The details of the anxiety test have been described previously (De Vry et al. 1993). In short, experiments were performed in standard, sound-proof chambers (Coulbourn Instruments, Lehigh Valley) equipped with an electrifiable grid and a microphone for the registration of USV in the range of 17–29 kHz (threshold level at 35 dB). On day 1 of the experiment, naive rats were put into operant chambers and during a 23 min session they received 20 inescapable footshocks (0.6 mA scrambled shocks of 2 s duration each). Daily training sessions continued until day 4. On days 5 and 6, rats were tested in a separate, sound-proof chamber. A test session started with a series of five shocks and immediately thereafter the total duration of USV was measured for 5 min. Animals emitting at least 150 s of USV during both sessions were selected for further experiments. Before each test, animals were randomly allocated to one of four groups (n = 8–10 per group), receiving either vehicle or drug(s). As USV responding remained stable for at least 3 months, rats were repeatedly tested, with wash-out periods of at least 48 h.

Drugs

All compounds were dissolved in vehicle (0.9% NaCl); if necessary, acetic acid (2 N) was added and the pH was adjusted with sodium hydroxide (2 N). The following drugs were tested: (±)-8-OH-DPAT [(±)-8-hydroxy-2-(di-*n*-propylamino)tetralin], DOI [1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane], mCPP-2HCl (*m*-chlorophenylpiperazine), mCPBG (*m*-chlorophenylbiguanadine), ketanserin and

TFMPP [1-(3-trifluorometylphenyl)piperazine), (RBI, Natick, Mass, USA); WAY-100635, ondansetron, GR 127935 and MDL 100,907 (synthesized by the Bayer Chemistry Department, Wuppertal, Germany); paroxetine and SB 200,646 (SmithKline Beecham Pharmaceuticals, Welwyn, UK), citalopram-HBr (Lundbeck, Copenhagen, Denmark), GR 125487D (Glaxo Wellcome, Stevenage, UK), ritanserin (Janssen Pharmaceutica, Beerse, Belgium), fluvoxamine-maleate (Solvay-Duphar, Weesp, The Netherlands), and fluoxetine-HCl (Sigma, St Louis, Mo., USA). Drugs were given intraperitoneally (IP) or subcutaneously (SC) in a volume of 1 ml/kg body weight. Orally (PO) administered drugs were given in a volume of 10 ml/kg. In the dose-response studies, all SSRIs were injected 60 min before testing. In antagonism studies, the SSRI or 5-HT agonist was injected 60 min before testing and the antagonist (or combination of antagonists) was injected 30 min before testing.

Statistics

Results are expressed as the duration of USV (in seconds). Means and SEMs were calculated for all groups. ED_{50} values and 95% confidence limits were calculated after log-probit transformation of the percent inhibition of USV (as compared to vehicle control) and calculation of the best fit regression line. A one-way ANOVA was used to analyse the dose-response data and a two-way ANOVA [factors Agonist (either 5-HT receptor agonist or SSRI), Antagonist and Interaction] was used to analyse the data obtained from antagonism studies. Following ANOVA, a Tukey post-hoc analysis was performed. Antagonism was considered to be complete if (1) the difference between agonist × antagonist- and agonist × vehicle-treated groups was statistically significant, (2) the difference between agonist × antagonist – and vehicle x vehicle-treated groups was not statistically significant, and (3) the mean values (in seconds) for USV indicated that the levels of USV were virtually identical between the agonist × antagonist – and the vehicle × vehicle-treated groups. If the first two criteria only were fulfilled, antagonist treatment was considered only to attenuate the agonist-induced reduction of USV.

Results

Effects of SSRIs upon USV (Fig. 1)

A dose-dependent reduction of USV was obtained with paroxetine [$F(3,71) = 11.80$, $P < 0.001$, ED_{50} (95% confidence limits in mg/kg, IP): 6.9 (2.3–20.3)], citalopram [$F(3,35) = 9.03$, $P < 0.001$, ED_{50}: 6.5 (2.8–14.7)], fluvoxamine [$F(4,43) = 13.00$, $P < 0.001$, ED_{50}: 11.7 (2.7–50.9)] and fluoxetine [$F(2,29) = 7.99$, $P < 0.01$, ED_{50}: not computable; Fig. 1]. Whereas paroxetine caused a marked decrease in USV, fluoxetine had only a weak effect at a dose of 30 mg/kg. Paroxetine (3.0 mg/kg) was therefore selected for further antagonism testing.

Single antagonist treatment (Figs. 2 and 3, Table 1)

Doses of the 5-HT_{1A} receptor agonist, 8-OH-DPAT, the 5-$HT_{1B/1D}$ receptor agonist, TFMPP, the 5-$HT_{2A/2C}$ receptor agonist, DOI, and the 5-HT_{2C} receptor agonist, mCPP, which induced an approximately

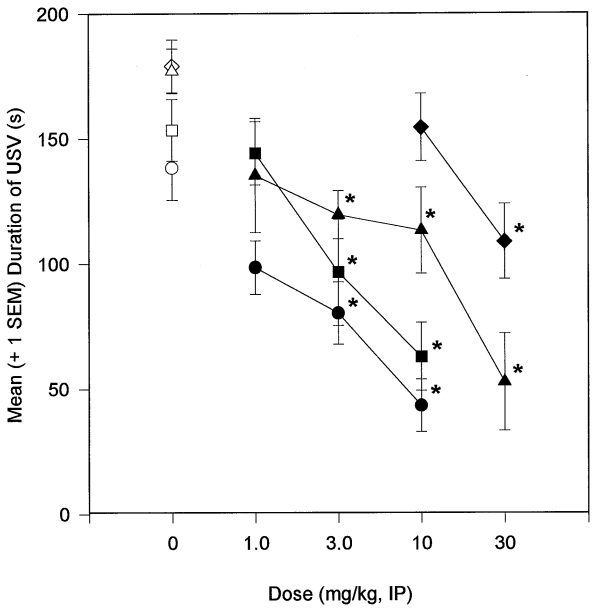

Fig. 1 Effects of the selective serotonin reuptake inhibitors, paroxetine, citalopram, fluvoxamine and fluoxetine, on shock-induced ultrasonic vocalization (*USV*) in young adult rats. $n = 8$–10 per dose, all drugs injected 60 min before testing. *$P < 0.05$ as compared to vehicle × vehicle treatment. ● Paroxetine, ■ citalopram, ▲ fluvoxamine, ◆ fluoxetine

50% reduction of USV (as compared with vehicle treatment) were selected from previous studies (De Vry et al. 1993). The appropriate dose of the antagonist was selected by testing whether the respective antagonist was able to block the effects of the selected agonist. Thus, the following combinations were tested: WAY-100635 versus 8-OH-DPAT (5-HT_{1A} receptor), GR 127935 versus TFMPP (5-$HT_{1B/1D}$ receptor) and ritanserin, ketanserin and MDL 100,907 versus DOI (5-HT_{2A} receptor). This approach could not be applied for the 5-HT_3 receptor antagonist, ondansetron, and the 5-HT_4 receptor antagonist, GR 125487D, because the 5-HT_3 receptor agonist, mCPBG, failed to reduce USV (maximal effect: +12% at 30 mg/kg, IP, data not shown) and a selective 5-HT_4 receptor agonist is not currently available. As a consequence, in the studies aimed at antagonizing the effects of paroxetine, high doses of ondansetron and GR 125487D were used to obtain full blockade of 5-HT_3 and 5-HT_4 receptors, respectively.

WAY-100635 completely antagonized the 8-OH-DPAT-induced reduction of USV (Fig. 2, upper panel). ANOVA revealed a significant effect for AGONIST [$F(1,33) = 10.49$, $P < 0.01$], Antagonist [$F(1,33) = 25.94$, $P < 0.001$] and Interaction [$F(1,33) = 8.30$, $P < 0.01$]. Post-hoc analysis showed a significant difference between the 8-OH-DPAT × vehicle-treated group, and the vehicle × vehicle- and 8-OH-DPAT × WAY-100635-treated groups. A lower dose of WAY-100635 (0.3 mg/kg, IP) was not effective against the 8-OH-DPAT-induced reduction of USV (data not shown).

GR 127935 (30 mg/kg, IP) tended to attenuate the anxiolytic effect of TFMPP (5 mg/kg, IP), but the effect failed to reach statistical significance (vehicle × vehicle: 150 ± 17 s, TFMPP × vehicle: 21 ± 10 s, vehicle × GR 127935: 157 ± 13 s, and TFMPP × GR 127935: 86 ± 23 s; Agonist [$F(1,34) = 35.78$, $P < 0.001$]; Antagonist [$F(1, 34) = 4.64$, $P < 0.05$]; Interaction [$F(1, 34) = 2.96$, $P = 0.09$]). Post-hoc analysis revealed a significant difference between the TFMPP × vehicle-treated group and both the vehicle × vehicle-treated group and the TFMPP × GR 127935 – treated group. The vehicle × vehicle-treated group and the TFMPP × GR 127935 – treated group were also significantly different.

The USV-reducing effects of the 5-HT_{2C} receptor agonist, mCPP (2.0 mg/kg, IP), were not affected by the 5-HT_{2C} receptor antagonist, SB 200,646 (20 mg/kg, PO; vehicle × vehicle: 158 ± 10 s, mCPP × vehicle: 91 ± 24 s, vehicle × SB 200,646: 149 ± 19 s, and mCPP × SB 200,646: 67 ± 26 s). ANOVA revealed only a significant effect for the main factor AGONIST [$F(1,31) = 14.92$, $P < 0.01$]. In view of the inactivity of SB 200,646 against mCPP, SB 200,646 was not tested further against paroxetine.

The 5-HT_{2A} receptor antagonists, ritanserin and MDL 100,907, abolished the USV reducing actions of DOI. For ritanserin, a significant effect for Agonist [$F(1,55) = 20.26$, $P < 0.001$], Antagonist [$F(1, 55) = 5.11$, $P < 0.05$] and Interaction [$F(1,55) = 13.54$, $P < 0.001$] was obtained (Fig. 2, middle panel). Post-hoc analysis showed a significant difference between the DOI × vehicle-treated group and the vehicle × vehicle- and DOI × ritanserin-treated groups. A significant effect for Agonist [$F(1,36) = 16.72$, $P < 0.001$], Antagonist [$F(1, 36) = 5.63$, $P < 0.05$] and Interaction [$F(1,36) = 5.77$, $P < 0.05$] was also obtained for MDL 100,907 (Fig. 2, lower panel). Post-hoc analysis again showed a significant difference between the DOI × vehicle-treated group and the vehicle × vehicle- and DOI × MDL 100,907 -treated groups. Lower doses of ritanserin (1.0 mg/kg, IP) and MDL 100,907 (0.01 mg/kg, IP) were not effective against the DOI-induced reduction in USV (data not shown). For ketanserin (1.0 mg/kg, IP; vehicle × vehicle: 119 ± 15 s, DOI × vehicle: 55 ± 17 s, vehicle × ketanserin: 146 ± 6.9 s, and DOI × ketanserin: 107 ± 14 s) significant effects were obtained for Agonist [$F(1,35) = 13.45$, $P < 0.001$] and Antagonist [$F(1,35) = 7.94$, $P < 0.01$]. Although the interaction between both main factors failed to reach significance, comparison of the magnitude of the USV reducing effects of treatment with DOI alone (% reduction of USV as compared to vehicle × vehicle-treatment: 54%) and DOI × ketanserin (10%) showed that ketanserin attenuated the effects of DOI.

In the next series of experiments, we tested whether 5-HT receptor antagonists were able to block the anxiolytic effects of paroxetine (3.0 mg/kg, IP). The

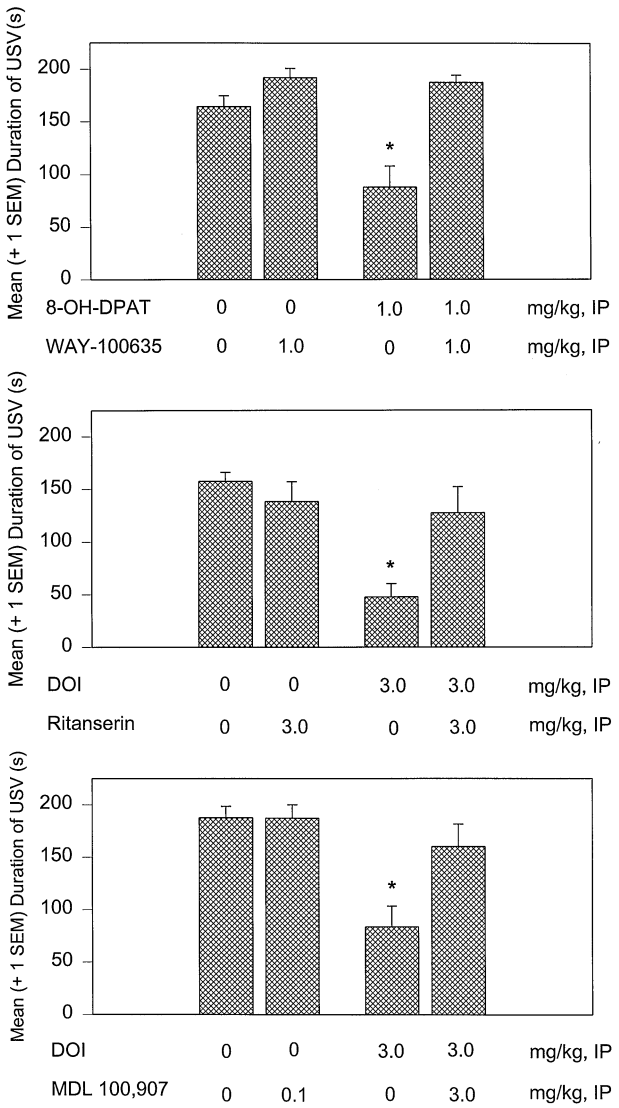

Fig. 2 Antagonism of the anxiolytic effects of selective 5-HT receptor agonists (t-60 min) by selective 5-HT receptor antagonists (t-30 min) in the shock-induced ultrasonic vocalization (*USV*) test in young adult rats. *Upper panel*: Antagonism of the USV-reducing effect of the 5-HT_{1A} receptor agonist, 8-OH-DPAT, by the 5-HT_{1A} receptor antagonist, WAY-100635. *Middle panel*: Antagonism of the USV-reducing effect of the 5-$HT_{2A/2C}$ receptor agonist, DOI, by the 5-$HT_{2A/2C}$ receptor antagonist, ritanserin. *Lower panel*: Antagonism of the USV-reducing effect of the 5-$HT_{2A/2C}$ receptor agonist, DOI, by the 5-HT_{2A} receptor antagonist, MDL 100,907. $n = 7$–15 per group. $^*P < 0.05$ as compared to vehicle × vehicle treatment

5-HT_{1A} receptor antagonist, WAY-100635, failed to block the actions of paroxetine [Agonist: $F(1,32) = 23.63$, $P < 0.001$]. When the 5-$HT_{1B/1D}$ receptor antagonist, GR 127935, was tested against paroxetine, a significant effect was only obtained for the factor Agonist [$F(1,32) = 14.40$, $P < 0.001$], suggesting that GR 127935 did not block the actions of paroxetine (Table 1).

Ritanserin did not antagonize the paroxetine-induced reduction of USV. A significant effect was only

Table 1 Effects of 5-HT_{1A}, 5-$HT_{1B/1D}$, 5-$HT_{2A/2C}$, 5-HT_3 and 5-HT_4 receptor antagonists on the anxiolytic activity of paroxetine in the shock-induced ultrasonic vocalization test in rats

Antagonist	SSRI	
(Dose in mg/kg, IP)	Vehicle mean ± SEM (s)	Paroxetine mean ± SEM (s)
Vehicle	182 ± 9	129 ± 16
WAY-100635 (3.0)	193 ± 11	120 ± 13
Vehicle	179 ± 15	144 ± 12
GR 127935 (30)	195 ± 8	141 ± 13
Vehicle	173 ± 14	112 ± 15
Ritanserin (3.0)	187 ± 13	103 ± 19
Vehicle	168 ± 10	114 ± 18
Ondansetron (0.1)	161 ± 12	139 ± 15
Vehicle	168 ± 10	94 ± 20
GR 125487D (3.0)[a]	173 ± 10	86 ± 15

[a] SC injection. Paroxetine was tested at a dose of 3.0 mg/kg, IP, t-60 min. Antagonists were administered 30 min before testing

Table 2 Effects of combined treatment with the selective 5-HT_{1A} receptor antagonist, WAY-100635, and the 5-$HT_{1B/1D}$ receptor antagonist, GR 127935, the 5-HT_3 receptor antagonist, ondansetron, or the 5-HT_4 receptor antagonist, GR 125487D, on the anxiolytic effects of paroxetine on shock-induced ultrasonic vocalization in rats

Antagonist combination	SSRI	
(Dose in mg/kg, IP)	Vehicle mean ± SEM (s)	Paroxetine mean ± SEM (s)
Vehicle	151 ± 10	85 ± 22
WAY-100635 (1.0) + GR 127935 (30)	213 ± 5	46 ± 22
Vehicle	172 ± 11	107 ± 18
WAY-100635 (1.0) + ondansetron (0.1)	194 ± 5	124 ± 25
Vehicle	171 ± 11	78 ± 15
WAY-100635 (1.0) + GR 125487D (3.0)[a]	168 ± 12	128 ± 22

[a] SC injection. Paroxetine was tested at a dose of 3.0 mg/kg, IP, t-60 min. Antagonists were administered 30 min before testing

obtained for the factor Agonist [$F(1,32) = 21.21$, $P < 0.001$, Table 2]. Ketanserin (1.0 mg/kg, IP) was also inactive against paroxetine (vehicle × vehicle: 164 ± 13 s, paroxetine × vehicle: 108 ± 26 s, vehicle × ketanserin: 135 ± 17 s and paroxetine × ketanserin: 85 ± 20 s). ANOVA again revealed only a significant effect for the factor Agonist [$F(1,28) = 8.32$, $P < 0.01$]. When tested alone, higher doses of ketanserin significantly decreased USV (3.0 mg/kg, IP, data not shown).

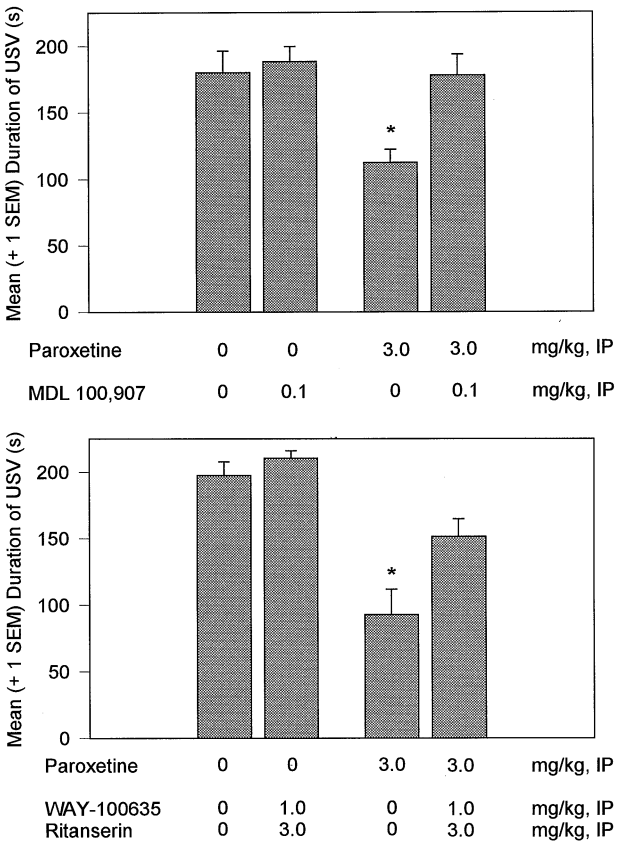

Fig. 3 Effects of (combined) treatment with different 5-HT receptor antagonists (t-30 min) on the anxiolytic effects of paroxetine (t-60 min) in the shock-induced ultrasonic vocalization (USV) test in young adult rats. *Upper panel*: Antagonism of the USV-reducing effect of paroxetine by the 5-HT_{2A} receptor antagonist, MDL 100,907. *Lower panel*: Effects of combined treatment with the 5-HT_{1A} receptor antagonist, WAY-100635, and the mixed 5-$HT_{2A/2C}$ receptor antagonist, ritanserin, on the USV-reducing effect of paroxetine. $n = 8$–10 per group. *$P < 0.05$ as compared to vehicle × vehicle treatment

In contrast to the negative findings for the non-selective 5-HT_{2A} receptor antagonists, ritanserin and ketanserin, the *selective* 5-HT_{2A} receptor antagonist, MDL 100,907, completely blocked the USV reducing activity of paroxetine [Agonist: $F(1,36) = 9.14$, $P < 0.01$; Antagonist: $F(1,36) = 8.06$, $P < 0.01$, and Intraction: $F(1, 36) = 4.89$, $P < 0.05$]. Post-hoc analysis revealed significant differences between the paroxetine × vehicle-treated group and both the vehicle x vehicle- and paroxetine × MDL 100,907-treated groups (Fig. 3, higher panel).

Although the 5-HT_3 receptor antagonist, ondansetron, tended to partially antagonize the paroxetine-induced reduction of USV, this effect failed to reach statistical significance and only a main effect for the factor Agonist [$F(1,35) = 8.22$, $P < 0.01$] was obtained. Likewise, the 5-HT_4 receptor antagonist, GR 125487D, did not antagonize the effects of paroxetine [Agonist: $F(1,33) = 34.39$, $P < 0.001$, Table 1].

Combined antagonist treatment (Fig. 3, Table 2)

In the final series of experiments, we tested whether combined treatment with WAY-100635 and either GR 127935, ritanserin, ondansetron, or GR 125487D resulted in a more pronounced blockade of the paroxetine-induced reduction of USV than treatment with either antagonist alone. Combined treatment with WAY-100635 and GR 127935 did not affect the actions of paroxetine. A significant effect was obtained for the factors Agonist [$F(1,34) = 55.15$, $P < 0.001$] and Interaction [$F(1,34) = 10.38$, $P < 0.01$]. Post-hoc analysis showed a significant difference between the paroxetine × vehicle-, paroxetine × WAY-100635/GR 127935- and vehicle × WAY-100635/GR 127935-treated groups, as compared with the vehicle × vehicle-treated control group.

For the combination WAY-100635 and ritanserin, a significant effect was obtained for the factors Agonist [$F(1,36) = 43.16$, $P < 0.001$] and Antagonist [$F(1,36) = 8.26$, $P < 0.01$], whereas Interaction was almost significant [$F(1,36) = 3.39$, $P = 0.07$; Fig. 3, lower panel]. Post-hoc analysis showed a significant difference between the paroxetine × vehicle-treated group, and both the paroxetine × WAY-100635/ritanserin- and vehicle × vehicle-treated groups. However, although combined treatment with WAY-100635 and ritanserin significantly attenuated the effects of paroxetine, the blockade was not complete.

Combination of WAY-100635 with the 5-HT_3 receptor antagonist, ondansetron, or the 5-HT_4 receptor antagonist, GR 125487D, did not block the USV-reducing effects of paroxetine. ANOVA revealed a significant effect for the factor Agonist [$F(1,34) = 17.00$, $P < 0.001$ and $F(1,33) = 19.75$, $P < 0.001$, for ondansetron and GR 125487D, respectively].

Discussion

In the present study, the SSRIs, paroxetine, citalopram, fluvoxamine and fluoxetine dose dependently reduced USV, the order of potency being similar to that found in other USV experiments with adult rats or rat pups (Mos and Olivier 1989; Winslow and Insel 1991a; Sanchez 1993; Sanchez and Meier 1997). Moreover, as this order of potency correlates with the inhibitory effect of these compounds on 5-HT reuptake in vitro (Stanford 1996 and references therein; Thomas et al. 1987), it appears that the anxiolytic activity of these SSRIs, as assessed in the USV test, is predominantly mediated by inhibition of 5-HT reuptake. In the rat elevated plus-maze, however, the anxiolytic effects of the SSRIs, fluoxetine, paroxetine, fluvoxamine and sertraline, could not be directly correlated with their inhibition of 5-HT reuptake in vitro or in vivo (Jackson et al. 1994). This discrepancy may involve the different profile of fluvoxamine in the elevated plus-maze and the USV test. Fluvoxamine was more potent in the elevated plus-maze than in the USV test and did not inhibit 5-HT reuptake at doses affecting open arm entries.

Different classes of 5-HT_{1A} receptor agonists show robust anxiolytic effects in a variety of USV paradigms (e.g., Winslow and Insel 1991b; De Vry et al. 1993; Sanchez 1993; Molewijk et al. 1995). Thus it can be postulated that indirect activation of 5-HT_{1A} receptors, as a result of an increased availability of 5-HT, may contribute to the USV-reducing activity of SSRIs. However, the 5-HT_{1A} receptor antagonist, WAY-100635 (Fletcher et al. 1996), did not block the paroxetine-induced reduction of USV in rats at doses effective against the 5-HT_{1A} receptor agonist, 8-OH-DPAT, as assessed under similar experimental conditions (this study; Maurel Remy et al. 1996; Bartoszyk et al. 1997). This finding suggests that 5-HT_{1A} receptors may not be critically involved in the USV-reducing effects of paroxetine. Interestingly, WAY-100635 attenuates the antidepressant-like effects of fluoxetine in the rat forced swimming test, suggesting that, in contrast to the anxiolytic effects, the antidepressive effects of SSRIs require stimulation of 5-HT_{1A} receptors (De Vry 1996). In a further series of experiments, the ability of combined blockade of 5-HT_{1A} receptors *and* either 5-$HT_{1B/1D}$, 5-$HT_{2A/2C}$, 5-HT_3 or 5-HT_4 receptors to attenuate more effectively the anxiolytic effects of paroxetine than selective blockade of either receptor subtype alone was tested. A marked attenuation of the effects of paroxetine was induced only by combined treatment with WAY-100635 and the mixed 5-$HT_{2A/2C}$ receptor antagonist, ritanserin. This finding suggests that simultaneous stimulation of 5-HT_{1A} *and* 5-HT_{2A} and/or 5-HT_{2C} receptors contributes to the USV-reducing activity of paroxetine. Therefore, we tested the possibility that combined treatment with 5-HT_{1A} and 5-$HT_{2A/2C}$ receptor agonists would result in a more pronounced anxiolytic effect as compared to single treatment with either agonist. However, preliminary data do not support this hypothesis, because USV was reduced to a similar extent by simultaneous injection of 8-OH-DPAT and DOI (3.0 and 0.3 mg/kg, IP, respectively) and single injection of either drug alone (data not shown).

By analogy to the effects of 5-HT_{1A} receptor agonists in the USV test, the fact that 5-$HT_{1B/1D}$ receptor agonists such as TFMPP reduce USV in both adult rats (Mos and Olivier 1989; De Vry et al. 1993) and mice (Nastiti et al. 1991) suggests that 5-$HT_{1B/1D}$ receptors could contribute to the USV-reducing activity of paroxetine. In the present study, the 5-$HT_{1B/1D}$ receptor antagonist, GR 127935 (Skingle et al. 1993), failed to modify the effects of paroxetine. However, it cannot be concluded that 5-$HT_{1B/1D}$ receptors are not critically involved in the anxiolytic effects of paroxetine because the reversal of the USV reducing effects of

TFMPP by GR 127935 was incomplete. Indeed, at the (high) dose of GR 127935 tested, its partial agonist activity at 5-$HT_{1B/1D}$ receptors may have confounded a full blockade of the effects of TFMPP. Furthermore, TFMPP is a equipotent agonist at 5-$HT_{1B/1D}$ and 5-$HT_{2A/2C}$ receptors (Schoeffter and Hoyer 1989). The reduction of USV found with the 5-$HT_{2A/2C}$ receptor agonist, DOI, suggests that agonist activity at 5-$HT_{2A/2C}$ receptors may contribute to the actions of TFMPP under the present conditions. The role of the latter mechanisms can be evaluated by testing GR 127935 against more potent and selective 5-$HT_{1B/1D}$ receptor agonists.

Combined injection of WAY-100635 and GR 127935 *increased* USV, suggesting that simultaneous blockade of 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors induces an anxiogenic effect. This appears paradoxical, because antagonism of presynaptic 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors has been shown to increase brain 5-HT levels (Millan et al. 1997), and this augmentation of serotonergic neurotransmission is thought to underlie the anxiolytic effects of SSRIs. However, the difference between the two treatments is the presumed additional blockade of postsynaptic 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors by the combined injection of the antagonists. Therefore, a possible, but highly speculative, explanation for this paradoxical finding may involve particular interactions of postsynaptic 5-HT receptors in the control of anxiety. Thus activation of an as yet unidentified 5-HT receptor subtype induces an anxiogenic effect which may be counteracted by activation of postsynaptic 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors. However, if 5-HT levels are increased in the presence of a blockade of postsynaptic 5-HT_{1A} and 5-$HT_{1B/1D}$ receptors, the counteracting effect produced by the latter receptors would be suppressed, and activation of the putative "anxiogenic" 5-HT receptor(s) would lead to an increase in USV. Further testing of this hypothesis may lead to identification of the putative "anxiogenic" 5-HT receptor(s) and to the development of new anxiolytics acting as antagonists at this (these) receptor(s).

We tested whether the USV-reducing activity of SSRIs is due to increased activation of 5-HT_{2A} receptors by carrying out antagonism experiments with the mixed 5-$HT_{2A/2C}$ receptor antagonist, ritanserin, the mixed 5-$HT_{2A/\alpha 1}$ adrenergic receptor antagonist, ketanserin (Marwood 1994), and the selective 5-HT_{2A} receptor antagonist, MDL 100,907 (Sorensen et al. 1993). Although these antagonists blocked the USV reducing effects of the mixed 5-$HT_{2A/2C}$ receptor agonist, DOI, only MDL 100,907 abolished the paroxetine-induced reduction of USV. As ritanserin, ketanserin, and MDL 100,907 all possess potent 5-HT_{2A} receptor antagonist activity in vivo (Schreiber et al. 1994, 1995; Kehne et al. 1996), we have no explanation why only MDL 100,907 was effective against paroxetine in the present paradigm. It is possible that the non-selective nature of ketanserin and ritanserin masks their 5-HT_{2A} receptor antagonist activity when tested against paroxetine. For example, ritanserin, but not MDL 100,907, has marked antagonist activity at 5-HT_{2C} receptors (e.g., Kehne et al. 1996), and a potential involvement of 5-HT_{2C} receptors in the behavioural effects of SSRIs is suggested by the functional down-regulation of both 5-HT_{2A} and 5-HT_{2C} receptors upon repeated administration of paroxetine in rats (Kennett et al. 1994b; Bijak et al. 1996). However, the existence and functional relevance of such a putative interaction between 5-HT_{2A} and 5-HT_{2C} receptors in the anxiolytic effects of paroxetine in the USV test remains to be demonstrated. Interestingly, we failed to block the USV reducing effects of the 5-HT_{2C} receptor agonist, mCPP, with the 5-HT_{2C} receptor antagonist, SB 200,646 (Kennett et al. 1994a). However, SB 200,646 and mCPP may be of limited value as pharmacological tools for studying 5-HT_{2C} receptors because the affinity of SB 200,646 for 5-HT_{2C} receptors is relatively low and the 5-HT_{2C}/5-HT_{2A} selectivity ratio of SB 200,646 is less than 2 orders of magnitude (Kennett et al. 1994a). mCPP, in contrast, has affinity for 5-HT_{2A} and 5-$HT_{1B/1D}$ receptors, and the activity of mCPP at the latter receptors may underlie its anxiolytic-like effects, as assessed in a conflict test in rats (Chojnacka-Wojcik and Klodzinska 1992). Notwithstanding the need for further characterization of the precise role of 5-HT_{2C} receptors in the anxiolytic effects of SSRIs and mCPP, our results with MDL 100,907 strongly suggest that activation of 5-HT_{2A} receptors is critically involved in the anxiolytic activity of paroxetine.

In line with the apparent insensitivity of the USV paradigm towards 5-HT_3 receptor ligands (De Vry et al. 1993; Sanchez 1993; Molewijk et al. 1995), we found that the 5-HT_3 receptor antagonist, ondansetron, did not abolish the anxiety-reducing effects of paroxetine. Similarly, the 5-HT_4 receptor antagonist, GR 125487D, was inactive against paroxetine. Although we used high doses of ondansetron and GR 125487D that were presumably adequate to block 5-HT_3 and 5-HT_4 receptors *in vivo*, respectively, we could not verify their antagonist efficacy in our experimental paradigm because the 5-HT_3 receptor agonist, mCPBG, was inactive (see also: Molewijk et al. 1995) and no selective agonists for the 5-HT_4 receptor were available. Nevertheless, it appears that 5-HT_3 and 5-HT_4 receptors are not critically involved in the anxiolytic effects of paroxetine.

To conclude, our results suggest that 5-HT_{2A} receptors are predominantly involved in the anxiety reducing effects of paroxetine in the rat USV test. Although the precise role of 5-$HT_{1B/1D}$, 5-HT_{2C}, 5-HT_3, and 5-HT_4 receptors remains to be elucidated, we postulated that the latter receptor subtypes are not critically involved in the USV reducing effects of SSRIs. Although it also seems unlikely that the 5-HT_{1A} receptor is of critical importance, our results suggest that simultaneous activation of 5-HT_{1A} *and* 5-HT_{2A} and/or

5-HT_{2C} receptors contributes to the anxiolytic efficacy of SSRIs. Since 5-HT_{2A} receptors are probably *not* critically involved in the antidepressive effects of SSRIs, as assessed in the rat forced swimming test (De Vry 1996 and unpublished data), different 5-HT receptor subtypes are probably involved in the antidepressive and anxiolytic effects of SSRIs. However, as full expression of the clinical efficacy of SSRIs requires at least 3–4 weeks of treatment, experiments with a repeated treatment regimen, as well as other SSRIs, should be performed before definite conclusions can be drawn about the role of 5-HT receptor subtypes in the therapeutic effects of SSRIs.

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References

Bartoszyk GD, Hegenbart R, Ziegler H (1997) EMD 68843, a serotonin reuptake inhibitor with selective presynaptic 5-HT_{1A} receptor agonistic properties. Eur J Pharmacol 322:147–153

Bel N, Artigas F (1993) Chronic treatment with fluvoxamine increases extracellular serotonin in frontal cortex but not in raphe nuclei. Synapse 15:243–245

Bijak M, Dziedzicka-Wasylewska M, Gogoz Z, Skuzy G, Tokarski T (1996) Effects of paroxetine given repeatedly on 5-HT receptor subpopulations in the rat brain. Psychopharmacology 127:73–82

Chojnacka-Wojcik E, Klodzinska A (1992) Involvement of 5-HT_{1B} receptors in the anticonflict effect of mCPP in rats. J Neural Transm 87:87–96

Detke MJ, Kirby L, Lucki I (1995) Serotonergic mechanisms involved in the effects of flouxetine in the rat forced swimming test (FST). Soc Neurosci Abstr 21:976

De Vry J (1996) 5-HT_{1A} receptors in psychopathology and the mechanism of action of clinically effective therapeutic agents. Drugs News Perspect 9:270

De Vry J, Benz U, Schreiber R, Traber J (1993) Shock-induced ultrasonic vocalization in young adult rats: a model for testing putative anti-anxiety drugs. Eur J Pharmacol 249: 331–339

De Vry J, Schreiber R, de Beun R (1995) Impulse control and serotonergic drugs: commonalities and differences in animal models. Eur Neuropsychopharmacol 5:177–178

Fletcher A, Forster EA, Bill DJ, Brown G, Cliffe IA, Hartley JE, Jones DE, McLenachan A, Stanhope KJ, Critchley DJP, Childs KJ, Middlefell VC, Lanfumey L, Corradetti R, Laporte AM, Gozlan H, Hamon M, Dourish C (1996) Electrophysiological, biochemical, neurohormonal and behavioural studies with WAY-100635, a potent, selective and silent 5-HT_{1A} receptor antagonist. Behav Brain Res 73:337–353

Gale JD, Green A, Darton J, Sargent RS, Clayton NM, Bunce KT (1994) GR 125487: a 5-HT_4 receptor antagonist with a long duration of action in vivo. Br J Pharmacol 113:120P

Gobert A, Rivet JM, Spedding M, Cistarelli L, Giradon S, Millan MJ (1996) Fluoxetine and duloxetine simultaneously release serotonin (5-HT), dopamine (DA) and noradrenaline (NAD) in the frontal cortex of freely-moving rats: WAY 100,635 selectively potentiates the induction of 5-HT release. Soc Neurosci Abstr 22:368.7

Ichimaru Y, Egawa T, Sawa A (1995) 5-HT_{1A} receptor subtype mediates the effect of fluvoxamine, a selective serotonin reuptake inhibitor, on marble-burying behavior in mice. Jpn J Pharmacol 68:65–70

Jackson HC, Cheetham SC, Luscombe GP, Mazurkiewicz SE, Viggers JA, Heal DJ (1994) The anxiolytic effects of selective 5-HT reuptake inhibitors in the elevated plus-maze are not explained by reuptake inhibition or affinity for 5-HT receptor subtypes. Br J Pharmacol 114 [Proc Suppl]:391P

Kehne JH, Baron BM, Carr AA, Chaney SF, Elands J, Feldman DJ, Frank RA, van Giersbergen PLM, McCloskey TC, Johnson MP, CcCarty DR, Poirot M, Senyah Y, Siegel BW, Widmaier C (1996) Preclinical characterization of the potential of the putative atypical antipsychotic MDL 100,907 as a potent 5-HT_{2A} antagonist with a favorable CNS safety profile. J Pharmacol Exp Ther 277:968–981

Kennett GA, Pittaway K, Blackburn TP (1994a) Evidence that 5-HT_{2C} receptor antagonists are anxiolytic in the rat Geller-Seifter model of anxiety. Psychopharmacology 114:90–96

Kennett GA, Lightowler S, Tulloch IF, Blackburn TP (1994b) Chronic paroxetine and fluoxetine administration in rats produces a desensitization of 5-HT_{2C} receptor function. A possible index to therapeutic efficacy in OCD. Neuropsychopharmacology 10 [Suppl]:261S

Kreiss DS, Lucki I (1995) Effects of acute and repeated administration of antidepressant drugs on extracellular levels of 5-hydroxytryptamine measured in vivo. J Pharmacol Exp Ther 274:866–876

Marwood JF (1994) Influence of alpha1-adrenoceptor antagonism of ketanserin on the nature of its 5-HT_2 receptor antagonism. Clin Exp Pharmacol Physiol 21:955–961

Maurel Remy S, Schreiber R, Dalmus M, De Vry J (1996) Somatodendritic 5-HT_{1A} receptors are critically involved in the anxiolytic effects of 8-OH-DPAT. Psychopharmacology 125:89–91

McNicoll C, Bill DJ, Fletcher A (1995) Potentiation of SSRI-induced inhibition of mouse marble burying by a selective 5-HT_{1A} antagonist. J Psychopharmacol 9:116

Millan MJ, Perrin-Monneyron (1997) Potentiation of fluoxetine-induced penile erections by combined blockade of 5-HT_{1A} and 5-HT_{1B} receptors. Eur J Pharmacol 321:R11–R13

Molewijk HE, van der Poel AM, Mos J, van der Heyden JAM, Olivier B (1995) Conditioned ultrasonic distress vocalizations in adult male rats as a behavioural paradigm for screening anti-panic drugs. Psychopharmacology 117:32–40

Mos J, Olivier B (1989) Ultrasonic vocalizations by rat pups as an animal model for anxiolytic activity: effects of serotonergic drugs. In: Bevan P, Cools AR, Archer T (eds) behavioural pharmacology of 5-HT. Lawrence Erlbaum, London, pp 361–365

Murphy DL, Pigott PA (1990) A comparative examination of a role for serotonin in compulsive obsessive disorder, panic disorder and anxiety. J Clin Psychiatry 51 [Suppl]:53–60

Nastiti K, Benton D, Brain PF, Haug M (1991) The effects of 5-HT receptor ligands on ultrasonic calling in mouse pups. Neurosci Biobehav Rev 15:483–487

Njung'e K, Handley SL (1991) Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. Br J Pharmacol 104:105–112

Sánchez C (1993) Effect of serotonergic drugs on footshock-induced ultrasonic vocalization in adult male rats. Behav Pharmacol 4:269–277

Sánchez C, Meier E (1997) Behavioral profiles of SSRI's animal models of depression, anxiety and aggression. Are they all alike? Psychopharmacology 129:197–205

Schoeffter P, Hoyer D (1989) Interaction of arylpiperazines with 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} receptors: do discriminatory 5-HT_{1B} receptor ligands exist? Naunyn Schmiedeberg's Arch Pharmacol 339:675–683

Schreiber R, Brocco M, Millan MJ (1994) Blockade of the discriminative stimulus effects of DOI by MDL 100,907 and the "atypical" antipsychotics, clozapine and risperidone. Eur J Pharmacol 264:99–102

Schreiber R, Brocco M, Audinot V, Gobert A, Veiga S, Millan MJ (1995) (1-(2,5-Dimethoxy-4 iodophenyl)-2-aminopropane)-induced head-twitches in the rat are mediated by 5-hydroxytryptamine (5-HT)$_{2A}$ receptors: modulation by novel 5-HT$_{2A/2C}$ antagonists, D$_1$ antagonists and 5-HT$_{1A}$ agonists. J Pharmacol Exp Ther 273:101–112

Schreiber R, Melon C, Dalmus M, De Vry J (1996) Characterization of the 5-HT receptor subtypes involved in the anxiolytic effects of selective serotonin reuptake inhibitors in the rat. Behav Pharmacol 7 [Suppl 1]:101

Skingle M, Scopes DIC, Feniuk W, Tyers MB, Connor HE, Carter MC, Clitherow JW (1993) GR 127935, a potent and orally active 5-HT$_{1D}$ receptor antagonist. Br J Pharmacol 110:9P

Sorensen SM, Kehne JH, Fadayel GM, Humphreys TM, Ketteler HJ, Sullivan CK, Taylor VL, Schmidt CJ (1993) Characterization of the 5-HT$_2$ receptor antagonist MDL 100907 as a putative atypical antipsychotic: behavioral, electrophysiological and neurochemical studies. J Pharmacol Exp Ther 266:684–691

Stanford SC (1996) Prozac: panacea or puzzle? Trends Pharmacol Sci 17:150–154

Thomas DR, Nelson DR, Johnson AM (1987) Biochemical effects of the antidepressant paroxetine, a specific 5-hydroxytryptamine uptake inhibitor. Psychopharmacology 93:193–200

Winslow JT, Insel TR (1991a) Infant rat separation is a sensitive test for novel anxiolytics. Prog NeuroPsychopharmacol Biol Psychiatry 15:745–757

Winslow JT, Insel TR (1991b) Serotonergic modulation of the rat pup ultrasonic isolation call: studies with 5-HT$_1$ and 5-HT$_2$ subtype-selective agonists. Psychopharmacology 105:513–520