

ORIGINAL INVESTIGATION

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Effect of 5-HT₂ receptor antagonists on a cranial nerve reflex in the rabbit: evidence for inverse agonism

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Abstract This study examined the role of the serotonin 5-HT₂ receptor in motor function by examining the effect of antagonists on the motor performance of a cranial nerve reflex, the nictitating membrane (NM) reflex of the rabbit. The NM reflex was elicited by varying intensities of a tactile stimulus and the magnitudes of the elicited responses were measured at each intensity. Dose-response curves were obtained for the effects of several 5-HT₂ receptor antagonists on response magnitude. *d*-Bromolysergic acid diethylamide (BOL), LY-53,857 and ketanserin had no significant effect on the magnitude of the NM reflex, indicating that they are neutral antagonists. However, the 5-HT₂ receptor antagonists ritanserin, MDL-11,939 and mianserin produced a significant reduction in response magnitude with no significant effects on response frequency, suggesting that they were acting as inverse agonists at the 5-HT₂ receptor. The reduction in reflex magnitude produced by mianserin (10 µmol/kg) was fully blocked by BOL (5.8 µmol/kg), supporting the conclusion that mianserin was producing a reduction in reflex magnitude through an effect at the 5-HT₂ receptor. The occurrence of inverse agonism suggests the possible existence of constitutive activity *in vivo*. We conclude that the 5-HT₂ receptor (either 2A or 2C) plays an important role in motor function, perhaps by providing a tonic influence on motor systems.

Key words Ritanserin · MDL-11,939 · Mianserin · Ketanserin · BOL · LY-53,857 · 5-HT_{2A/2C} receptor antagonists · Nictitating membrane · Motor performance · Rabbit

Introduction

A number of studies have demonstrated that serotonin plays an important role in learning (Harvey 1996) and

motor performance (Jacobs and Fornal 1993). Drugs that are agonists at the 5-HT₂ receptor produce a robust enhancement of associative learning as measured by the rabbit's nictitating membrane (NM) response (Romano et al. 1991; Romano and Harvey 1994; Harvey 1996) or by the conditioned avoidance response in rats (Alhaider et al. 1993). 5-HT_{1A} receptor agonists had no effect or produced a slight retardation of learning (Alhaider et al. 1993; Harvey 1996; Welsh et al. 1998b). The enhancement of associative learning produced by 5-HT₂ agonists was found in varying situations to be accompanied by an increase in the amplitude of the unconditioned response (Schindler et al. 1985; Harvey et al. 1988; Romano et al. 1991; Romano and Harvey 1993, 1994). More recently, we found that the 5-HT₂ antagonists ritanserin and MDL-11,939 produced a retardation of learning and a decrease in the amplitude of the unconditioned response, effects that were opposite to those of the 5-HT₂ agonists, while another 5-HT₂ antagonist, LY-53,857, had no effect on either measure (Welsh et al. 1998a). The effects of these three antagonists on learning were measured as a function of three doses of drug (0.067, 0.67 and 6.7 µmol/kg); however, effects on motor performance were obtained with fixed doses of the antagonists and at a fixed intensity of the unconditioned stimulus, a puff of air to the cornea.

Several *in vitro* studies had demonstrated that antagonists at the 5-HT_{2C} receptor could act either as neutral antagonists that had no effect on phosphoinositide hydrolysis, or as inverse agonists that decreased phosphoinositide hydrolysis (Kenakin 1996). This raised the possibility that we were obtaining a similar effect *in vivo*, with LY-53,857 acting as a neutral antagonist and ritanserin and MDL-11,939 acting as inverse agonists at the 5-HT₂ receptor. The present study examined the effects of 5-HT₂ antagonists on the performance of the NM response in greater detail. To this end, we employed six 5-HT₂ antagonists: BOL (*d*-bromolysergic acid diethylamide); ketanserin; LY-53,857; MDL-11,939; mianserin; and ritanserin. These drugs were chosen because they have been demonstrated to have equal potencies *in vivo* (Table 1). *In vitro*, ketanserin and MDL-11,939 have a

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Table 1 Receptor affinities and in vivo potencies

Antagonist	Receptor affinities (k_i ; nm)			In vivo potencies ($\mu\text{mol/kg}$)	
	5-HT _{2A}	5-HT _{2B}	5-HT _{2C}	Rat ID ₅₀	Rabbit ID ₄₀
BOL	0.48 ¹	12.6 ¹	7.14 ¹	0.58 ³	>5.8 ¹⁰
Ketanserin	0.38 ²	3240 ¹	54 ²	0.04–1.36 ^{3,4,5,6,9}	>10.0 ¹⁰
LY-53,857	1.70 ²	6.87 ¹	4.2 ²	0.56–1.60 ^{3,8,9}	>6.7 ¹¹
MDL-11,939	0.76 ²	4700 ¹	550 ²	0.38 ⁹	3.0 ¹¹
Mianserin	1.50 ²	52.5 ¹	4.3 ²	0.20–0.76 ^{5,6,7,9}	0.4 ¹⁰
Ritanserin	0.24 ²	5.18 ¹	0.6 ²	0.19–0.68 ^{4,5,8,9}	0.67–1.0 ^{11,12}

¹ Using [¹²⁵I]DOI in a transformed cell line expressing human 5-HT_{2A} receptors (Wainscott et al. 1996)

² Using [³H]ketanserin in rat cortex (Leysen 1992)

³ Antagonism of LSD (0.186 $\mu\text{mol/kg}$) discriminative stimulus cue in rats (Cunningham and Appel 1987)

⁴ Antagonism of DOI (1.76 $\mu\text{mol/kg}$) discriminative stimulus cue in rats (Kleven et al. 1997)

⁵ Antagonism of DOI (1.76 $\mu\text{mol/kg}$) discriminative stimulus cue in rats (Schreiber et al. 1994)

⁶ Antagonism of quipazine (2.2 $\mu\text{mol/kg}$) discriminative stimulus cue in rats (Yamamoto et al. 1991)

⁷ Antagonism of quipazine (5.6 $\mu\text{mol/kg}$) discriminative stimulus cue in rats (Friedman et al. 1984)

⁸ Antagonism of DOM (4 $\mu\text{mol/kg}$) discriminative stimulus cue in rats (Glennon and Hauck 1985)

⁹ Antagonism of quipazine ($\mu\text{mol/kg}$) induced corticosterone elevation in rats (Fuller and Snoddy 1990)

¹⁰ Retardation of CR acquisition (A. G. Romano, H. Hood and J. A. Harvey, unpublished data)

¹¹ Retardation of CR acquisition (Welsh et al. 1998a)

¹² Retardation of CR acquisition (Welsh et al. 1998b)

greater affinity for the 5-HT_{2A} as compared with the 5-HT_{2C} or the 5-HT_{2B} serotonin receptor subtypes, while the other four drugs have essentially equivalent affinities at these three sites (Table 1). The effects of these drugs on motor performance were examined by measuring the frequency and amplitude of the NM response to four different intensities of an air puff stimulus. Finally, as a test for possible inverse agonist action, we examined whether one of the 5-HT₂ receptor antagonists having no effect on response magnitude (BOL) would block the effects of a 5-HT_{2A/2C} receptor antagonist that produced the largest decreased response magnitude (mianserin).

Materials and methods

Subjects

New Zealand White rabbits of both sexes, weighing 1.8–2.2 kg upon arrival, were housed individually with free access to rabbit chow and water under a 12/12-h light/dark cycle in an AAALAC-approved colony maintained at 22°C. Rabbits were given 5 days of adaptation to the laboratory before initiation of experiments. All animal experiments were carried out in accordance with the National Institute of Health guide "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985).

Drugs

Ritanserin, LY-53,857 maleate, ketanserin tartrate, and mianserin hydrochloride were purchased from Research Biochemicals Int. (Natick, Mass., USA). MDL-11,939 was a gift from Marion Merrell Dow Pharmaceuticals and *d*-2-bromolysergic acid diethylamide hydrogen tartrate (BOL) was supplied by the National Institute on Drug Abuse. Mianserin, ketanserin, LY-53,857 and BOL were dissolved in distilled water, which also served as the vehicle control for these drugs. Ritanserin and MDL-11,939 were dissolved in distilled water with a minimum quantity of acetic acid and the pH adjusted to 5.5 with NaOH, and their vehicle controls received distilled water adjusted to a pH of 5.5. Because of its short duration of action (Cunningham and Appel 1987), BOL was injected 20 min prior to testing, while all other drugs were injected

1 h prior to testing. Injections of drug or vehicle were subcutaneous, between the scapula, in a volume of 4 ml/kg. BOL was injected at doses of 0.06, 0.58 and 5.8 $\mu\text{mol/kg}$ while all other drugs were injected at doses of 0.1, 1.0 and 10 $\mu\text{mol/kg}$. Dosages of drug were based on previous data obtained in the rabbit (Welsh et al. 1998a,b) and covered the range of in vivo potencies that have been reported in the rat (Table 1).

Apparatus

The apparatus, IBM PC-AT and ASYST software for stimulus control and data acquisition have been described in detail elsewhere (Romano et al. 1991). Standard procedures were employed to measure the NM reflex. Briefly, each animal was placed in a Plexiglas restrainer and fitted with a headmount that supported a potentiometer which was coupled directly to a suture placed in the right NM. The headmount also supported a 2-mm diameter metal tube positioned 5–7 mm from the center of the right cornea for delivery of varying intensities of an air puff. The air puff was calibrated so that a 100-ms duration puff would produce a pressure of 200 g/cm² as measured at the tip of the metal tube. The intensity of the air puff was manipulated by altering its duration. Movements of the NM were transduced to DC voltages and digitized every 5 ms with a resolution of 0.03 mm NM movement per analog-to-digital count. A response was defined as a 0.5 mm or greater extension of the NM occurring within 500 ms of air puff onset.

Elicitation of the NM reflex as a function of stimulus duration

Animals were first placed in the conditioning chambers for a 70-min adaptation session, during which time no stimuli were presented and no drug or vehicle was injected. One day later, the animals were given the first of four testing sessions. Subsequent sessions were spaced 2 days apart. On the first testing session all animals received an injection of vehicle while the next three sessions consisted of injecting a low, medium and high dose, respectively, of each drug or drug combination. The number of animals in each group was: BOL, 12; ketanserin, 11, LY-53,857, 12; MDL-11,939, 11; mianserin, 12; and ritanserin, 12. Vehicle control animals ($n=24$) received only vehicle on the four testing sessions. Twelve controls received the distilled water vehicle and 12 the distilled water vehicle adjusted to pH 5.5. All testing sessions were 70 min in duration and began with ten presentations of a 100-ms air puff. This was followed by 60 trials during which the intensity of the air

puff was varied by manipulating its duration. Thus, the 60 trials consisted of 15 blocks of four trials each, with each four-trial block consisting of the pseudorandom presentations of a 10-, 25-, 50- and 100-ms air puff. Trials were spaced 60 s apart (range 55–65 s).

Antagonism of mianserin by BOL

This experiment was carried out exactly as described above. Four groups of animals ($n=12/\text{group}$) were employed and each group received two injections spaced 1 h apart. Thus, animals received vehicle+vehicle, vehicle+BOL, mianserin+vehicle, or mianserin+BOL. The second injection of vehicle or BOL occurred 20 min prior to initiation of testing. There were 4 testing days. On the first day all four groups were injected with vehicle. The vehicle+vehicle group continued to receive vehicle injections on each of the following three testing sessions. The injections of BOL were constant at a dose of $5.8 \mu\text{mol}/\text{kg}$ on each of the subsequent three testing sessions. Finally, mianserin was injected at doses of 0.1, 1.0 and $10 \mu\text{mol}/\text{kg}$, on testing days 2, 3 and 4, respectively. This procedure provided a replication of the effects of increasing doses of mianserin on response magnitude (the mianserin+vehicle group), the effects of repeated doses of BOL ($5.8 \mu\text{mol}/\text{kg}$) on response magnitude (the vehicle+BOL group), and finally the ability of BOL ($5.8 \mu\text{mol}/\text{kg}$) to block the effects of each of the three doses of mianserin (0.1, 1.0 and $10 \mu\text{mol}/\text{kg}$) on response magnitude (the mianserin+BOL group). Differences among the groups could then be compared with the performance of control animals (the vehicle+vehicle group).

Data analysis

Data were analyzed by repeated measures analyses of variance (ANOVA) using SYSTAT 7.0 for Windows (SPSS Inc., Chicago, Ill., USA). Statistical significance was set at $P<0.05$. Responses were analyzed in terms of frequency of occurrence and peak amplitude at each of the four stimulus intensities. On trials in which there was no response within 500 ms of stimulus onset, an amplitude of 0 mm was recorded and included in the calculation of mean response magnitudes. Because there were no significant differences between the two groups of vehicle controls, their data were combined.

Results

Effect of repeated testing on the NM reflex of vehicle controls

The vehicle control group ($n=24$) demonstrated a significant increase in response magnitude ($F_{3,105}=140.7$) and frequency ($F_{3,105}=156.5$) as a function of stimulus duration (Fig. 1). Response magnitude did not change across the 4 days of testing ($F_{3,105}<1$). However, there was a small but significant increase in the frequency of responses ($F_{3,105}=4.33$) across the 4 days of testing. There were no significant interactions between stimulus intensity and days of testing for response magnitude or frequency.

Effect of BOL, LY-53,857 and ketanserin on response magnitudes and frequency

All animals that were employed to examine the effects of 5-HT₂ antagonists on response magnitude were first tested

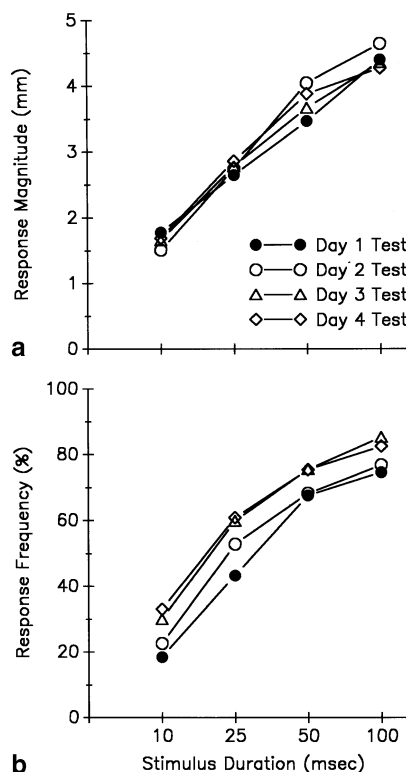


Fig. 1a, b Effect of repeated testing of vehicle controls on response magnitude (a) and response frequency (b) as a function of air puff duration. Rabbits were injected with vehicle prior to each testing day. Injections and testing occurred every second day. Each point is the mean of 24 animals. ● Day 1 test, ○ day 2 test, △ day 3 test, ◇ day 4 test

following injection of vehicle and then by increasing doses of drug. BOL ($F_{3,33}=1.99$), LY-53,857 ($F_{3,33}=1.65$) and ketanserin ($F_{3,30}=1.96$) had no significant effect on response magnitude (Fig. 2). There was no significant interaction between dose and air puff duration on response magnitude for animals receiving either BOL ($F_{9,99}=1.14$) or LY-53,857 ($F_{9,99}<1$). However, ketanserin did produce a significant interaction between stimulus duration and dose of drug ($F_{9,90}=2.62$), which was due to the fact that the doses of ketanserin differentially reduced response magnitude only at the 50-ms stimulus duration ($F_{3,30}=3.72$). LY-53,857 ($F_{3,33}<1$) and ketanserin ($F_{3,30}<1$) failed to produce any significant effects on response frequencies. As noted above, vehicle controls produced a small but significant increase in response frequency ($F_{3,105}=156.5$) and this effect was also seen after injection of BOL ($F_{3,33}=11.25$). The effect of BOL on response frequencies was not significantly different from that of the vehicle controls (data not shown).

Effect of ritanserin, MDL-11,939 and mianserin on response magnitudes and frequency

As shown in Fig. 3, ritanserin ($F_{3,33}=6.56$), MDL-11,939 ($F_{3,30}=9.52$), and mianserin ($F_{3,33}=17.63$) produced a

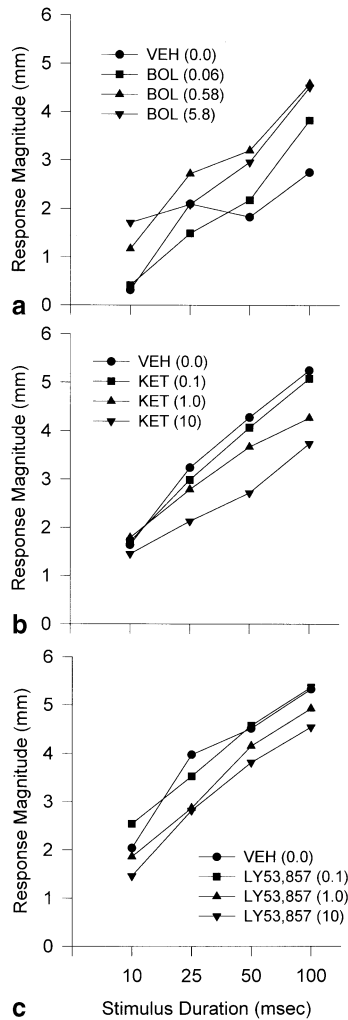


Fig. 2a–c Drugs having no significant effect on response magnitude elicited by differing durations of an air puff US. Rabbits were injected with vehicle (*VEH*) on test day 1 and then every second day with increasing doses of **a** *d*-bromolysergic acid diethylamide (*BOL*), ($n=12$); **b** ketanserin (*KET*), ($n=11$); or **c** LY53,857, ($n=12$). Numbers in parentheses indicate the dose of drug as $\mu\text{mol/kg}$

significant reduction in response magnitudes as a function of drug dose. There was a significant interaction between stimulus duration and dose of drug for MDL-11,939 ($F_{9,90}=2.99$) and mianserin ($F_{9,99}=7.81$), and inspection of Fig. 3 indicates that the reduction in response magnitude produced by MDL-11,939 and mianserin was proportionately greater at higher drug doses and at the longer stimulus durations. This intensity by dose interaction just missed significance for ritanserin ($F_{9,99}=2.34$, $P=0.057$). There was no significant effect of ritanserin ($F_{3,33}=1.16$), MDL-11,939 ($F_{3,30}<1$) or mianserin ($F_{3,33}=2.1$) on response frequency.

In order to present the effects of ritanserin, MDL-11,939 and mianserin on response magnitude and frequency as a function of drug dose, we collapsed the response magnitude and response frequency data across the four stimulus durations and expressed these mean values as a percent change from the first day of testing when all

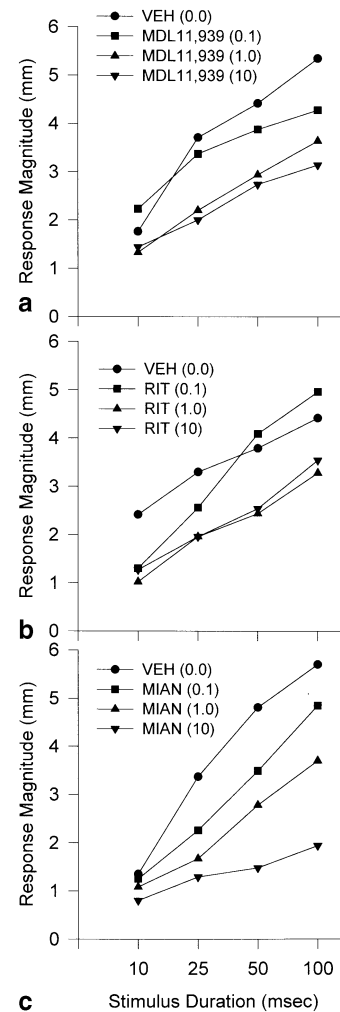


Fig. 3a–c Drugs that significantly reduced response magnitude elicited by differing durations of an air puff US. Rabbits were injected with vehicle (*VEH*) on test day 1 and then every second day with increasing doses of either: **a** MDL11,939, ($n=11$); **b** ritanserin (*RIT*), ($n=12$); or **c** mianserin (*MIAN*), ($n=12$). Numbers in parentheses indicate the dose of drug as $\mu\text{mol/kg}$

animals had received injections of vehicle. These data are presented as a function of drug dose in Fig. 4. The data taken from Fig. 1 for vehicle controls are also presented in the same manner for comparison purposes.

Antagonism of the effects of mianserin on response magnitude by BOL

This experiment was carried out in order to determine whether the reduction in response magnitude produced by mianserin was due to an action at the 5-HT₂ receptor. The results are presented in Fig. 5. In agreement with the data of Figs. 1 and 2, injections of vehicle alone or vehicle and BOL (5.8 $\mu\text{mol/kg}$) had no effect on response magnitudes, while mianserin produced a significant, dose-dependent reduction in magnitude which was proportionately greater at the longer air puff durations. BOL

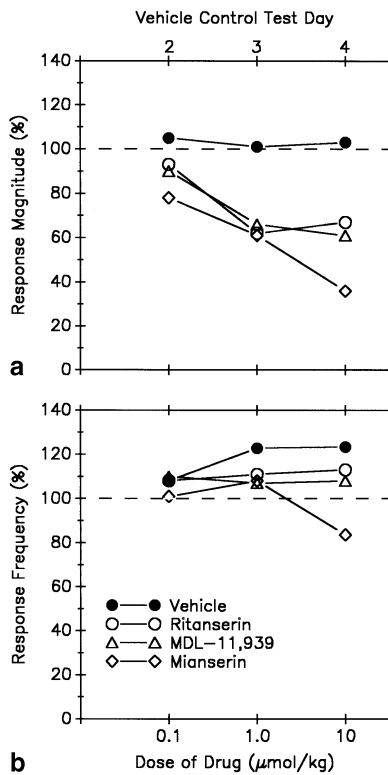


Fig. 4a–b Dose-response functions for the effects of ritanserin, MDL-11,939 and mianserin on response magnitude (a) and response frequency (b), averaged across the four air puff durations, were obtained from the data of Fig. 3 and are expressed, at each dose, as a percentage of the mean value obtained after saline injections on test day 1. For comparison purposes, the data for the 24 vehicle control animals (see Fig. 1) who received vehicle on each of the four test days is also presented in the same manner, i.e., the mean response magnitudes and frequencies obtained on test days 2, 3 and 4 are expressed as a percentage of the mean values obtained on test day 1. ● Vehicle, ○ ritanserin, △ MDL-11,939, ◇ mianserin

(5.8 $\mu\text{mol/kg}$) completely blocked the reduction in response magnitudes produced by even the highest dose of mianserin (10 $\mu\text{mol/kg}$). An ANOVA indicated that there was a significant difference in response magnitude between the four groups of animals ($F_{3,44}=2.962$), as well as a significant three way interaction ($F_{27,396}=1.94$) between group, stimulus intensity and dose of mianserin. Follow-up tests revealed that the significant group difference was localized to testing day 4, when the dose of mianserin was 10 $\mu\text{mol/kg}$ ($F_{3,44}=4.02$). A Tukey HSD multiple comparison indicated that on day 4, the mianserin (10 $\mu\text{mol/kg}$)+vehicle group demonstrated a significantly lower response magnitude than all other groups at the 100-ms stimulus duration.

Discussion

Role of serotonin in motor function

The results of this study demonstrate that the serotonergic system plays an important role in regulating the per-

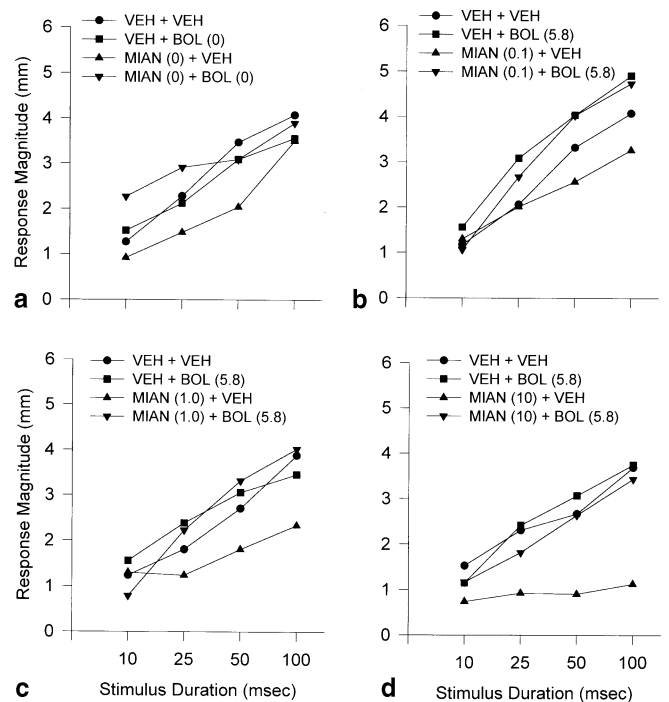


Fig. 5a–d Antagonism by BOL (5.8 $\mu\text{mol/kg}$) of the effects of mianserin on response magnitude. All animals received vehicle injections on day 1 of testing (a). On days 2 (b), 3 (c) and 4 (d) of testing: the vehicle controls (VEH+VEH) continued to receive injections of vehicle only; the VEH+BOL group received a constant dose of 5.8 $\mu\text{mol/kg}$; the MIAN+VEH group received increasing doses of 0.1, 1.0 and 10 $\mu\text{mol/kg}$ mianserin as indicated; and the MIAN+BOL group received the increasing doses of mianserin as indicated plus the constant dose of BOL (5.8 $\mu\text{mol/kg}$). Each point is the mean of 12 animals. VEH, vehicle; MIAN, mianserin. Numbers in parentheses indicate the dose of drug as $\mu\text{mol/kg}$. For further details see Materials and methods

formance of the NM reflex. We reported elsewhere that the magnitude of the NM reflex can be significantly enhanced by agonists at the 5-HT₂ receptor (Harvey et al. 1988; Romano and Harvey 1994), while in the present study, several 5-HT₂ receptor antagonists were able significantly to decrease response magnitude. Our results are in general agreement with a number of studies that have suggested that serotonin may play an important role in the regulation of motor function (Jacobs and Fornal 1993; Wallis 1994) and that have identified the 5-HT₂ receptor as the mediator of these effects (McCall and Aghajanian 1980; Sugihara et al. 1995). For example, serotonin excites facial motor neurons through an action at the 5-HT₂ receptor (McCall and Aghajanian 1980). A similar action on motor neurons in the accessory abducens nucleus which regulates the NM reflex (Marek et al. 1983) could account for the effects of ritanserin, MDL-11,939 and mianserin on response magnitude in this study. On a more global scale, neurons in the nucleus raphe obscurus and pallidus have been reported to fire at a low and regular rate when an animal is not moving, but increase their firing rate in a manner directly related to the speed of locomotion (Jacobs and Fornal 1993). Serotonergic projections from these medullary nuclei can

regulate motor movements through their innervation of the inferior olivary nucleus (Bishop and Ho 1986; Compoint and Buisseret-Delmas 1988), a brain region that is also importantly involved in the regulation of the NM reflex (Harvey and Welsh 1996). 5-HT₂ agonists can induce generalized motor movements through activation of the olive in vivo (Barragan et al. 1985; Wiklund et al. 1981) and in vitro studies have confirmed that this action is mediated by the 5-HT₂ receptor (Sugihari et al. 1995).

Two classes of 5-HT₂ receptor antagonists

The results of this study indicate that 5-HT₂ receptor antagonists can be divided into two groups. One group typified by the 5-HT₂ antagonists ritanserin, MDL-11,939 and mianserin produced decreases in response magnitude that were highly significant and dose-dependent. The ability of these antagonists to have an effect opposite to that of 5-HT₂ agonists suggests that they may be acting as inverse agonists at the 5-HT₂ receptor. It is not possible to determine from these actions whether the effects obtained might be due to specific actions at either the 5-HT_{2A} or the 5-HT_{2C} receptor subtype. As can be seen from Table 1, ritanserin and mianserin have equivalent affinities for these two receptor subtypes. Although MDL-11,939 has a much greater affinity for the 5-HT_{2A} as compared with the 5-HT_{2C} receptor subtype as measured in vitro, one would not expect any specificity at the doses of drug employed in vivo.

In contrast, with the effects of ritanserin, MDL-11,939 and mianserin noted above, three other antagonists, BOL, LY-53,857 and ketanserin, were neutral in that they had no overall significant effect on response magnitudes. It should be noted that the results with ketanserin were somewhat ambiguous in that, based on a significant interaction between dose and US duration, ketanserin, at the highest dose, was found to produce a significant decrease in response magnitude at the 50 ms stimulus duration. However, this effect was not systematic in that there were no significant effects at the 10-, 25- or 100-ms durations of the US. This marginal effect of ketanserin on response magnitude could reflect some weak actions of the drug at the 5-HT₂ or at the NE α ₁ receptor. Unlike the other 5-HT₂ antagonists employed in this study, ketanserin is also a potent NE α ₁ receptor antagonist (Fozard 1982).

It has recently been demonstrated for a number of receptor systems, including the 5-HT_{2C} receptor, that some antagonists are neutral, while others are active and can be classified as inverse agonists (Sanders-Bush and Breeding 1991; Barker et al. 1994; Lebreque et al. 1995; Westphal and Sanders-Bush 1994; Kenakin 1996). As noted above, it was quite possible that ritanserin, mianserin and MDL-11,939 were acting as inverse agonists in this study. Alternatively, the differences between BOL, LY-53,857 and ketanserin on the one hand and ritanserin, MDL-11,939 and mianserin on the other hand might reflect some unknown actions of the latter three

drugs on other receptor systems. To examine this possibility we examined the interaction of BOL the neutral antagonist, with mianserin the most potent of the putative inverse agonists. The finding was clear; BOL a highly selective 5-HT₂ antagonist could completely block the effects of mianserin. This strongly suggests that mianserin was producing its effects through an action at the 5-HT₂ receptor, and thus may have been acting as an inverse agonist at this receptor. If our results are indeed due to inverse agonism, then this in turn suggests the existence of constitutive activity at the 5-HT₂ receptor in vivo, perhaps serving to maintain a constant level of tonic influence on motor systems.

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