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Ketanserin-sensitive depressant actions of 5-HT receptor agonists in the neonatal rat spinal cord

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1 The monosynaptic reflex (MSR), recorded *in vitro* from the neonatal rat spinal cord, was depressed by 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT), methysergide and R(+)-8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), and also by the selective 5-HT_{1D} agonists, sumatriptan and N-methyl-3-(1-methyl-1-piperidinyl)-1H-indole-5-ethane sulphonamide (GR 85548).

2 Ketanserin (1 μM) and methiothepin (1 μM) reduced the duration of depressions elicited by 5-CT, but not those produced by 5-HT, sumatriptan, GR 85548, methysergide or 8-OH-DPAT.

3 The IC_{50} for MSR depression by 5-CT was 3.6, 2.1-6.2 nM ($n=4$), by sumatriptan was 15.2, 12.9-18.0 nM ($n=32$), by GR 85548 was 18.4, 11.7-29.1 nM ($n=12$), by methysergide was 29.8, 10.2-87.1 nM ($n=4$) and by 8-OH-DPAT was 0.21, 0.11-0.43 μM ($n=3$) (geometric means and 95% confidence limits).

4 Ketanserin (0.1 or 1 μM) antagonized competitively responses to sumatriptan (apparent pA_2 7.8 $\pm$ 0.1, $n=5$), GR 85548 (apparent pA_2 7.6, unpaired data, $n=5$), methysergide (apparent pA_2 7.9 $\pm$ 0.12, $n=4$) and 8-OH-DPAT (apparent pA_2 8.3 $\pm$ 0.1, $n=3$). Concentration-response curves to 5-CT showed a smaller, parallel shift to the right (apparent pA_2 6.8 $\pm$ 0.1, $n=4$), but responses to 5-HT were unaffected by ketanserin (1 μM) ($n=4$).

5 Methiothepin (1 μM) antagonized competitively responses to GR 85548 (apparent pA_2 7.7, unpaired data, $n=5$).

6 Mianserin (0.3μM), a concentration sufficient to cause substantial block of 5-HT_{2C}-mediated responses but have only a small effect on 5-HT_{1D}-mediated actions, caused a small, non-parallel shift of the concentration-response curve to sumatriptan.

7 Depression of the MSR by sumatriptan was not blocked by (±)-cyanopindolol (0.1 μM), (±)-propranolol (0.5 or 1 μM) or spiroxatrine (0.1 μM), and depression of MSR by 8-OH-DPAT was not blocked by spiroxatrine (0.1 μM). (±)-Cyanopindolol (0.1 and 1 μM) itself induced a slow depression of the MSR.

8 The novel 5-HT_{1D} antagonist, N-[4-methyl-1-piperazinyl) phenyl]2'-methyl-4'-(5-methyl-1, 2, 4-oxadiazol-3-yl) [1, 1-biphenyl]-4-carboxamide (GR 127935, 30 nM to 1 μM) caused a concentration-related depression of the reflex (up to 50%) usually slow in onset. Neither with these concentrations nor with concentrations in the range 1-3 nM was there any unequivocal blockade of responses to sumatriptan.

9 It is concluded that sumatriptan, GR 85548, methysergide and 8-OH-DPAT depress the MSR in the neonate rat spinal cord via ketanserin-sensitive receptors, which have some similarities to 5-$HT_{1D\alpha}$ receptors but which are not blocked by GR 127935. 5-HT released by tryptaminergic pathways may act via the same receptors to depress the MSR. 5-HT applied to the cord probably acts via a different, possibly novel 5-HT receptor to depress the MSR.

Keywords: 5-Hydroxytryptamine; spinal monosynaptic reflex; 5-$HT_{1D\alpha}$ receptors; rat spinal cord.

Introduction

Stimulation of a dorsal root in neonate rat spinal cord evokes a monosynaptic reflex (MSR) in the ventral root (Otsuka & Konishi, 1974). The MSR is depressed by 5-hydroxytryptamine (5-HT) and certain 5-HT receptor agonists (Saito *et al.*, 1982; Wang & Dun, 1990; Crick & Wallis, 1991), the potency of 5-HT being greatly increased by incubation with citalopram, a blocker of neuronal 5-HT uptake. The IC_{50} changes from 9.5 μM to 30 nM (Crick & Wallis, 1991). The observations that 5-HT-induced synaptic depression is not accompanied by a concomitant depression of glutamate-evoked depolarizations (Wu *et al.*, 1991) and that 5-HT agonists have similar effects to lowering $[Ca^{2+}]$ in paired-pulse testing (Yomono *et al.*, 1992) strongly suggest that 5-HT acts via presynaptic receptors. The action of 5-HT is not antagonized by ketanserin (Crick & Wallis, 1991), although descending inhibition of the MSR mediated via endogenous 5-HT is ketanserin-sensitive. 5-HT released on stimulation of latero-ventral spinal descending tracts, or accumulating as a result of chemical treatment, depresses the MSR via receptors blocked by 5-HT_2 receptor antagonists, such as ketanserin and ritanserin, and also by spiperone and methiothepin (Wallis *et al.*, 1993a,b). Crick *et al.* (1994) proposed that an avid uptake mechanism for 5-HT effectively protects synaptic 5-HT receptors from exogenous 5-HT but does not prevent released 5-HT, which presumably transiently reaches much higher concentrations, from activating ketanserin-sensitive receptors. At higher concentrations, 5-HT directly depolarizes spinal motoneurones (EC_{50} 1.4 μM in the presence of citalopram, Elliott & Wallis, 1992) via an action at 5-HT_2 receptors (Wang & Dun, 1990). On certain motoneurones a second 5-HT receptor with a distinct pharmacology which mediates depolarization may also be involved (Larkman & Kelly, 1991; 1992).

These observations indicate that depression of the MSR and increases in motoneurone excitability due to depolarization might involve two or more 5-HT receptor subtypes. Further, the finding that depression of the MSR by certain 5-HT agonists could be blocked by 5-HT antagonists, whereas the action of 5-HT could not (Crick *et al.*, 1994), suggested that 5-HT_{1A}, 5-HT_{1B} or 5-HT_2 receptors, or 2 or more of these,

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might mediate reflex depression. 5-HT applied to the cord acts through a receptor incapable of being blocked by a combination of 5-HT_1, 5-HT_2 and 5-HT_3 antagonists, and probably does not involve 5-HT_4 receptors (Crick *et al.*, 1994). The discovery that ketanserin was able to block the actions of sumatriptan on the reflex (Manuel & Wallis, 1994) hinted at a novel mode of 5-HT receptor-mediated synaptic depression in the rat. Sumatriptan is one of a number of selective 5-HT receptor agonists which have a potent depressant action on the MSR (Hendrikse *et al.*, 1992; Crick *et al.*, 1994). We describe here recent experiments with sumatriptan, the related ligand N-methyl-3-(1-methyl-4-piperidinyl)-1H-indole-5-ethane sulphonamide (GR 85548) and 5-carboxamidotryptamine (5-CT) which attempt to examine whether 5-HT_{1D} rather than 5-HT_{1B} receptors might be responsible for some of the reported actions of 5-HT receptor agonists. The actions of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) and methysergide in depressing the MSR have also been rexamined. The rat 5-HT_{1D} receptor is the rodent equivalent of the human 5-$HT_{1D\alpha}$ receptor (Boess & Martin, 1994). Although there is no specific antagonist for the 5-$HT_{1D\alpha}$ receptor, it has been proposed that ketanserin may permit discrimination between 5-HT_{1B} or 5-$HT_{1D\beta}$ and 5-$HT_{1D\alpha}$ receptors (Kaumann *et al.*, 1993; Tilford & Baxter, 1994). Furthermore, a potent and selective 5-HT_{1D} receptor ligand is now available in N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2'-methyl-4'-(5-methyl-1, 2, 4-oxadiazol-3-yl) [1,-1-biphenyl]4-carboxamide (GR 127935); this is reported to display nanomolar affinity for 5-HT_{1D} receptors (Skingle *et al.*, 1995).

A preliminary account of this work has been presented to the British Pharmacological Society (Manuel & Wallis, 1994).

Methods

Preparation

Neonatal Wistar rats, 4–8 days old of either sex, were anaesthetized with ether. Following decapitation, the spinal column with the rib cage attached was removed and pinned to a Sylgard base in a dish filled with oxygenated, modified Krebs solution at room temperature. A laminectomy was performed from the ventral surface, and the spinal roots cut where they entered the spinal column. Once the spinal cord was cut at the thoracic and sacral ends, it could be lifted out of the spinal column after cutting the underlying connective tissue. The spinal cord was re-pinned in a second dish, the dura removed from both sufaces, the ganglia cut away and the spinal roots untangled. A sagittal hemisection was performed from the ventral surface. The spinal cord was allowed 1 h to recover from surgical trauma in the gassed medium at room temperature before experimentation.

Recording and stimulation

The hemisection was pinned, cut surfaces downwards, to the Sylgard base of a perfusion chamber (volume 1 ml) and superfused (2–3 ml min^{-1} at 25°C) with modified Krebs solution. A segmental dorsal and ventral root pair (L3, L4 or L5) were gently drawn into a stimulating and recording suction electrode, respectively.

The dorsal root was stimulated supramaximally every 15 s (10–35 V, pulse width 0.1 ms). The reflex response (Figure 1a) consists of a brief, short latency monosynaptic reflex (MSR, duration 3–5 ms, latency 5–8 ms) and a slower polysynaptic response (PSR, duration up to 2 s, latency 10–12 ms) (Crick & Wallis, 1991). The response was amplified and fed into a peak height detector (see Crick & Wallis, 1991), which measured the peak amplitude of the MSR. A signal proportional to the peak voltage was relayed to a chart recorder and the reflex response monitored on a storage oscilloscope. A separate chart recorder monitored the d.c. potential recorded from the ventral root to detect any depolarizing action of the drugs employed. The amplitude of the MSR varied from 1 to 5 mV, but a preparation was not used unless amplitude was stable and in excess of 1 mV for at least 30 min.

Perfusion and drug application

The superfusion medium was a modified Krebs solution (mM: NaCl 118, KCl 3, KH_2PO_4 1.2, $CaCl_2$ 1.2, $MgSO_4.H_2O$ 0.6, $NaHCO_3$ 25 and glucose 11) which was gassed with 5% CO_2 in O_2. Agonists were applied in the superfusion medium. The half-time for the replacement of the medium in the recording bath was about 12 s. At least 30 min was allowed between applications, depending on the time course of action of the agent. In cases where response amplitude failed to recover to control values, the drug was not re-applied until a steady reflex amplitude, above 1mV, was observed for more than 20 min. Antagonist studies took two forms. A concentration-response curve to the agonist was constructed prior to incubation with the antagonist for at least 60 min and the concentration-response curve was then repeated in the presence of the antagonist. In some experiments, a control concentration-response curve was obtained from one preparation and a concentration-response curve in the presence of antagonist obtained from another preparation. The long period required for recovery from the depressant action of agonists, e.g. sumatriptan, methysergide, and also the long incubation times required for antagonists to equilibrate with receptors (see Wallis *et al.*, 1993a,b), prevented application of more than one concentration of antagonist to a preparation.

The drugs used were 5-hydroxytryptamine creatinine sulphate (5-HT, Sigma); 5-carboxamidotryptamine maleate (5-CT, RBI/Semat), methiothepin mesylate (RBI/Semat); R (+)-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide (8-OH-DPAT, RBI/Semat); (±)-propranolol hydrocholride (RBI/Semat), spiroxatrine (RBI/Semat) and mianserin hydrochloride (RBI/Semat). The following drugs were generously donated: (±)-cyanopindolol (Sandoz); N-methyl-3-(1-methyl-4-piperidinyl)-1H-indole-5-ethane sulphonamide. HCl (GR 85548, Glaxo); N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide hydrochloride monohydrate (GR 127935, Glaxo); methysergide bimaleate (Sandoz); ketanserin tartrate (Janssen); sumatriptan succinate (GR 43175, Glaxo). Drugs were initially dissolved in distilled water, except spiroxatrine which was dissolved in dimethyl sulphoxide (DMSO). To dissolve GR 127935 in water, it was sometimes necessary to add a few drops of glacial acetic acid.

Statistical analysis

The action of an agonist was expressed as percentage change in MSR amplitude. The temporal dispersion of the reflex was not altered by agonists, so that peak height was assumed to be an appropiate measure of reflex depression (see also Deshpande & Warnick, 1988). The IC_{50} for an agonist was determined as the geometric mean of the IC_{50}s from individual concentration-depression curves and variability expressed as 95% confidence limits. Statistical comparisons were made by taking the IC_{50} of individual experiments before and after antagonist application using a paired *t*-test or by comparison of IC_{50} values from unpaired data using an unpaired *t* test. An apparent pA_2 was estimated from IC_{50} values and the Schild equation.

Results

Depression of MSR by 5-carboxamidotryptamine (5-CT)

The actions of 5-CT, the 5-HT receptor ligand most potent in depressing MSR (Crick & Wallis, 1991) were re-examined. Figure 1a shows the depression of the MSR by 3 nM 5-CT and the extent to which ketanserin (1 μM) blocked the depressant

action of a ten fold higher concentration of 5-CT. The antagonism of 5-CT by ketanserin had an interesting feature in that the prolonged depression of the MSR by 5-CT was reduced in duration in the presence of ketanserin (Figure 1b). This was also the case for blockade of 5-CT depressions by methiothepin (Figure 1c). In Figure 1b,c, responses have been matched to compare the effects of concentrations of 5-CT causing depressions of comparable magnitude. Depression of the MSR by 5-CT seemed to comprise an early phase more resistant to blockade by ketanserin or methiothepin than the later phase of depression. Table 1 shows that the durations and times to half-recovery were significantly shorter in the presence of ketanserin or methiothepin. Since higher concentrations of agonist had to be used when an antagonist was present, the more rapid occurrence of peak depression was to be expected. The reductions in the time course of 5-CT-induced depressions were not seen with depressions induced by other agonists, such as 5-HT, sumatriptan, 8-OH-DPAT or methysergide, in the presence of an antagonist.

Antagonism by ketanserin of different 5-HT receptor agonists

There were clear differences in the ability of ketanserin to block depressions of the MSR induced by 5-HT and 5-HT agonists. In confirmation of our earlier findings (Crick & Wallis, 1991), ketanserin (1 μM) had no significant effect on concentration-response (CR) curves to 5-HT (Figure 2a). There was, however, a clear antagonism of the action of 5-CT (Figure 2b) with a parallel displacement of the CR curve to the right, giving an apparent pA_2 of 6.8 ± 0.1 (mean $\pm$ s.e.mean, $n=4$). 5-CT in the concentrations used did not cause any measurable depolarization of motoneurones, as measured by the d.c. record from the ventral root. Ketanserin (1 μM) caused a greater rightward shift of the CR curve for methysergide, which was a full agonist on this tissue (Figure 2c). Full control CR curves to methysergide were not attempted because of the prolonged depressant action of 0.1 μM methysergide, which then made it difficult to construct a second curve in the presence of an antagonist. The apparent pA_2 for blockade of the action of methysergide by ketanserin was 7.9 ± 0.1 (mean $\pm$ s.e.mean, $n=4$).

Agonist actions of sumatriptan and GR 85548

In confirmation of a previous report (Crick *et al.*, 1994), sumatriptan was found to depress potently the MSR (IC_{50} 15.2 nM, 12.9–18.0 nM, $n=32$, geometric mean and 95% confidence limits). The inhibition by sumatriptan was readily blocked by ketanserin (1 μM) with a parallel displacement to the right of the CR curves (Figure 3a). In constructing control CR curves, higher concentrations of sumatriptan, e.g. 0.1 μM, were generally avoided because depression by the agonist persisted long after removal of the drug. The apparent

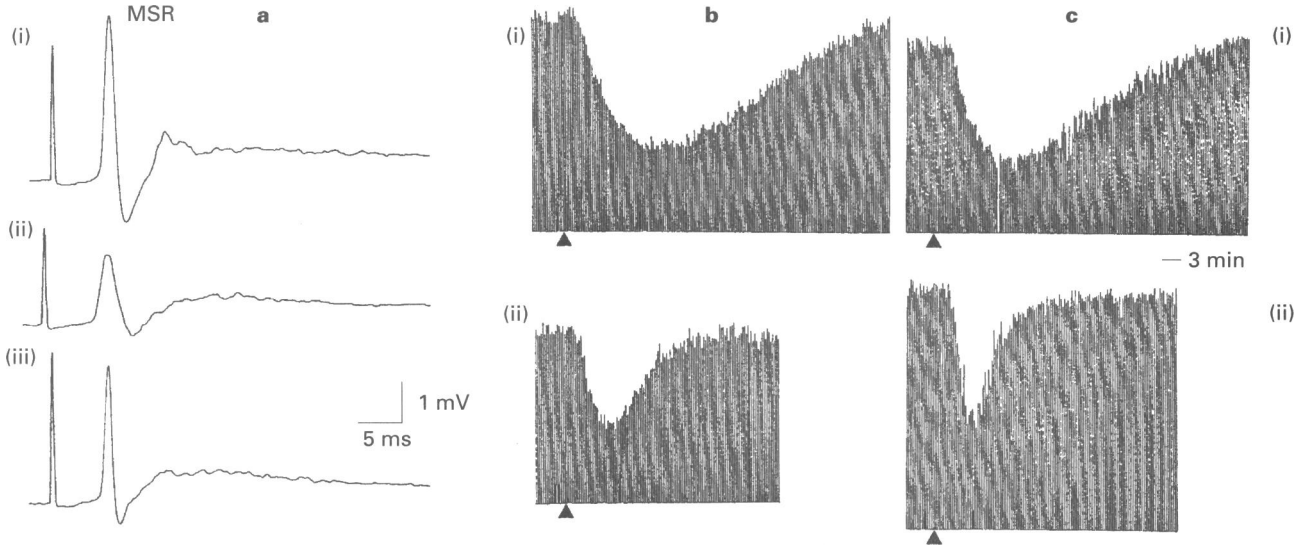

Figure 1 The depressant effect of 5-carboxamidotryptamine (5-CT) on the monosynaptic reflex (MSR) and its reversal by ketanserin and methiothepin. (a) Chart records from oscilloscope digital store of reflex response recorded by a suction electrode from a ventral root on stimulation (30 V, 0.1 ms) of the segmental dorsal root. The initial component following the stimulus artifact is the MSR. (i) Control, (ii) during maximal depression by 5-CT (3 nM), (iii) during maximal depression by 5-CT (30 nM) in the presence of ketanserin (1 μM). (b,c) Shortening of the time courses of depressions by the antagonists. Chart recorder traces of peak height detector output (arbitrary units) corresponding to peak voltage (2–4 mV) of each MSR generated at 15 s intervals. Agonist added at (▲). (b(i)) Response to 3 nM 5-CT, (b(ii)) response to 30 nM 5-CT in the presence of 1 μM ketanserin, (c(i)) response to 3 nM 5-CT, (c(ii)) response to 30 nM 5-CT in the presence of 1 μM methiothepin. (a) and (b) from the same, (c) from a different experiment.

Table 1 Duration of depressions of monosynaptic reflex (MSR) by 5-carboxamidotryptamine (5-CT) alone or in the presence of ketanserin (Ket) or methiothepin (Meth)

	Depression (%)	*Time to peak* (min)	$\frac{1}{2}$ *Recovery* (min)	*Duration* (min)	n
5-CT (10 nM)	69.3 ± 3.4	10.5 ± 0.7	17.7 ± 1.3	52.4 ± 3.1	6
5-CT (100 nM) + Ket (1 μM)	83.6 ± 3.0	4.9 ± 0.2***	8.0 ± 0.5***	25.6 ± 1.5***	4
5-CT (100 nM) + Meth (1 μM)	84.9 ± 2.4	4.9 ± 0.4***	5.5 ± 0.6***	21.3 ± 1.0***	4

Values are means ± s.e.mean. Values for ketanserin and methiothepin experiments compared with experiments with 5-CT alone. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's unpaired t test.

pA_2 for this action of ketanserin estimated from individual dose-ratios was 7.8 ± 0.1 (mean ± s.e. mean, $n=5$).

GR 85548 is a potent selective agonist at intracranial and other vascular 5-HT_{1D} receptors in the dog; it depresses neurally-induced contractions of the saphenous vein with an IC_{50} of 10 nM (Connor *et al.*, 1993). GR 85548 was potent in depressing the MSR (Figure 3b, c) with an IC_{50} of 18.4 nM (11.7–29.1, geometric mean and 95% confidence limits, $n=12$). The polysynaptic component of the reflex was not significantly depressed by either GR 85548 or sumatriptan. The depressant action of GR 85548 was antagonized by ketanserin (1 μM) and the CR curve displaced to the right in a parallel fashion (Figure 3b). The IC_{50} for GR 85548 in the presence of ketanserin was 710.2 nM (378.4–1333.5, geometric mean and 95% confidence limits) and the apparent pA_2 for the antagonism estimated from unpaired data was 7.6. Methiothepin (1 μM) also blocked the action of GR 85548 (Figure 3c). The IC_{50} was shifted from 18.4 nM (11.7–29.1, geometric mean and 95% confidence limits, $n=12$) to 908 nM (157–5260, geometric mean and 95% confidence limits, $n=5$, $P<0.001$). There was a parallel shift in the CR curve (Figure 3c) and the apparent pA_2 estimated from unpaired data was 7.7.

The potency displayed by ketanserin in antagonizing the effects of sumatriptan is consistent with an action at 5-HT_{2C} receptors (see Hoyer & Schoeffter, 1991). In five experiments, the effect of mianserin on depression of the MSR by sumatriptan was examined because this antagonist possesses a 40 fold selectivity for 5-HT_{2C} over 5-HT_{1D} receptors (pK_i 8.0 and 6.4, respectively, Hoyer & Schoeffter, 1991). Mianserin (0.3 μM) produced a small, non-parallel shift of the CR curve, the upper part of the curve being shifted to the right (not-illustrated). The IC_{50} for sumatriptan was significantly changed from 15.2 nM (12.9–18.0, $n=32$) to 32.5 nM (18.4–57.5, geometric means and 95% confidence limits, $n=5$, $P<0.01$, unpaired data).

Lack of blockade of sumatriptan by (±)-cyanopindolol, (±)-propranolol and spiroxatrine

Evidence was sought to establish whether sumatriptan was acting at 5-HT_{1A} or 5-HT_{1B} receptors; previously we had suggested that an effect at 5-HT_{1B} receptors was a plausible explanation (Crick *et al.*, 1994). In five experiments, the potent 5-$HT_{1A/1B}$ antagonist (±)-cyanopindolol, 0.1 μM, displayed no antagonist effect against sumatriptan, while in four experiments another 5-$HT_{1A/1B}$ antagonist, (±)-propranolol (0.5 to 1 μM) had no blocking action (not illustrated). (±)-Cyanopindolol itself, however, caused some reflex depression; 0.1 μM (±)-cyanopindolol depressed MSR by 27% (range 12 to 43%) in 5 experiments, and 1 μM (±)-cyanopindolol depressed MSR by 58% (range 52 to 64%) in 3 experiments. A selective 5-HT_{1A} antagonist, spiroxatrine (Nikam *et al.*, 1988), had no significant antagonist action against sumatriptan at a concentration of 1 μM, although in 5 of 8 experiments this high concentration caused some depression of the maximum (not illustrated).

Ketanserin antagonism of R(+)-8-OH-DPAT depression

Both the MSR (Saito *et al.*, 1982) and the synaptic response of motoneurones to stimulation of a dorsal root (Wu *et al.*, 1991) are known to be depressed by 8-OH-DPAT. The blockade of this action by spiperone is usually interpreted (see Wu *et al.*, 1991; Crick *et al.*, 1994) as interaction with 5-HT_{1A} receptors. However, as can been seen from Figure 4, this interpretation may be incorrect, since R (+)-8-OH-DPAT depression of the MSR was unaffected by the selective 5-HT_{1A} antagonist, spiroxatrine (0.1 μM), but very effectively antagonized by ketanserin (0.1 μM). Ketanserin caused a parallel shift of the CR curve to the right and the apparent pA_2 was 8.3 ± 0.1 (mean ± s.e. mean, $n=3$).

GR 127935

Confirmation of the involvement of 5-HT_{1D} receptors was sought by use of the novel, potent 5-HT_{1D} antagonist, GR 127935 (Skingle *et al.*, 1993). Agonist action was a substantial problem in assessing any antagonism of sumatriptan. Over the concentration-range 30 nM to 1 μM, there was a concentration-

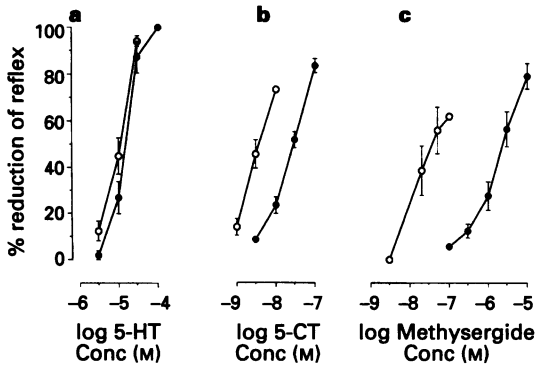

Figure 2 Effect of ketanserin on depression of the monosynaptic reflex (MSR) by 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT) and methysergide. (○) Controls and (●) after incubation with ketanserin (1 μM) for at least 1 h. Points show mean depression measured from point of maximal depression with s.e. mean. (a) 5-HT: The IC_{50} for the control curve was 10.9 μM (6.7–17.7 μM) and for the curve in the presence of ketanserin 15.3 μM (8.6–27.2 μM, $n=4$, geometric means and 95% confidence limits, $P>0.05$). (b) 5-CT: The IC_{50} for the control curve was 3.6 nM (2.1–6.2 nM) and for the curve in the presence of ketanserin 27.2 nM (21.0–35.2 nM, $n=4$, $P<0.001$). (c) Methysergide: The IC_{50} for the control curve was 29.8 nM (10.2–87.1 nM) and for the curve in the presence of ketanserin 2366 nM (1059–5297 nM, $n=4$, $P<0.001$).

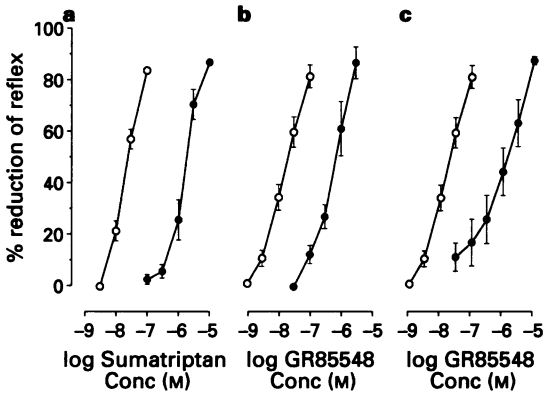

Figure 3 Antagonism by ketanserin of the depressions of the monosynaptic reflex (MSR) by sumatriptan and N-methyl-3-(1-methyl-4-piperidinyl)-1H-indole-5-ethane sulphonamide (GR 85548) and antagonism by methiothepin of the action of GR 85548. Pooled data, (○) controls, (●) after incubation with the antagonist for at least 1 h. Points show mean depression with ± s.e. mean. (a) Antagonism of the action of sumatriptan by ketanserin (1 μM). Pooled data from 5 experiments. The IC_{50} for the control curve was 24.3 nM (18.1–32.7 nM) and for the curve in the presence of ketanserin 1.6 μM (0.9–2.7 μM, geometric means and 95% confidence limits, $P<0.001$). (b) Antagonism of the action of GR 85548 by ketanserin (1 μM). The control data (○) were pooled from 12 experiments and (●) show data pooled from 5 experiments in the presence of ketanserin. The IC_{50} for the control curve was 18.4 nM (11.7–29.1 nM) and for the curve in the presence of ketanserin 710.2 nM (378.4–1333.5 nM, geometric means and 95% confidence limits, $P<0.001$). (c) Antagonisim of the action of GR 85548 by methiothepin (1 μM). Control curve shows pooled data from 12 experiments; 5 preparations were subsequently incubated with methiothepin (1 μM) for at least 1 h to give curve represented by (●). The IC_{50} for the control curve was 18.4 nM (11.7–29.1 nM) and for the curve in the presence of methiothepin 908 nM (157–5,260 nM, geometric means and 95% confidence limits, $P<0.001$).

related depression of the MSR from about 10% to about 50%. The depression was usually slow in onset and gradually increased over a period of 1 to 2h. An initial depression (up to 60%) and recovery, before the depression of slow onset, was seen in a minority of preparations. In one of these experiments (see Figure 5), ketanserin (1 μM) was also applied and was found to reverse the depression by GR 127935. In eleven trials with these concentrations of GR 127935 an apparent antagonism was seen on only one occasion despite an incubation time of up to 2 h. Lower concentrations of GR 127935 (1–3 nM) were also tested in 9 experiments and found not to depress responses to 15 nM or 30 nM sumatriptan.

Absence of direct effects on motoneurones

None of the agents tested caused a depolarization or a hyperpolarization of motoneurones measurable from the ventral root, unlike 5-HT itself which caused depolarizations of around 1 mV (not illustrated, see Wallis *et al.*, 1991).

Discussion

The results add further weight to the view that 5-HT receptor agonists depress MSR without changing motoneurone membrane potential. Further, the rank order of potency for MN depolarization (5-HT > α-methyl-5-hydroxytryptamine (α-Me-5-HT) > 5-CT > 5-methoxytryptamine (5-MeOT) > > 8-OH-DPAT, sumatriptan, methysergide, Wallis *et al.*, 1991) is very different from the rank order for reflex depression (5-CT > sumatriptan > methysergide > 5-HT > 8-OH-DPAT > 5-MeOT > α-Me-5-HT, Crick *et al.*, 1994). Nevertheless, the EC_{50} for 5-HT depolarization is 1.4 μM (Elliott & Wallis, 1992), while the IC_{50} for 5-HT depression of MSR is 30 nM (Crick & Wallis, 1991).

The finding that the action of several 5-HT receptor agonists was readily blocked by ketanserin should help clarify the nature of the receptors through which reflex depression is achieved. In particular, ketanserin displayed a surmountable antagonism of sumatriptan (apparent pA_2 7.8), of GR 85548 (apparent pA_2 7.6), of methysergide (apparent pA_2 7.9) and of 8-OH-DPAT (apparent pA_2 8.3). There was also a surmountable antagonism of the depressant action of 5-CT, but a somewhat lower pA_2 was estimated (6.8). The later phase of depression by 5-CT appeared to be blocked more readily by ketanserin or methiothepin than the early phase of (peak) depression. As with the other agonists, antagonism was routinley measured at peak depression. It is possible that depression by 5-CT comprised a ketanserin-sensitive and a ketanserin-insensitive component, and similarly a methiothepin-senstive and a methiothepin-insensitive component.

The ability of ketanserin to antagonize a number of 5-HT agonists, which are relatively selective for different 5-HT receptor subtypes, suggested that reflex depression might be mediated by a single subtype activated by the various agonists. Thus, we considered whether any of the 5-HT_1 receptor subtypes or the recently described 5-ht_5, 5-ht_6, or 5-ht_7 receptors could be mediating reflex depression. The observed high affinity of sumatriptan ruled out many of these. Sumatriptan has a high affinity for 5-HT_{1D} and 5-HT_{1B} receptors (Hoyer *et al.*, 1994) with a pK_i at rat 5-HT_{1D} and 5-HT_{1B} receptors expressed in COS-7 cells of 8.0 and 7.35, respectively (Hamblin *et al.*, 1992); at 5-ht_{1F} receptors the pK_i is 7.2 (Boess & Martin, 1994). Crick *et al.* (1994) estimated a pK_A of 7.9 for sumatriptan as a depressant of the MSR in rat spinal cord (cf. pIC_{50} 7.8 this paper). Previously it had been proposed that sumatriptan may activate either 5-HT_{1B} or 5-HT_{1D} receptors in rat spinal cord (Hendrikse *et al.*, 1992; Crick *et al.*, 1994). Since a pK_i of less than 6.0 is reported for 5-HT_{1A}, 5-ht_{1E} and 5-ht_{5B} receptors and less than 5.0 for 5-HT_{2A}, 5-HT_{2C}, 5-HT_4 and 5-ht_{5A} receptors (data from Hoyer & Schoeffter, 1991; Boess & Martin, 1994), these receptors may be eliminated. No value for 5-ht_6 receptors is given in these papers, but ketanserin has a pK_i less than 5.0 at 5-ht_6 receptors. The 5-ht_7 receptor (pK_i for sumatriptan 6–7, Boess & Martin, 1994) also seems an unlikely candidate amongst recently cloned receptors.

The affinity of 5-ht_{1F} receptors for both ketanserin and methiothepin is less than 6.0 (Boess & Martin, 1994). Since the action of sumatriptan was blocked by ketanserin with a pA_2 of 7.8 and by methiothepin with a pA_2 of 7.4 methiothepin (Crick *et al.*, 1994, Table 2), 5-ht_{1F} receptors cannot be involved. Although the 5-ht_7 receptor is expressed in the rat central nervous system (Lovenberg *et al.*, 1993a,b), its relatively low affinity for ketanserin (6.7) and high affinity for methiothepin (9.0, Hoyer *et al.*, 1994) do not match our data (see Table 2). Further, the pK_D of 6.2 for sumatriptan and the reported lack of agonist activity of sumatriptan at 5-ht_7 receptors at 10 μM (Hoyer *et al.*, 1994) eliminate this receptor as a likely candidate.

Thus, 5-HT_{1D} or 5-HT_{1B} receptors remain the most likely mediators of MSR depression. It was previously thought that

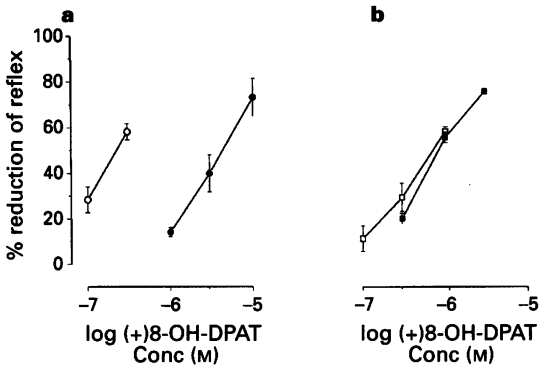

Figure 4 The depression of the monosynaptic reflex (MSR) by R (+)-8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) was antagonized by ketanserin but not by spiroxatrine. Pooled data, (○) controls and (●) after incubation with the antagonist for at least 1 h. Points show mean depression with ± s.e. mean. (a) Antagonism of the action of 8-OH-DPAT by ketanserin (0.1 μM). Pooled data from 3 experiments. The IC_{50} for the control curve was 0.21 μM (0.11–0.43 μM) and for the curve in the presence of ketanserin 4.0 μM (1.37–11.9 μM, geometric means and 95% confidence limits, $P<0.001$). (b) Failure to antagonize the action of 8-OH-DPAT with spiroxatrine (0.1 μM). Pooled data from 4 experiments. The IC_{50} for the control curve was 0.61 μM (0.31–1.2 μM) and for the curve in the presence of spiroxatrine 0.82 μM (0.62–1.07 μM, geometric means and 95% confidence limits).

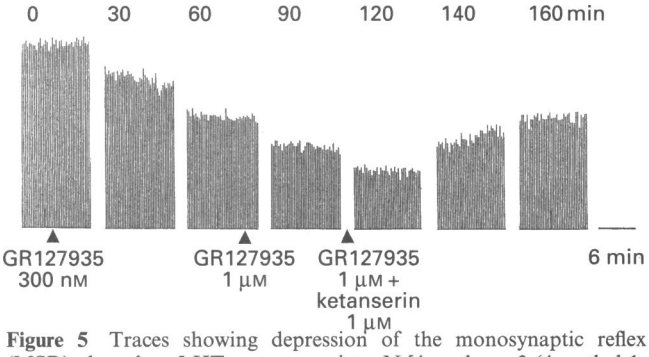

Figure 5 Traces showing depression of the monosynaptic reflex (MSR) by the 5-HT_{1D} antagonist, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide (GR 127935) and reversal of the depression by ketanserin. Chart recorder traces of peak height detector output (arbitrary units) corresponding to peak voltage of each MSR (initially 1.5 mV) generated at 15 s intervals. Selected portions of the trace at the times (min) indicated illustrate the control and point of application of GR 127935, depression of the MSR by GR 127935 (300 nM), further depression by GR 127935 (1 μM) and recovery of the response in the presence of GR 127935 (1 μM) plus ketanserin (1 μM).

5-HT_{1B} and 5-HT_{1D} receptors represented species variants controlled by a single gene, 5-HT_{1B} receptors being expressed in rodents and 5-HT_{1D} receptors in other species (Hoyer & Middlemiss, 1989). However, both 5-HT_{1B} and 5-HT_{1D} recognition sites are present in rat brain (Herrick-Davis & Titeler, 1988; Bruinvels *et al.*, 1992) and distinct 5-HT_{1B} and 5-HT_{1D} receptor genes have been identified (Hamblin *et al.*, 1992). In man, two genes encode two receptors with 5-HT_{1D} pharmacology (Hamblin *et al.*, 1992; see Hoyer *et al.*, 1994), which Weinshank *et al.* (1992) have termed 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. The rat 5-HT_{1D} receptor has an almost identical pharmacological profile to the human 5-$HT_{1D\alpha}$ receptor and *in situ* hybridization histochemistry has demonstrated its presence in rat brain, e.g. dorsal raphe, basal ganglia, vestibular nucleus, etc. (Hamblin *et al.*, 1992; Bruinvels *et al.*, 1994). Both sumatriptan and GR 85548, high affinity ligands for 5-HT_{1D} receptors, blocked the MSR at nanomolar concentrations. This does not suggest a 5-HT_{1B} action since the affinity (pK_i) of sumatriptan for 5-HT_{1B} receptors is 6.4–6.8 (Van Wijngaarden *et al.*, 1990; Hoyer & Schoeffter, 1991). Further, Bruinvels *et al.* (1992) report a pK_D of 7.8 for sumatriptan binding to 5-HT_{1D} receptors in rat brain, the same as our value (Table 2). Both sumatriptan and GR 85548 were antagonized by ketanserin, an antagonist thought to allow discrimination between 5-HT_{1B} and 5-HT_{1D} effects. Ketanserin has a similarly low affinity for 5-HT_{1B} and 5-$HT_{1D\beta}$ receptors ($pK_i < 6.0$ for both receptors, Boess & Martin, 1994). For human and rat 5-$HT_{1D\alpha}$ receptors there may be differences in affinity depending on the expression system, e.g. $pK_i < 6.0$ in COS-7 or CHO cells (Boess & Martin, 1994), but for receptors expressed in HEK 293 cells a pK_d of 7.0–7.5 was determined (Kaumann *et al.*, 1993). The latter values are consistent with pA_2 values estimated for rabbit basilar artery (7.3, Tilford & Baxter, 1994) and with the apparent pA_2 determined in the present experiments of 7.6–7.8 (summarized in Table 2). Thus, the data suggest an interaction of sumatriptan and GR 85548 with 5-$HT_{1D\alpha}$ receptors.

May methysergide and 8-OH-DPAT activate the same receptors to depress the MSR? Methysergide was a full agonist with a pIC_{50} of 7.5 (cf. pK_i 8.2 at rat cloned 5-$HT_{1D\alpha}$ receptors, Bach *et al.*, 1993, Table 2) and was antagonized by ketanserin with a pA_2 consistent with mediation by 5-$HT_{1D\alpha}$ receptors. The pIC_{50} for depression of MSR by 8-OH-DPAT was 6.2–6.7 (see Figure 4), whereas the pEC_{50} at 5-HT_{1A} receptors is 8.2 (Hoyer *et al.*, 1994) and the pK_i at rat 5-$HT_{1D\alpha}$ receptors is 7.0 (Hamblin *et al.*, 1992). Although 8-OH-DPAT was blocked by spiperone (pA_2 6.3, Crick *et al.*, 1994), this ligand is not slective at μmolar concentrations. Thus, spiperone displays a pK_B at 5-HT_{1A} receptors of 7.2 (Hoyer & Schoeffter, 1991) and an identical pK_i at rat 5-$HT_{1D\alpha}$ receptors (Hamblin *et al.*, 1992). Crucially, the selective 5-HT_{1A} antagonist, spiroxatrine, failed to block the action of 8-OH-DPAT, whereas ketanserin was an effective competitive antagonist with an apparent pA_2 of 8.3 (Table 2). Although a high value, this can be interpreted as interaction with 5-$HT_{1D\alpha}$ receptors. Since the depressant action of 5-methoxytryptamine (5-MeOT) on MSR is also blocked by ketanserin (pA_2 7.0, Crick *et al.*, 1994), this might also be an action at 5-$HT_{1D\alpha}$ receptors.

Methiothepin was an effective, competitive antagonist of the depressant actions of GR 85548 and sumatriptan on the MSR. An apparent pA_2 of 7.7 was estimated for antagonism of GR 85548, while we previously reported an apparent pA_2 of 7.4 for antagonism of sumatriptan by methiothepin (Table 2). Methiothepin is a potent but non-selective antagonist at 5-HT_1 and 5-HT_2 receptors (Hoyer *et al.*, 1994), but the reported affinity values for 5-HT_{1D} receptors vary substantially for different species and tissues (Wilkinson & Middlemiss, 1992). From functional studies using human tissue, the estimated pA_2 for blockade of 5-HT_{1D}-evoked responses ranged from 7.3 (Bax *et al.*, 1992) to 8.8 (Parsons *et al.*, 1989). Thus the actions of methiothepin against sumatriptan and GR 85548 are also consistent with blockade of 5-$HT_{1D\alpha}$ receptors. Further, mianserin, which at a concentration of 0.3 μM would be expected to cause substantial blockade of 5-HT_{2C}-mediated responses but have only small effects on 5-HT_{1D}-mediated effects, displayed only weak antagonism against sumatriptan consistent with a 5-HT_{1D} effect.

By elimination it can be deduced that MSR depression elicited by sumatriptan, GR 85548, methysergide, 8-OH-DPAT, and perhaps 5-MeOT, is likely to be mediated by 5-$HT_{1D\alpha}$ receptors. However, the potent and selective 5-HT_{1D} antagonist, GR 127935, failed to produce any unequivocal antagonism at concentrations ranging from 1 nM to 1 μM, but did display agonist properties at the higher concentrations tested. Agonist properties have been reported for GR 127935 at human cloned 5-$HT_{1D\alpha}$ receptors (Pauwels & Golpaert, 1995; Watson *et al.*, 1995). The long incubation times necessary for full equilibration with the tissue (Skingle *et al.*, 1995) make it difficult to assess antagonism adequately in isolated spinal cord. Nevertheless, the agent is an effective 5-HT_{1D} antagonist in rat central nervous system. Recently, GR 127935 (0.05 μM) has been shown to be an antagonist of sumatriptan in rat brain slices but not to block 5-HT_{1B} autoreceptors (Davidson & Stamford, 1995). Given the crucial importance of GR 127935 as an identifying ligand, the identity of the ketanserin-sensitive receptor as a 5-$HT_{1D\alpha}$ receptor must remain unconfirmed. For convenience it will be referred to as '5-HT_{1D}-like' below.

Actions of 5-HT

If 5-HT_{1D}-like receptors were to mediate depression of MSR, a confusion in the literature regarding the action of descending

Table 2 Potency of 5-HT receptor agonists in depressing the monosynaptic reflex and apparent pA_2 values for antagonists compared with reported affinity at 5-$HT_{1D\alpha}$ receptors

Agent	*Agonist* (pIC_{50})	*Antagonist* (pA_2)	*Rat 1Dα* (pK_i)	*Human 1Dα* (pK_i)
Sumatriptan	7.9[a]	–	8.0[b]	8.5[c]
Methiothepin	–	7.4[a]	9.2[b]	7.3–8.8[d,e]
Ketanserin	–	7.8	7.3[f]	<6.0[g]
GR 85548	7.7	–	[dog 7.0–8.0][h]	–
Methiothepin	–	7.7	9.2[b]	7.3–8.8[d,e]
Ketanserin	–	7.6	7.3[f]	<6.0[g]
Methysergide	7.5	–	8.2[i]	8.4[c]
Ketanserin	–	7.9	7.3[f]	<6.0[g]
5-CT	8.4	–	9.4[b]	9.1[c]
Ketanserin	–	6.8	7.3[f]	<6.0[g]
8-OH-DPAT	6.2–6.7	–	7.0[b]	6.9[c]
Ketanserin	–	8.3	7.3[f]	<6.0[g]
Spiperone	–	6.3[a]	7.2[b]	6.0[c]

[a]Crick *et al.*, 1994; [b]Hamblin *et al.*, 1992; [c]Weinshank *et al.*, 1992; [d]Bax *et al.*, 1992; [e]Parsons *et al.*, 1989; [f]Tilford & Baxter, 1994; [g]Boess & Martin, 1994; [h]Connor *et al.*, 1993; [i]Bach *et al.*, 1993.

5-HT-containing pathways might be explained. The long-lasting inhibition of MSR evoked on stimulation of later-oventral descending pathways to the ventral horn (Miyata *et al.*, 1987; Yomono *et al.*, 1992) is reportedly due to 5-HT acting at 5-$HT_{2A/2C}$ receptors (Yomono *et al.*, 1992; Wallis *et al.*, 1993a,b), despite the presence on motoneurones of 5-HT_{2A} receptors increasing cell excitability (Jackson & White, 1990; Wang & Dun, 1990; Elliott & Wallis, 1992). The antagonists which block this endogenous 5-HT-mediated inhibition (ketanserin, ritanserin, spiperone, methiothepin and yohimbine) would be capable of blocking 5-HT_{1D}-like receptors (Table 2) at the concentrations used (usually 1 μM). The pK_i for ritanserin at 5-$HT_{1D\alpha}$ receptors is 8.8 (Bach *et al.*, 1993) and for yohimbine 7.5 (Hamblin *et al.*, 1992). Wallis *et al.* (1993a) used several concentrations of ketanserin in an attempt to assess its potency against endogenous inhibition elicited from later-oventral tracts. The pIC_{50} value for ketanserin in blocking this descending inhibition ranged from 6.9 to 7.5, consistent with an action at 5-HT_{1D}-like receptors. Further, several agents which failed to block descending inhibition, e.g. ICS 205-930 and mesulergine (both at 1 μM, Yomono *et al.*, 1992), propranolol (0.1 μM, Wallis *et al.*, 1993a) display pK_i values < 6.0 at 5-$HT_{1D\alpha}$ receptors (Boess & Martin, 1994).

A second aspect of depression of MSR by indoleamines remains unresolved. The receptors through which applied 5-HT acts to depress the reflex are not 5-HT_{1D}-like (Crick *et al.*, 1994), since a combination of ketanserin (1 μM), methiothepin (1 μM) and ondansetron (1 μM) failed to block them. The present paper again demonstrates the resistance of the 5-HT action to ketanserin and shows that a component of the action of 5-CT may also be resistant. Crick *et al.*, (1994) concluded that applied 5-HT could not be acting via 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 receptors and mediation by a novel receptor was a possibility. Although 5-HT released from tryptaminergic projections may activate synaptic 5-HT_{1D}-like receptors, the high affinity uptake system in tryptaminergic terminals may prevent applied 5-HT from reaching these receptors in an effective concentration (Crick *et al.*, 1994). Thus, the uncharacterized or novel 5-HT receptor may be located remote from the synapse, possibly on primary afferent terminals.

The possibility that applied 5-HT might release an inhibitory transmitter has been explored previously (Crick & Wallis, 1991; Wallis *et al.*, 1993a). Although this question was not fully resolved, we concluded that mediation by glycine, $GABA_A$ and NMDA receptors, or release of enkephalins or noradrenaline were not responsible for the depression by 5-HT.

In conclusion these results suggest that sumatriptan, GR 85548, methysergide and 8-OH-DPAT, as well as descending tryptaminergic pathways, depress the MSR in the neonate rat spinal cord via receptors which have some similarities to 5-$HT_{1D\alpha}$ receptors, but are not blocked by the selective 5-HT_{1D} ligand, GR 127935. 5-HT applied to the cord may act via a different, possibly novel receptor to inhibit the MSR.

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