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Voltage-dependent block of NMDA responses by 5-HT agonists in ventral spinal cord neurones

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1 Modulation by 5-hydroxytryptamine receptor agonists of the NMDA responses of ventral spinal cord neurones was studied by use of the whole-cell patch-clamp technique.

2 In a Mg-free solution containing tetrodotoxin and glycine, 5-hydroxytryptamine (5-HT, 10–100 μM) reduced the NMDA response, the block increasing with hyperpolarization. Kainate responses were little affected.

3 Some classical agonists of 5-HT receptors induced similar blocking effects. At 10 μM, both a selective agonist of 5-HT_2 receptors, (±)-2,5-dimethoxy-4 iodo amphetamine (DOI), and a selective agonist of some 5-HT_1 receptors, (±)-8-hydroxy-2(n-dipropyl amino) tetralin (8-OH-DPAT), induced pronounced blocking effects, of 48% and 33% respectively at −100 mV, whereas another 5-HT_1 agonist, 5-carboxamidotryptamine (5-CT) was ineffective. At 100 μM, 5-methoxytryptamine (5-MeOT) induced a complete block of the NMDA responses recorded at −100 mV. The order of potency was: 5-MeOT ≃ DOI > 8-OH-DPAT > 5-HT > 5-CT.

4 Neither spiperone nor ketanserin (1 μM) prevented the blocking effect of 5-HT or DOI.

5 Prolonged preincubations with 5-HT did not block the response if NMDA was applied without 5-HT. When 5-HT agonists were applied both by preincubation and with NMDA, the degree of block increased during the NMDA application.

6 Lowering the NMDA concentration (from 100 to 20 μM) slightly decreased the blocking effect of 5-MeOT.

7 External Mg^{2+} ions (1 mM) also reduced the blocking effects of 5-HT and 5-MeOT.

8 The blocking effects described appear to be independent of classical 5-HT receptors. Their voltage-dependence suggests a mechanism of open channel block consistent with all the results obtained.

Keywords: NMDA; 5-HT; motoneurones; DOI; 8-OH-DPAT

Introduction

Glutamate appears to be the transmitter mediating excitatory postsynaptic potentials of rat motoneurones in response to dorsal root stimulation (see Ziskind-Conhaim, 1990 and references therein). At early stages of development, in day 16 rat embryos, sensorimotor synaptic transmission seems to be mediated mostly by NMDA receptors (Ziskind-Conhaim, 1990; see also Kalb *et al.*, 1992) showing the transient expression of NMDA receptors in the developing spinal cord ventral horn. At later stages, AMPA/low-affinity kainate receptors are also clearly involved (see Jahr & Yoshioka, 1986; Konnerth *et al.*, 1990).

The ventral horn of adult mammalian spinal cord is known to receive 5-hydroxytryptaminergic (5-HT) projections (from the raphe nuclei and medial reticular formation of the medulla). Both 5-HT projections and electrophysiological responses to 5-HT have been observed early during development in the rat ventral spinal cord (Ziskind-Conhaim *et al.*, 1993). It has been reported that 5-HT is the mediator of a slow excitatory postsynaptic potential (e.p.s.p.) in a population of motoneurones (Wang & Dun, 1990) and several studies have shown both excitatory and inhibitory effects of 5-HT on lamprey spinal cord neurones (Wallen *et al.*, 1989) and on turtle or rat motoneurones (see Hounsgaard & Kiehn, 1989; Wang & Dun, 1990; Takahashi & Berger, 1990; Berger & Takahashi, 1990; Berger *et al.*, 1992; Elliott & Wallis, 1992; Larkman & Kelly, 1992; Garratt *et al.*, 1993; Ziskind-Conhaim *et al.*, 1993).

In the lamprey spinal cord, NMDA-gated channels are required for generating the bursting patterns involved in the execution of regular movements (Grillner & Matsushima, 1991) and 5-HT modulates the central pattern generator for locomotion (Harris-Warrick & Cohen, 1985). In the neonatal rat spinal cord, both NMDA and 5-HT are able to induce fictive locomotion (Kudo & Yamada, 1987; Smith & Feldman, 1987; Cazalets *et al.*, 1992; Cowley & Schmidt, 1994).

5-HT has long been known to facilitate synaptically or glutamate-induced excitation of adult facial (McCall & Aghajanian, 1979) and spinal (White & Neuman, 1980) mammalian motoneurones. This facilitatory effect of 5-HT partly results from a generalised increase in excitability of motoneurones (VanderMaelen & Aghajanian, 1980). In contrast, in immature rat motoneurones and in lamprey motoneurones, negative modulatory effects of 5-HT have been observed and were shown to be presynaptic (Wu *et al.*, 1991; Buchanan & Grillner, 1991). In embryonic rat motoneurones, a series of various 5-HT agonists (in the 10–100 μM concentration range) has been shown to depress the amplitude of dorsal root-evoked potentials (Ziskind-Conhaim *et al.*, 1993); however, the origin of this effect, pre- or postsynaptic, has not been established.

Interactions between 5-HT and glutamate agonists have also been investigated at the postsynaptic level on various types of motoneurones. 5-HT (25–50 μM) has been reported occasionally to enhance glutamate-induced depolarization in immature rat motoneurones (Wu *et al.*, 1991). In frog spinal cord, different results have been obtained according to the receptor-specificity of the 5-HT agonist used. Whereas low concentrations of the 5-HT_1 agonist, 8-OH-DPAT (0.01 μM) could potentiate NMDA responses (Holohean *et al.*, 1992a), high concentrations of 5-HT (>10 μM) reduced both synaptic

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ventral root potentials and postsynaptic responses to NMDA and kainate in the presence of TTX (Holohean *et al.*, 1992b). The recent demonstration that 5-HT and glutamate are co-released by single raphe neurones (Johnson, 1994) further stimulates this research area.

In the present study, performed in cultured neurones from embryonic rat ventral spinal cord, using voltage-clamp whole-cell recording and extracellular fast perfusion, we investigated modulations by various 5-HT receptor agonists of the response to exogenous NMDA applications.

Methods

The experiments were performed at room temperature (20–23°C) on primary cultures of ventral spinal cord neurones from rat embryos, by use of the whole-cell configuration of the patch-clamp technique.

Cell preparation

The spinal cord of E16 rat embryos (OFA, Iffa Credo) was taken out and dissected under a microscope in a phosphate buffer saline (PBS without Ca and Mg, Gibco) supplemented with glucose (33 mM). The meninges were first removed. Then, in order to increase the proportion of motoneurones, the ventral part of the spinal cord was separated from the dorsal part and cut in small pieces in order to be dissociated as described below.

The tissues were first incubated for 15 min at 37°C in PBS-glucose containing 10% trypsin-EDTA (10× from Gibco). After three successive washes with PBS-glucose, the tissues were further incubated for 15 min at 37°C in PBS-glucose containing dexoyribonuclease I (DN-25 from Sigma, 0.03 mg ml^{-1}), $MgCl_2$ (0.1 mg ml^{-1}) and bovine serum albumin (A2058 from Sigma, 1 mg ml^{-1}). This step was followed by gentle mechanical dissociation. The cells were then washed in PBS-glucose by two successive centrifugations (7 min at 700 r.p.m.) and the pellet was resuspended in 1 ml of culture medium (DMEM-F12 from Gibco supplemented with 2 mM glutamine, 10 mM HEPES buffer, 9 mM sodium bicarbonate, 33 mM glucose, 5 iu ml^{-1} penicillin/streptomycin from Specia and 0.25 μg ml^{-1} fungizone from Gibco).

The cells were seeded at a density of $1-2 \times 10^5$ cells per dish on the centre of 35 mm dishes which had been precoated first with polyornithine (1.5 μg ml^{-1} for one night at 37°C followed by three washes) and then with 20% foetal calf serum (in culture medium, for 1 h at 37°C followed by two washes). Cells were allowed to settle for 15 to 30 min at 37°C. Then 1 ml of complete culture medium containing 5% foetal calf serum and 5% horse serum (heat-inactivated Gibco) was added to each dish. Between days 5 and 7, the cells were treated with 5-fluoro-2'-deoxyuridine-5'-monophosphate (FUDR) (12 μM) and uridine (40 μM).

The cells were used for electrical recording between day 11 and day 15.

Clearly, our cultures contained various types of neurones, including interneurones. In order to increase our chances of recording from motoneurones, we usually selected the largest neurones in the dish (showing a cell body diameter $\geqslant 20$ μm). In any case, the results described below were reproducible and did not appear to depend on the type of neurone used.

Experimental solutions and drugs

Before recording, the culture medium was replaced by the external solution to be used during the recording. In most experiments this solution was the '0 Mg' external solution containing (in mM): NaCl 160, KCl 2.5, $CaCl_2$ 1.8 and HEPES-NaOH 10, pH 7.4. Some experiments were also performed with the '1 Mg' external solution containing in addition 1 mM $MgCl_2$, or with the '1 Mg + glucose' external solution containing (in mM): NaCl 150, glucose 20, KCl 2.5, $CaCl_2$ 1.8, $MgCl_2$ 1 and HEPES-NaOH 10. The internal solution used to fill the recording electrode contained (in mM): Cs methanesulphonate 145, CsCl 15, $MgCl_2$ 1, EGTA 0.1, ATP-Mg 3, GTP-Na 0.3 and HEPES-CsOH 10, pH 7.2.

A stock solution of 1 mM 5-HT-creatinine sulphate (Sigma) or 10 mM 5-HT-hydrochloride (RBI) was prepared in the external solution plus tetrodotoxin (TTX) (0.2 μM) and glycine (1 or 10 μM). Stock solutions of 5-HT oxalate (Sigma), 5-methoxytryptamine hydrochloride (5-MeOT, RBI), 5-carboxamido-tryptamine maleate (5-CT, RBI), (±) 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT, Sigma) were prepared at 10 mM in distilled water. (±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI, RBI) was dissolved at 20 mM in distilled water. Ketanserin tartate (RBI) was dissolved at 1 mM in distilled water and spiperone (Sigma) was dissolved at 0.66 mM in 10 mM HCl. Stocks of 5-HT and 5-HT receptor agonists were prepared daily.

Perfusion system

The culture dish was continuously perfused with the external solution. In addition, a fast perfusion system usually made up of 4 to 6 identical and parallel barrels (made of glass and Teflon tubing, each barrel connected to a glass syringe via a Teflon tap) was used for rapid application of NMDA and 5-HT-agonists. The recorded cell was continuously perfused with one of these barrels (solution flowing by gravity) and the fast perfusion system was moved laterally to apply the desired solution to the cell. Lateral movements of this system were controlled by a computer-driven motor. The control barrel contained the external solution plus tetrodotoxin (0.2 μM) and glycine (either 1 or 10 μM). Other barrels contained in addition NMDA (100 μM) or a 5-HT receptor agonist or a 5-HT receptor agonist plus NMDA.

In some experiments (such as that of Figure 7), the fast perfusion system was made up of only two glass barrels and each of these barrels could be used to apply two different solutions into the recording chamber (barrel 1 could be used to apply either the control solution or e.g. 5-HT, whereas barrel 2 could be used to apply NMDA or e.g. NMDA + 5-HT). Two identical Teflon valves were used (one for each barrel); each valve had two inputs and two outputs, and any one of the two input solutions could flow either through the output connected to the glass barrel into the recording chamber, or through the second output into another chamber. Thus, all solutions flowed continuously and the lateral movement made in order to apply NMDA was always exactly the same whether or not e.g. 5-HT was present, avoiding any possible mechanical artefact.

Recording

Patch-clamp micropipettes were made from hard glass (Kimax 51); the shank of each pipette was covered with Sylgard and the tip was fire-polished. The resistance of these electrodes filled with the internal solution was between 5 and 10 MΩ. The cells were voltage-clamped by an EPC7 List amplifier, controlled by a TANDON 38620 computer, via a Cambridge Electronic Design (CED) 1401 interface, using CED patch- and voltage-clamp software. The current monitor output of the amplifier was filtered at 0.3 kHz before being sampled on-line at 0.6 kHz. The bath was connected to the ground via an agar bridge.

The series resistance (*Rs*) was systematically measured several times during each experiment. Particular care was taken to eliminate experiments in which *Rs* changed suddenly. *Rs* was between 10 and 20 MΩ. These values are high enough to introduce a difference of a few mV between the voltage applied to the electrode and that actually applied to the inside of the cell (error of maximum 10 mV for the largest responses). However, the current modulations observed occurred without any simultaneous change of *Rs* and thus cannot result from changes in the applied voltage.

Results

Voltage-dependent block of NMDA responses by 5-HT

The response to an application of NMDA (100 μM) of 2 s duration was tested every 40 s in the continuous presence of a saturating concentration of glycine (1 or 10 μM) in the 0 Mg extracellular solution (see Methods). The holding potential was usually −60 mV. In order to measure the NMDA response at −100 mV, in a series of experiments (such as that of Figure 1a), a 4 s long voltage-jump to −100 mV was applied a few hundreds of ms before each NMDA application. In some other experiments (such as that of Figure 1b and c), the cell was held at −100 mV for several minutes; however, this often resulted in a progressive deterioration of the cell.

When the control NMDA response had been repetitively measured at either −60 mV or −100 mV (using the control and NMDA barrels of the fast perfusion system: see Methods), the effects of 5-HT on the basal current and on the NMDA response were tested by alternately perfusing the cell with the barrel containing 5-HT and with the barrel containing 5-HT+NMDA. For concentrations of 5-HT between 10 and 100 μM, a dose-dependent reduction of the NMDA response was observed. In most cells, the current recorded in the absence of NMDA was not affected by 5-HT under our experimental conditions (see also Figure 2). This can be explained by the fact that the internal solution that we used was K^+-free (Cs^+-containing) and thus minimized previously described K^+-current modulations by 5-HT.

The inhibitory effect of 20 μM 5-HT at −100 mV is illustrated in Figure 1a. The 5-HT-induced reduction of the NMDA response was completely reversible, as illustrated in the experiment of Figure 1b and c, in which a higher concentration of 5-HT (100 μM) was used, inducing a stronger block of the NMDA response.

In some cells (such as that of Figure 1b, c and d) which could be recorded during long enough periods without any deterioration (showing no change in conductance, no change in series resistance, and stable NMDA responses), the effect of 5-HT could be studied successively at different holding potentials. At −60 mV (Figure 1d) 5-HT also reduced the NMDA response, but the reduction was clearly less pronounced than at −100mV (Figure 1c).

In order to be able to compare the effects of 5-HT at different membrane potentials during the same 5-HT application, in some experiments we used the voltage-jump protocol illustrated by Figure 2. The holding potential was −60 mV and jumps of 500 ms duration were alternately applied from −60 to −100 or −30 mV in the absence of NMDA and during the NMDA applications. This protocol allowed the measurement of the NMDA responses at −30, −60 and −100 mV by subtracting the traces recorded in the absence of NMDA from those recorded in the presence of NMDA. This same protocol was used when 5-HT was co-applied. It allowed comparison of both the basal conductance and the NMDA response in the absence and presence of 5-HT. Figure 2a and b shows four superimposed current traces, the traces recorded at −60 mV and during the voltage-jumps (to −100 mV and −30 mV respectively), in control, 5-HT, NMDA and NMDA+5-HT. Figure 2c (for jumps to −100 mV) and 2d (for jumps to −30 mV) shows the NMDA responses obtained after subtraction and averaging of three similar traces. Whereas it is clear that 5-HT itself did not affect the conductance of this cell between −100 and −30 mV, it reduced the NMDA response by about 53% at −100 mV, 30% at −60 mV and by only 16% at −30 mV. The voltage-dependence of the 5-HT-induced block was such that in the presence of 5-HT, the NMDA response did not increase between −60 mV and −100 mV.

Results similar to those illustrated in Figure 2 were obtained in 12 cells using 5-HT of various origins (5-HT oxalate salt or 5-HT creatinine sulphate complex from Sigma, 5-HT hydrochloride from RBI). In a few cells, a slight decrease of the basal conductance was also induced by 100 μM 5-HT.

Table 1 gives the mean values of the percentage reduction of the NMDA response for different concentrations of 5-HT (and other agonists, see below) at different membrane potentials.

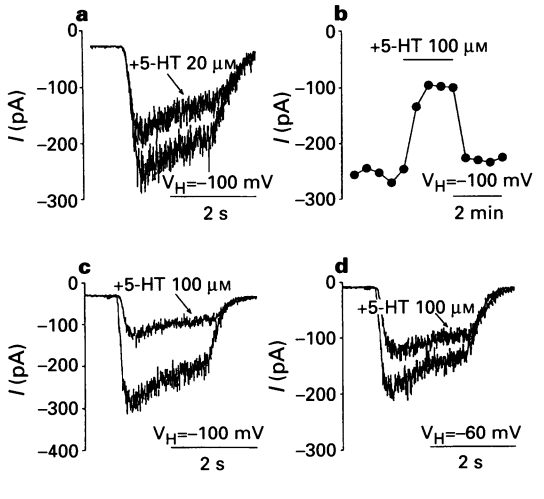

Figure 1 Reversible block of the NMDA response by 5-HT. (a) Mean of three successive NMDA responses in the absence and presence (arrow) of 20 μM 5-HT at −100 mV. When applied, 5-HT was present both before, between and during NMDA applications (see Methods). (b) Plot of the successive peak values of the NMDA response of another cell held at −100 mV in the absence or presence (bar) of 100 μM 5-HT. (c) Same cell as in (b). Mean of the three last responses recorded at −100 mV without or with 5-HT. (d) Mean of three NMDA responses recorded in the same cell before and during a previous application of 100 μM 5-HT performed at a holding potential of −60 mV. Mean responses were obtained by computer averaging.

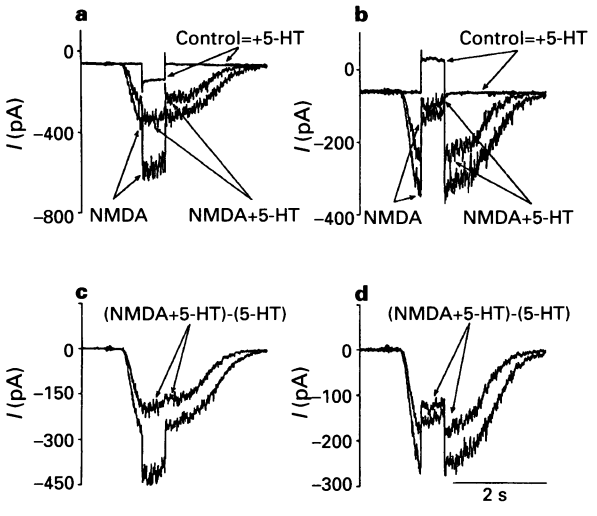

Figure 2 Voltage-dependence of the blocking effect of 5-HT (100 μM). Holding potential −60 mV; 500 ms voltage jumps to −100 mV (a and c) and −30 mV (b and d) were regularly applied in the absence of NMDA ('Control' and '+5-HT traces') and at the peak of the NMDA responses recorded either in the absence ('NMDA') or in the presence ('NMDA+5-HT') of 5-HT (a and b). Raw current records; note that the control and +5-HT traces are superimposed at the holding potential as well as at −100 mV (a) or −30 mV (b). (c and d) NMDA responses derived by subtraction. At all potentials, the largest responses are the NMDA responses obtained in the absence of 5-HT, by subtracting 'Control' traces from 'NMDA' traces, whereas the reduced responses are those obtained in the presence of 5-HT, by subtracting '5-HT' traces from 'NMDA+5-HT' traces. Averages of three responses. Same time scale for all panels.

All the cells which showed a response to NMDA also showed a response to kainate (100 μM). In 9 cells in which we recorded alternately the NMDA and kainate responses with the voltage-jump protocol of Figure 2, we found that the voltage-dependent block induced by 100 μM 5-HT was specific for the NMDA response: the response to kainate was only slightly reduced (by 5±4% (9)), in a voltage-indepenent manner (Figure 3).

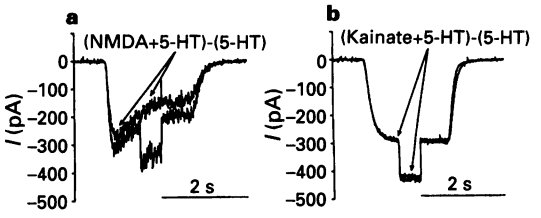

Figure 3 5-HT does not affect kainate responses in the same way. Same voltage-jump protocol as in Figure 2. Responses to 100 μM NMDA (a) and 100 μM kainate (b) were alternately recorded from the same cell. The currents obtained after subtraction of the leak current and averaging two responses are illustrated at −60 mV and during voltage jumps to −100 mV, in the absence and presence of 100 μM 5-HT. This experiment was performed with a six barrel fast-perfusion system (NMDA, control, kainate, kainate + 5-HT, 5-HT, NMDA + 5-HT). Voltage-jumps were applied at the plateau of the kainate responses. In the case of the NMDA responses, some desensitization had already occurred at the time of the voltage-jump.

Block of NMDA responses by other 5-HT agonists is not correlated with their affinities for 5-HT receptors

In an attempt to decide whether the blocking effect of 5-HT on the NMDA response was mediated by a known 5-HT receptor, we tested a few 5-HT agonists. Since rather high concentrations of 5-HT (≥10 μM) were required to produce this effect, 5-HT receptors of highest affinity do not appear to be likely candidates.

It has been proposed that 5-HT_2-like receptors account for some of the excitatory effects of 5-HT (involving a decrease in K^+ conductance) in neonatal rat spinal motoneurones (Wang & Dun, 1990; Elliott & Wallis, 1992). Thus, possible effects of the 5-HT_2 agonist, DOI, were tested. DOI (10 μM) induced a pronounced block of the NMDA response recorded at −100 mV (Figure 4). Like the effect of 5-HT, the blocking effect of DOI was rapidly reversed after a few seconds wash (Figure 4b). This blocking effect was also markedly voltage-dependent, being more pronounced at −100 mV than at −60 and −30 mV (Figure 4c and d, same voltage-jump protocol as in Figure 2). Table 1 gives the mean results derived from 7 experiments.

The broad-spectrum 5-HT agonist, 5-MeOT, has been reported to be less potent than 5-HT (Connell & Wallis, 1988), but to mimic the effects of 5-HT when used at high concentrations (Connell & Wallis, 1988; Wang & Dun, 1990; Cazalets *et al.*, 1992) in rat motoneurones. It was found to be very efficient in blocking the NMDA response. As shown by Figure 5a, the effect at −100 mV was detectable with only 1 μM 5-MeOT. Figure 5a and b shows the effects of four different concentrations of 5-MeOT (1, 2, 5 and 10 μM) successively tested on the same cell. The results derived from 6 similar experiments are given in Table 1. NMDA responses recorded in control and in the presence of 10 μM 5-MeOT are illustrated in Figure 5b. 5-MeOT reduced the NMDA response without affecting the basal current recorded at −100 mV in the absence of NMDA. The blocking effect of 5-MeOT was voltage-dependent as was that of 5-HT or DOI (Figure 5c and

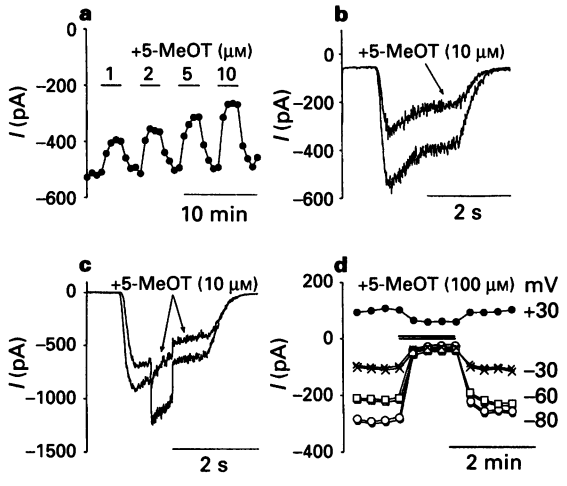

Figure 4 DOI induces a reversible voltage-dependent block of the NMDA response. (a and b) Mean of three successive NMDA responses recorded before, during (+DOI) and after (wash) an application of 10 μM DOI at −100 mV. (c and d) NMDA responses recorded in the absence or presence of 10 μM DOI in another cell held at −60 mV using 500 ms voltage-jumps to −100 mV (c) or −30 mV (d). Same protocol as in Figure 2.

Table 1 Percentage of block of the NMDA response by various 5-HT agonists

		V (mV)				
Agonists	*Concentration* (μM)	−100	−80	−60	−30	+30
5-HT	1	0 (3)		0 (3)		
	10	12±5 (6)				
	20	26±7 (8)		16±2 (3)		
	100	51±8 (16)		27±7 (17)	16±3 (4)	
DOI	10	48±6 (7)		28±6 (7)	22±4 (7)	
5-MeOT	1	14±5 (6)				
	2	19±5 (4)				
	5	29±5 (4)				
	10	42±6 (6)				
	100	92–97 (3)	88±1 (5)	82±4 (10)	69±5 (8)	39±2 (5)
5-CT	10	0 (4)				
	100	18±6 (4)				
8-OH-DPAT	10	33±6 (3)		21±0.5 (3)	15±1.5 (3)	
	100	81 (3)		58±2 (3)	41±11 (3)	

Mean value±s.d. (number of cells). Peak NMDA responses recorded in the '0 Mg' external solution (1–10 μM glycine, 100 μM NMDA); 5-HT agonists always applied both between and during the NMDA applications. The absence of effect of 1 μM 5-HT on the NMDA response was confirmed in the presence of external $MgCl_2$ and glucose at −40 or −50 mV (3 cells, examined in the '1 Mg + glucose' solution).

d). The experiment of Figure 5c was performed with the same voltage-jump protocol as in Figure 2. In another series of experiments, of which an example is shown in Figure 5d, the effect of 100 μM 5-MeOT was tested successively at different holding potentials. Similar results were obtained with both protocols, and are summarised in Table 1.

The blocking effect of 100 μM 5-MeOT was strong enough to be detected even at positive holding potentials, at which the NMDA-induced current was outward (see Figure 5d, +30 mV).

The blocking effects of 5-HT, DOI and 5-MeOT could be compatible with the involvement of a 5-HT_2-like receptor. We therefore tried to antagonize the effects of 5-HT and DOI with spiperone or ketanserin. As shown by Figure 6, preincubation for more than 30 min with 1 μM spiperone did not prevent the voltage-dependent blocking effect of 100 μM 5-HT (result confirmed in 2 other experiments). Similarly, 1 μM ketanserin did not prevent the blocking effect of 10 μM DOI (2 cells).

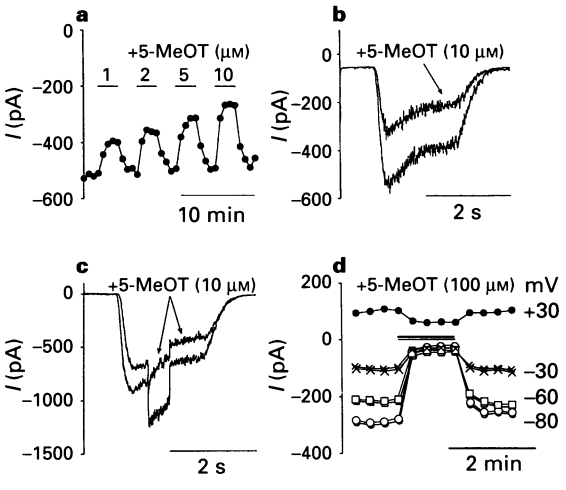

Figure 5 5-MeOT is a potent, reversible and voltage-dependent blocker of the NMDA response. (a) Successive peak values of the NMDA responses recorded at −100 mV in the absence or in the presence (bars) of increasing concentrations of 5-MeOT (1, 2, 5 or 10 μM). (b) Mean of three responses recorded in the same cell in the absence or presence of 10 μM 5-MeOT. (c and d) Voltage-dependence of the blocking effect of 5-MeOT. (c) Holding potential −60 mV and 500 ms voltage-jumps to −100 mV regularly applied; same protocol as in Figure 2. (d) 5-MeOT 100 μM was applied several times in another cell successively held at different membrane potentials (+30 (●), −30 (×), −60 (□) and −80 mV (○)). The successive peak values of the NMDA responses recorded at these membrane potentials, in the absence or presence (bar) of 5-MeOT, are illustrated on the same plot.

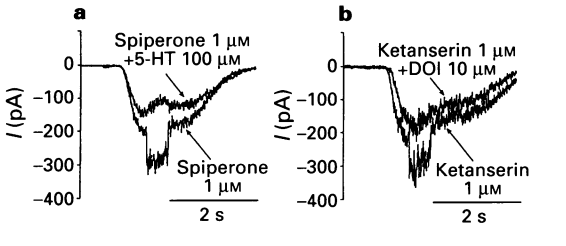

Figure 6 Spiperone and ketanserin do not prevent the blocking effects of 5-HT or DOI. The NMDA responses illustrated were recorded at −60 mV and during voltage jumps to −100 mV (same protocol as in Figure 2). (a) All solutions contained 1 μM spiperone and the cell had been preincubated for 34 min with spiperone before the 5-HT application. (b) Different cell. All solutions contained 1 μM ketanserin and the cell had been preincubated for 72 min with ketanserin before the DOI application.

We also tried to mimic the blocking effect of 5-HT with other agonists known to be less potent at 5-HT_2 receptors than 5-HT or DOI.

5-CT, 1 μM ($n=2$) or 10 μM ($n=4$) did not affect the NMDA response. At 100 μM, 5-CT did reduce this response in a voltage-dependent way, but to a much lesser extent than did 5-HT, 5-MeOT or DOI (see Table 1).

In contrast, 8-OH-DPAT appeared to be more effective than 5-HT in blocking the NMDA response (see Table 1). 8-OH-DPAT reduced the NMDA response at −100 mV by 33±6% (3) at 10 μM and by 81% (3) at 100 μM.

Possible mechanism of the blocking effect of 5-HT agonists on the NMDA response

The effects that we observed seem to be independent of the activation of a specific 5-HT receptor. Their voltage-dependence leads us to propose that 5-HT agonists block the NMDA-gated channel by entering this channel after it has been opened. The following experiments were performed in order to test such a hypothesis.

The blocking effect of 5-HT agonists on the NMDA response could be observed without preincubation, by applying the 5-HT agonist simultaneously with NMDA (not illustrated). In contrast, if the 5-HT agonist was applied before NMDA but not in the presence of NMDA, its blocking effect was no longer detectable at the end of the NMDA application. This is illustrated in Figure 7. In this experiment, in which the NMDA response was alternatively measured at −60 mV and −100 mV, two successive tests of the effect of 5-HT (100 μM) were performed. The first one (a) was performed by applying 5-HT only before (and not during) the NMDA applications, whereas the second (b) was performed by applying 5-HT both before and during the NMDA applications. While in Figure 7b the usual block was observed, in Figure 7a, only the rising phase of the response was slightly affected.

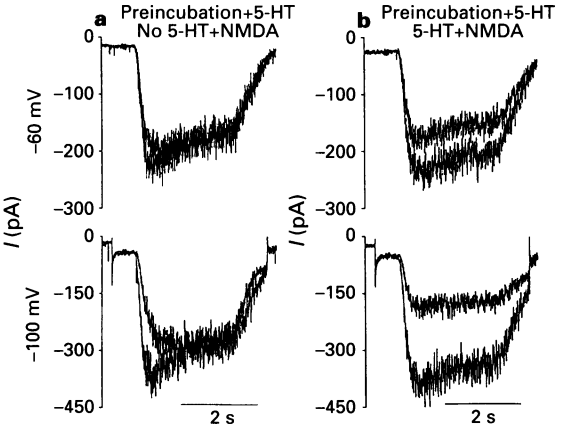

Figure 7 Preincubation with 100 μM 5-HT is not sufficient to block the NMDA response. In this cell, NMDA responses were alternately measured at −60 mV (upper records) and at −100 mV, using 4 s voltage jumps from −60 mV (lower records; the small current jumps seen at the beginning and at the end of these lower records correspond to the voltage jumps between −60 and −100 mV). 5-HT was first applied only by preincubation, between the NMDA applications, all performed in the absence of 5-HT (5-HT was washed by the NMDA containing solution). The mean of two successive NMDA responses recorded before and after the 5-HT preincubation is illustrated at −60 and −100 mV in (a). A second continuous application of 5-HT was then performed, by applying 5-HT both between and during the NMDA applications. The mean of two NMDA responses recorded before and during this 5-HT application is illustrated at each membrane potential in (b). The usual blocking effect was observed and was reversible (not shown).

Previous figures showed control responses and responses recorded when the blocking effect of the 5-HT agonist was maximum. The first NMDA response observed in the presence of the 5-HT agonist (at the peak of the response) was always larger than the following one, even though in most cases this first application was preceded by a long preincubation of several tens of seconds with the 5-HT agonist. This is illustrated by Figure 8a for DOI (10 μM) and by Figure 8b for 5-MeOT (10 μM). In addition, Figure 8 shows that the first NMDA response recorded in the presence of the 5-HT agonist (after preincubation) decreased faster in the continuous presence of NMDA than the control response (see Discussion).

If 5-HT agonists block the channel after the binding of NMDA to its receptor, the degree of block might increase with the concentration of NMDA. In a series of 8 experiments, we compared the effect of 10 μM 5-MeOT on the responses to 20 and 100 μM NMDA recorded alternately at -100 mV on the same cell. In each of these experiments, the response to 100 μM NMDA was slightly more sensitive to 5-MeOT than the response to 20 μM NMDA. Average values of the percentage block (by 10 μM 5-MeOT) of the responses to 20 μM and 100 μM NMDA were $43 \pm 7\%$ (8) and $51 \pm 6\%$ (8) respectively (the responses being measured at the end of the NMDA applications).

The above experiments were all performed in the absence of extracellular Mg^{2+} ions, in order to amplify the NMDA responses in the negative voltage range. A few experiments were performed in the presence of 1 mM extracellular $MgCl_2$. As shown in Table 2 and Figure 9, under these conditions, the blocking effects of 5-HT and 5-MeOT (100 μM) were weaker than in the absence of extracellular Mg^{2+} ions. The difference between the blocking effects of 100 μM 5-MeOT observed in the absence and presence of Mg^{2+} ions was more pronounced at -60 mV than at -30 mV. These results could be explained by some competition between Mg^{2+} ions and 5-MeOT in blocking the open NMDA channel (see Discussion).

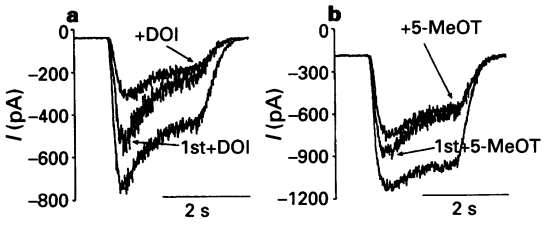

Figure 8 The DOI (a) or 5-MeOT (b)-induced block increases with time during the NMDA application. (a) Same cell as in Figure 4a. DOI (10 μM) was applied continuously in the absence and presence of NMDA. The NMDA responses illustrated were obtained before the DOI application (mean of three responses), after 30 s preincubation with DOI (first NMDA response obtained in the presence of DOI: '1st + DOI'), and at the maximum of the effect of DOI (mean of three responses: '+DOI'). (b) Same as in (a) in the case of another cell to which 10 μM 5-MeOT was applied. Holding potential -100 mV in (a) and (b).

Discussion

A classical 5-HT receptor is unlikely to be responsible for the blocking effect of 5-HT agonists on the NMDA response

In the present study, we have shown that, at micromolar concentrations, several 5-HT agonists can block the NMDA response in a voltage-dependent manner. The order of potency of the agonists that we used does not match any of the sequences expected for known 5-HT receptors (see Zifa & Fillion, 1992; Hoyer *et al.*, 1994). DOI and 5-MeOT were found to be more potent than 5-CT and 8-OH-DPAT. However, not only DOI and 5-MeOT, but also 8-OH-DPAT were more potent than 5-HT itself (see Table 1). Note that 8-OH-DPAT is known to be inactive on 5-HT_3 and 5-HT_4 receptors and much less active than 5-HT and DOI on 5-HT_{2A} and 5-HT_{2C} receptors; furthermore the high affinity of most other 5-HT receptors for 5-HT excludes the notion that these receptors could be responsible for the effects described. The hypothesis that a 5-HT_{2A} receptor could be involved would require that in addition, 8-OH-DPAT induces the same type of effect as other agonists, but in an independent manner. Such an hypothesis, very unlikely, is contradicted by the inability of spiperone and ketanserin to antagonize the effects of 5-HT or DOI. Hypotheses concerning the possible involvement of 5-HT_3 or 5-HT_4 receptors are also unlikely (even though the antagonists that we used are inactive on 5-HT_4 receptors) since in these cases 5-HT should be at least as effective as 5-MeOT.

Thus, we favour the idea that the blocking effect of 5-HT agonists on the NMDA response is independent of classical 5-HT receptors.

Even though the concentrations of 5-HT agonists that we used may seem rather high, they are in the range of concentrations previously tested on motoneurones. For example, in the case of the K^+ conductance decrease induced by superfusion with 5-HT in motoneurones of spinal cord slices, the threshold concentration for 5-HT was 10 μM (Wang & Dun, 1990); the EC_{50} for the 5-HT-induced depolarization in motoneurones of a hemisected spinal cord preparation has been reported to be 32 μM (Elliott & Wallis, 1992). In addition, in another *in vitro* preparation where only 10 μM NMDA (N-methyl-D,L-aspartate) was sufficient to elicit fictive locomotion, the concentrations of 5-HT necessary to evoke similar responses were in the 25–100 μM range (Cazalets *et al.*, 1992). However, in such preparations, 5-HT uptake is likely to occur and to be partly responsible for the high EC_{50} values measured (see Elliott & Wallis, 1992).

DOI has been found to be even less potent than 5-HT in previous studies performed on motoneurones, only occasionally inducing a small depolarization at 10 μM (Elliott & Wallis, 1992) or no depolarization but a decrease in synaptic responses at 20–100 μM (Ziskind-Conhaim, 1993). DOI has also been reported to be less effective than 5-HT in increasing the excitability of facial motoneurones (Garratt *et al.*, 1993) and has been considered as a partial agonist at 5-HT_2 receptors (see references in Garratt *et al.*, 1993), able to induce long duration excitatory effects at high concentrations (see Garratt *et al.*, 1993, using 10 μM, and Wang & Dun, 1990, using 200 μM).

Table 2 Effect of external Mg^{2+} ions on the percentage of block of the NMDA response by 5-HT or 5-MeOT (100 μM)

Agonist	*V* (mV)	*'0 Mg' external solution*	*'1 Mg' external solution*
5-HT	−60	26 ± 7 (12)	11 ± 3 (5)
5-MeOT	−60	82 ± 4 (10)	45 ± 4 (6)
	−30	69 ± 5 (8)	54 ± 2 (6)

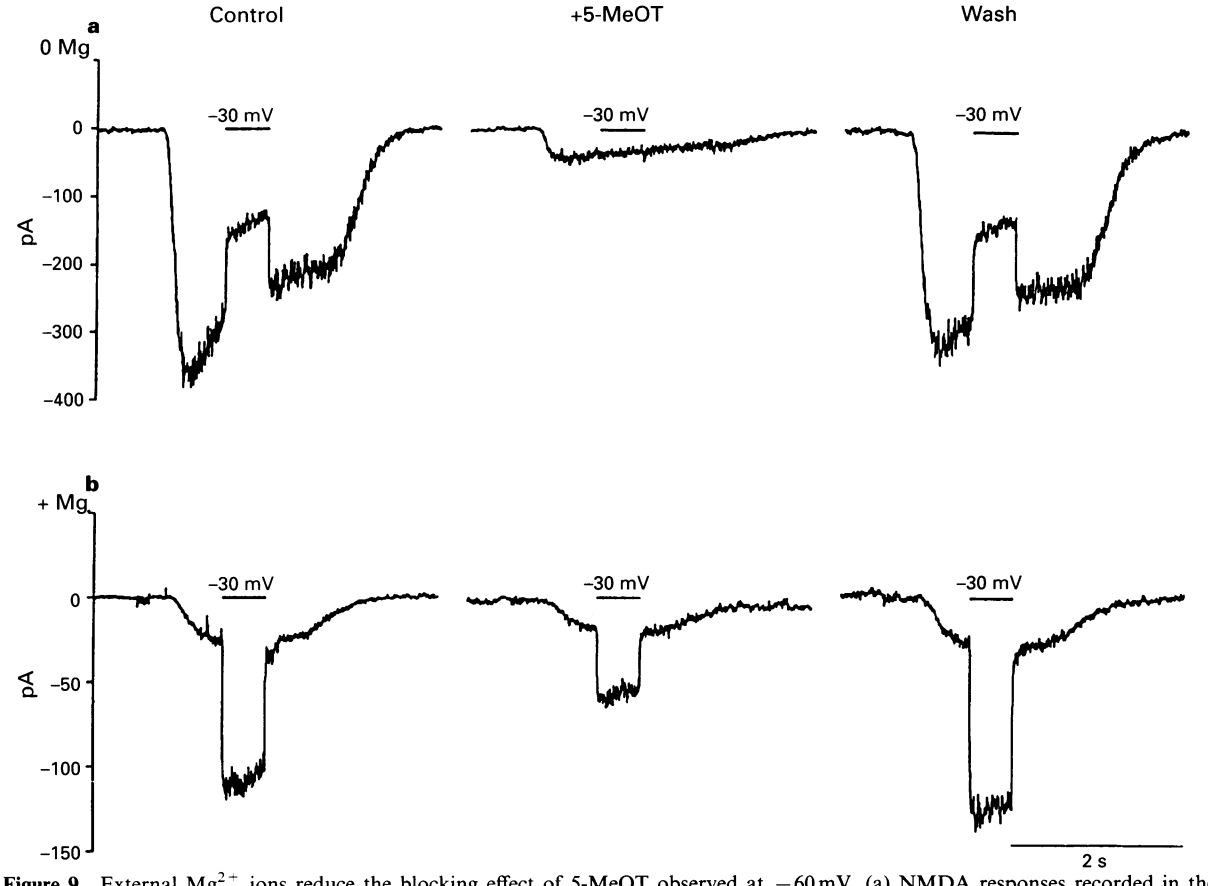

Figure 9 External Mg^{2+} ions reduce the blocking effect of 5-MeOT observed at −60 mV. (a) NMDA responses recorded in the absence of external Mg^{2+} ions at −60 mV and during voltage jumps to −30 mV (bars, same protocol as in Figure 2), before, during (+5-MeOT) and after (Wash) a continuous application of 100 μM 5-MeOT (mean of three responses). (b) NMDA responses recorded from another cell, in the presence of 1 mM extracellular $MgCl_2$, during the same experiment (mean of three responses). Note that under these conditions the response is larger at −30 mV than at −60 mV, due to the classical voltage-dependent block induced by external Mg^{2+} ions. At −60 mV, the 5-MeOT-induced block is much less pronounced in (b) than in (a) (see also Table 2).

The possible physiological importance of the blocking effects described here is difficult to evaluate. It depends on the local concentration of 5-HT around activated NMDA receptors under physiological conditions, in particular at the sites where glutamate and 5-HT are coreleased. Furthermore, we have to note that our results were obtained on cultured neurones and not on neurones developing *in vivo*. However, other authors using hemisected spinal cord have made observations that are consistent with our results (see e.g. Ziskind-Conhaim *et al.*, 1993).

An open channel block could account for our results

In the absence of external Mg^{2+} ions, hyperpolarization of the membrane markedly increased the block of the NMDA response by 5-HT agonists.

This voltage-sensitivity could be explained by the positive charge of these agonists if their site of action was located inside the membrane, the most likely site being the non selective cationic channel opened by NMDA. NMDA-gated channels are well-known to be blocked in a voltage-dependent manner by extracellular Mg^{2+} ions (Nowak *et al.*, 1984) and this was confirmed in the particular neurones that we used (block induced by 0.1 mM Mg^{2+} >90% at −100 mV in 6 cells; not shown). The possibility that the blocking effect of 5-HT agonists might be due to some Mg^{2+} contamination is excluded in view of the marked dilution of the stock solutions that we used (e.g. 1/10 000 in the case of 1 μM 5-MeOT) and since a variety of agonists from different sources had similar effects. Furthermore, a blocking effect persisted in the presence of 1 mM external $MgCl_2$.

In the presence of external Mg^{2+} ions, the blocking effect of 5-HT and 5-MeOT was weaker than in the absence of Mg^{2+} ions. In addition, in the case of 5-MeOT, we showed that the influence of Mg^{2+} ions on its blocking effect was more pronounced at −60 mV than at −30 mV. These results (Table 2) are in favour of the idea that Mg^{2+} ions could compete with the 5-HT agonist inside the open channel, this competition being more pronounced at more negative membrane potentials, because of the stronger charge of the divalent Mg^{2+} ions. This type of interpretation has already been proposed in order to explain similar properties of the blocking effect of MK-801 (Huettner & Bean, 1988).

Other aspects of our results are also in agreement with the proposal that 5-HT agonists block the open channel.

Application of 5-HT during the NMDA application was shown to be necessary for block of the final NMDA response (Figure 7; the small blocking effect remaining during the initial phase of the response in Figure 7a can be explained by the fact that some NMDA reached the cell at a time where 5-HT had not yet been completely washed away). Such a result indicates that 5-HT does not affect the response through a long lasting intracellular chemical modification.

During its first simultaneous application with NMDA (and also, but to a lesser extent, during the following ones), even if it had been applied for several tens of seconds before, the 5-HT agonist was less active on the peak NMDA response than on the response recorded at the end of the NMDA application. (The difference between the first and following responses recorded in presence of the 5-HT agonist could be explained if the blocked open channels are trapped in the blocked state during the intermediate wash without NMDA).

The response to 100 μM NMDA was shown to be slightly more sensitive to 5-MeOT than the response to 20 μM NMDA. This conflicts with the predictions of a model involving competition between NMDA and 5-MeOT, but can be explained by non-competitive models involving open channel block. In a similar way, bicuculline methiodide has been shown to reduce NMDA responses in a non-competitive way; however, the voltage-dependence of this block was not as clear (see Wright & Nowak, 1992; Mußhoff *et al.*, 1994).

Single channel recordings could be performed in order to study in more detail the mechanism(s) responsible for the blocking effects of 5-HT agonists.

Possible relationships with previous results

Inhibitory effects of 5-HT on the postsynaptic potentials evoked by dorsal root stimulation have been described previously (see Introduction). In some cases, these inhibitory effects were clearly distinct from the effects described in the present paper. For example, the inhibitory effects described by Wu *et al.* (1991) are clearly presynaptic and mediated by receptors of the 5-HT_1 family; 5-HT_1 receptors also seem responsible for the inhibitory effects reported by Elliott & Wallis (1992) (effects induced by 5-HT, 8-OH-DPAT and 5-CT, but not by DOI).

However, in some other studies, inhibitory effects have been described which might be partly related to the effects that we observed. For example, using hemisected spinal cord from rat embryos and from very young neonatal rats, Ziskind-Conhaim *et al.* (1993) have shown that all 5-HT agonists (including 5-HT_2 agonists) reduced synaptic potentials in more than 65% of motoneurones. If the contribution of NMDA receptors to the synaptic responses recorded in this case was larger than in studies performed on preparations from older animals (even though immature: Wu *et al.*, 1991; Elliott & Wallis, 1992), our results could explain some of the differences between these previous studies. The postsynaptic inhibitory effect of 5-HT (> 10 μM) on NMDA responses described by Holohean *et al.* (1992) might also be partly related to our results. However, in this case, high concentrations of 5-HT_2 antagonists were able to attenuate the inhibitory effect of 5-HT and an inhibitory effect of 5-HT on responses to kainate was also observed.

Some results obtained on neurones from other brain areas could also be partly related to our observations: see e.g. Hicks *et al.* (1989) for cerebellar Purkinje cells. More recently, Staubli & Otaky (1994) reported a suppressive effect of high concentrations of 5-HT on the induction of LTP in rat hippocampus, occurring via an action on NMDA responses.

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