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Serotonin-induced inhibition of locomotor rhythm of the rat isolated spinal cord is mediated by the 5-HT_1 receptor class

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The neurotransmitter serotonin (5-HT) induces rhythmic motor patterns (fictive locomotion) of the neonatal rat spinal cord *in vitro;* this is a useful experimental model to study the generation of a motor programme at exclusively spinal level. Nevertheless, 5-HT slows down the fictive locomotion typically elicited by activation of NMDA glutamate receptors, suggesting a complex action of this monoamine. By means of electrophysiological recordings from multiple ventral roots we demonstrated that the decrease caused by 5-HT in NMDA-induced periodicity was dose-dependent, enhanced after pharmacological blocking of 5-HT_2 excitatory receptors, and imitated by pharmacological agonists of the 5-HT_1 receptor family. Selective blockers of the 5-HT_{1A} or 5-$HT_{1B/D}$ receptor classes, either alone or in combination, largely (but not completely) attenuated this inhibitory action of 5-HT. It is concluded that the principal inhibitory action of 5-HT on the spinal locomotor network was mediated by certain subtypes of the 5-HT_1 receptor class, which tends to oppose the 5-HT_2 receptor-mediated excitation of the same network.

Keywords: central pattern generator; 5-hydroxytryptamine; locomotion; *N*-methyl-D-aspartate; motor programme; serotonin

1. INTRODUCTION

The neonatal rat spinal cord *in vitro* can produce rhythmic patterns of motor activity (termed fictive locomotion) even in the absence of sensory or supraspinal inputs (reviewed by Rossignol & Dubuc 1994). These patterns, recorded from ventral roots (VRs), are induced by several excitatory agents such as serotonin (5-HT) (Cazalets *et al.* 1992), *N*-methyl-D-aspartate (NMDA) (Kudo & Yamada 1987), or high extracellular potassium solution (Bracci *et al.* 1998), and consist of rhythmic bursts alternating between left–right and flexor–extensor motor pools. Fictive locomotion can also be evoked by electrical stimulation of the brainstem with concomitant massive release of 5-HT from descending terminals (Fyda *et al.* 1997), thus indicating an important role of this endogenous transmitter in the activation of the network responsible for locomotion (the so-called central pattern generator, CPG). Although NMDA, high-potassium solution or 5-HT might all activate the CPG through widespread depolarization of the network (Bracci *et al.* 1998), the role of 5-HT is complicated by the large heterogeneity of its receptors coupled to distinct effector mechanisms (Anwyl 1990). In particular, 5-HT is known to exert contrasting effects on the excitability of motoneurons, namely 5-HT_2 receptor-mediated depolarization, a dramatic decrease in spontaneous synaptic activity (Elliott & Wallis 1992) and inhibition of the dorsal root evoked reflex (Wallis *et al.* 1993); the latter two actions are thought to be due partly to 5-HT_1 receptors and partly to an unconventional receptor class yet to be identified. This complex action may account for the multifarious effects of 5-HT on the operation of the CPG: in fact, even though 5-HT *per se* elicits a locomotor pattern, its pattern is always slower than the one induced by NMDA (Cazalets *et al.* 1992). Furthermore, application of 5-HT during the NMDA-induced pattern decreases the cycle period (Bracci *et al.* 1998; Sqalli-Houssaini *et al.* 1993). These inhibitory effects of 5-HT on the locomotor network are enhanced after blocking of the 5-HT_2-mediated depolarization (Bracci *et al.* 1998), but have not yet been characterized in terms of their receptor pharmacology. Autoradiographic studies have revealed the presence of distinct 5-HT receptor subtypes in the spinal cord of the cat (Pubols *et al.* 1992) and the rat (Marlier *et al.* 1991), with a predominance of the 5-HT_1 over the 5-HT_2 class in the lumbar region (Marlier *et al.* 1991). Identification of the receptor classes mediating excitatory and inhibitory actions of 5-HT on the CPG would pave the way to the use of pharmacological tools to up- or downregulate the operation of the locomotor network.

For this purpose the present study examined whether 5-HT-induced rhythm inhibition might be due to activation of the 5-HT_1 receptor subtype (negatively coupled to adenylyl cyclase (Hoyer *et al.* 1994)) as they are responsible for hyperpolarization of rat hippocampal pyramidal cells (Beck 1989), inhibition of calcium currents in rat brainstem motoneurons (Bayliss *et al.* 1995) and presynaptic inhibition of glutamatergic transmission onto rat motoneurons (Singer *et al.* 1996). The 5-HT_1 receptor

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family (which comprises various subtypes (Hoyer *et al.* 1994)) seems thus to be a suitable system to mediate the main inhibitory action of 5-HT on locomotor patterns. Our rationale in approaching this investigation was to test the effect of 5-HT on NMDA-evoked fictive locomotion before and after adding a 5-HT_2 antagonist to assess the degree of 5-HT induced inhibition and to compare it with the action of different agonists selective for 5-HT_1 receptor subclasses. In this case, close mimicry of effects would suggest that a certain receptor subclass was involved in the depression of locomotive patterns. This initial observation was subsequently extended by applying selective 5-HT_1 antagonists to prevent the inhibitory effect of 5-HT. The use of several highly specific 5-HT_1 receptor agents enabled us to interpret the observed changes in locomotor patterns as due to activation or blocking of this receptor group.

2. METHODS

Experiments were performed on neonatal Wistar rats (0–2 days old). The spinal cord was dissected and isolated (from the mid-thoracic region to the *conus medullaris*) according to standard procedures (Ballerini *et al.* 1997), and then fixed to the bottom of a recording chamber (5-ml volume) and continuously superfused (7.5 ml min^{-1}) with artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 113; KCl, 4.5; $MgCl_2$, 7; H_2O, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucoses, 11; gassed with 95% O_2–5% CO_2; pH 7.4 at room temperature. All drugs were bath-applied at the concentrations mentioned in the text. Simultaneous VR recordings (usually from pairs of L2 and L5 VRs) were obtained with monopolar glass suction microelectrodes containing a pellet of Ag–AgCl. The signals were amplified by using a custom-made DC amplifier (0.1–30 kHz bandpass) and then displayed online on a computer and a chart recorder, and stored on a DAT recorder (11 KHz acquisition rate; this may have reduced the amplitude of high-frequency action potentials of VRs). Period measurements were performed over 30 consecutive cycles as previously described (Beato *et al.* 1997). NMDA and 5-HT were always applied for more than 15 min; only those applications that induced a persistent pattern of alternating activity (lasting more than 10 min) were considered for analysis. 5-HT agonists and antagonists were always applied at receptor-saturating concentrations (Hoyer *et al.* 1994; Audinot *et al.* 1997). 5-HT agonists were administered via the bathing solution for more than 15 min; the ensuing changes in rhythm periodicity were measured only after the period had become stable (usually after more than 5 min). 5-HT antagonists were superfused for more than 30 min before induction of the pattern by NMDA. Comparison of results before and after addition of 5-HT and related drugs was always performed with the use of the same preparations as the internal control. Data are presented as means ± s.d.; statistical significance was assessed by using the ANOVA test ($p = 0.01$ was taken as the level of significance). 5-HT was purchased from Sigma; NMDA, 1-(2-methoxyphenyl)-4-(4-phthalimidobutyl)piperazine (NAN-190) and 4-[3-(benzotriazol-1-yl)propyl]-1-(2-methoxy)piperazine (PIP) were purchased from Tocris; GR 125,743X was kindly donated by Glaxo–Wellcome. 5-carboxamido-tryptamine maleate (5-CT), CGS 12066A and ritanserin were a kind gift from Professor R. Corradetti (Department of Pharmacology, University of Florence).

3. RESULTS

Recordings were obtained from 38 spinal cord preparations. DC-coupled recordings allowed us to observe both slow polarization changes in VRs (corresponding to changes in membrane potential of the motoneuronal population) and firing (detected as a thickening of the baseline and corresponding to spiking activity of motoneurons (Ho & O'Donovan 1993; Bracci *et al.* 1996)). NMDA applications (2.5–8.0 μM) evoked persistent alternating motor patterns in 36 out of 38 preparations (average period 1.4 ± 0.4 s), which were subsequently tested with 5-HT.

(a) *NMDA-induced locomotion in the presence of 5-HT_1 receptor class agonists*

After induction of locomotor-like patterns by NMDA, the effect of 5-HT was compared with that of two different 5-HT_1 receptor agonists on ten preparations. An example of a rhythmic pattern (induced by 6 μM NMDA) is shown in figure 1*a* with simultaneous recordings from L2 and L5 VRs (containing mainly flexor and extensor motor axons, respectively). All four VRs produced rhythmic bursts with a period of 1.8 ± 0.3 s, accompanied by an increase in action potential firing on top of each oscillation. After a wash for over 20 min, the same concentration of NMDA was co-applied first with 10 μM (not shown) and then with 20 μM 5-HT (figure 1*b*). In the latter case there was an increase in the cycle period (2.5 ± 0.2 s) in accordance with previous observations (Bracci *et al.* 1998; Sqalli-Houssaini *et al.* 1993). Note, however, that high-frequency spike activity persisted in all ventral roots. Data pooled from all preparations are represented by the first two bars in the histogram of figure 1*e*. Cycle period, expressed as percentage of the cycle period observed in the presence of NMDA alone, was significantly ($p < 0.001$) longer in the presence of 10 μM 5-HT (142 ± 5%) or 20 μM 5-HT (171 ± 9%). The decelerating effect of 5-HT is enhanced after blocking of 5-HT_2 receptors (Bracci *et al.* 1998), which are known to be excitatory through a positive link with the phosphoinositol transduction system (Hoyer *et al.* 1994). As shown by the third bar of figure 1*e*, application of 5-HT in the presence of 1 μM ritanserin (a selective 5-HT_2 receptor antagonist) decreased the cycle period by 198 ± 8%, a value significantly different ($p < 0.001$, $n = 6$) from the decrease observed before block of the ritanserin-sensitive 5-HT receptors. This finding confirmed that the 5-HT inhibitory component was usually masked by an excitatory effect apparently mediated through 5-HT_2 receptors.

To reveal this inhibitory component pharmacologically we co-applied NMDA and a selective agonist for one of the two main subclasses of 5-HT_1 receptor, namely 5-HT_{1A} and 5-$HT_{1B/D}$. Figure 1*c* shows that 6 μM NMDA and 50 μM CGS 12066A (a selective agonist for 5-$HT_{1B/D}$ receptors (Neale *et al.* 1987)) induced a regular alternating pattern (period 2.0 ± 0.2 s) slower than the one observed with NMDA alone (figure 1*a*). Data pooled from six preparations are represented in the fourth bar of the histogram of figure 1*e* and show that the cycle period was significantly ($p < 0.001$) larger (153 ± 20%) than the control one. As shown in figure 1*d*, the same effect was observed on co-applying 6 μM NMDA and 1 μM 5-CT (a

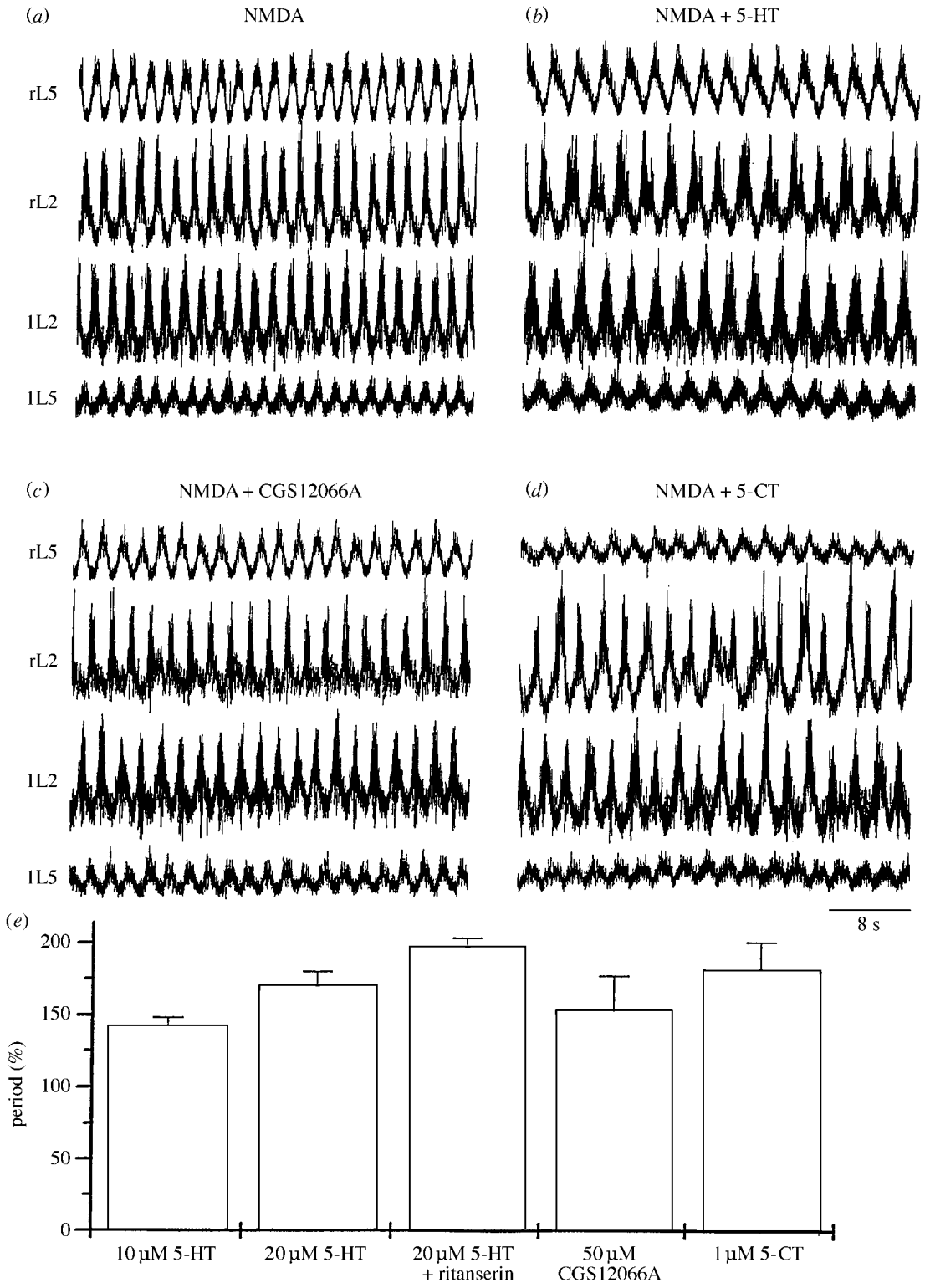

Figure 1. Inhibition of locomotor rhythm by 5-HT and 5-HT_1 receptor agonists. (*a*)–(*d*) Simultaneous records from four lumbar roots (identified as l, left, or r, right) from two segments (L2 or L5) of the same preparation. Locomotor oscillations evoked by 6 μM NMDA (*a*) are slowed when 6 μM NMDA is co-applied with 20 μM 5-HT (*b*), 50 μM CGS 12066A (*c*), or 1 μM 5-CT (*d*). A histogram of pooled data from various spinal cords (*e*) indicates lengthening of pattern period (expressed as percentage of control in NMDA solution) during application of 10 μM 5-HT, 20 μM 5-HT, 20 μM 5-HT plus 1 μM ritanserin, 50 μM CGS 12066A and 1 μM 5-CT. For number of preparations and statistical significance see text.

broad-spectrum 5-HT_1 agonist with a reportedly higher selectivity for 5-HT_{1A} receptors (Hoyer *et al.* 1994)): the resulting cycle period was clearly slower (2.3 ± 0.4 s) than the one in figure 1*a*. Data pooled from eight preparations (last bar in figure 1*e*) show that the cycle period was $181 \pm 18\%$ with respect to the control one ($p < 0.001$). These results suggest that both 5-HT_{1A} and 5-$HT_{1B/D}$ were involved in the decelerating action of 5-HT.

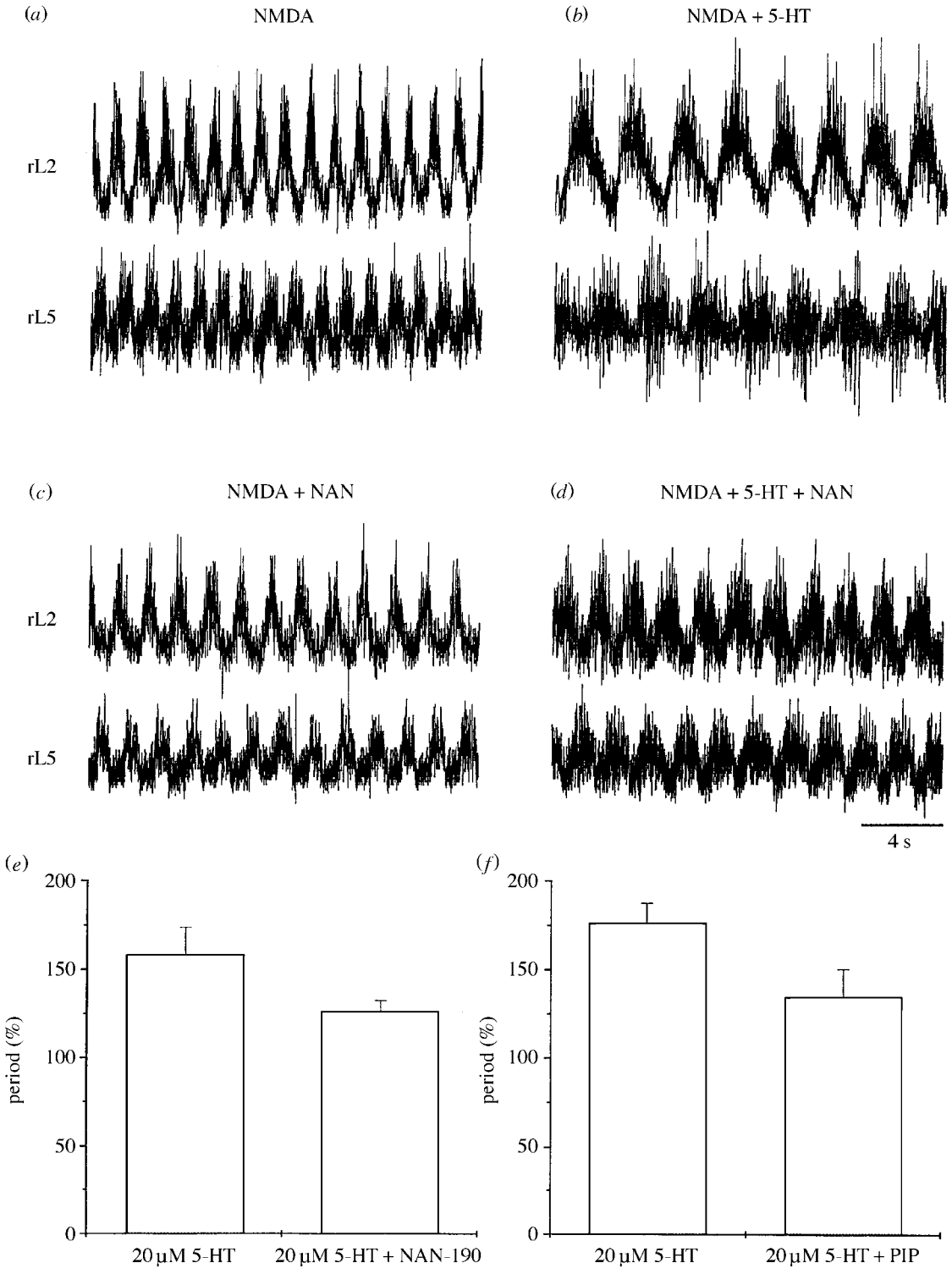

Figure 2. Inhibitory action of 5-HT is decreased by the 5-HT_{1A} receptor antagonists NAN-190 or PIP. Control locomotor rhythm induced by 3 μM NMDA (*a*), is slowed by 20 μM 5-HT (*b*), or by 1 μM NAN-190 (*c*), which also decreases the decelerating action of 20 μM 5-HT (*d*); all these responses are from the same spinal cord. (*e*) Histogram of pooled data from seven preparations showing that the deceleration by 20 μM 5-HT is significantly ($p<0.001$) reduced by 1 μM NAN-190. (*f*) Histogram ($n=5$ preparations) showing that the slowing down by 20 μM 5-HT is decreased ($p<0.001$) by 1 μM PIP.

(b) *NMDA-induced locomotion in the presence of 5-HT_1 receptor class antagonists*

To block the inhibitory effect of 5-HT, 5-HT_{1A} receptor antagonists were first tested ($n=12$). Figure 2*a* shows a rhythmic pattern induced by 3 μM NMDA (cycle period 1.2±0.3 s) that is slowed after the application of 20 μM 5-HT (cycle period 2.3±0.4 s; see figure 2*b*). NMDA application was repeated in the presence of 1 μM NAN-190, a selective blocker of 5-HT_{1A} receptors, which slightly slowed the oscillator (cycle period 1.4±0.3 s) (figure 1*c*). This effect (the averaged period of which was 110±5% of control) was consistently observed in the other six preparations tested with NAN-190, although such a small difference was not statistically significant.

Because NAN-190 (like other selective blockers of 5-HT receptors) is known to be a partial agonist on 5-HT_{1A} receptors (Fletcher *et al.* 1993), we tested whether its decelerating activity was dose-dependent. In four preparations we applied the same concentration of NMDA in control solution and in the presence of 1 μM or 10 μM NAN-190. The cycle period was 112±8% or 128±12%, respectively (the latter value was significantly different from control; $p<0.01$).

Figure 2*d* shows the effect of 20 μM 5-HT in the presence of NAN-190 and NMDA; although the cycle period was slower (1.8±0.4 s) than the one observed with NMDA plus NAN-190 solution (1.4±0.3 s), it is apparent that 5-HT was now much less effective in inhibiting the rhythm (compare figure 2*b* and 2*d*). Figure 2*e* shows that, on average, NAN-190 significantly ($p<0.001$) reduced the action of 20 μM 5-HT from 159±9% to 125±6% (a value that, however, remained slower than the one in NMDA solution alone; $p<0.01$).

The same protocol was used with another 5-HT_{1A} antagonist, PIP ($n=5$ preparations), known to act at both postsynaptic and presynaptic 5-HT_{1A} receptors (Mokrosz *et al.* 1994). Application of 1 μM PIP yielded results analogous to those with NAN-190. Figure 2*f* shows that in the presence of PIP the decelerating effect of 20 μM 5-HT (173±9%) was significantly reduced (132±12%, $p<0.001$) although not abolished.

The effects of a high-affinity, newly synthesized 5-$HT_{1B/D}$ receptor antagonist, GR125,743 (Audinot *et al.* 1997) were next investigated ($n=7$). Once a control pattern was obtained with 3 μM NMDA (figure 3*a*), application of 20 μM 5-HT increased the period from 1.1±0.3 s to 2.3±0.4 s (figure 3*b*). The same application of NMDA and 5-HT in the presence of 1 μM GR125,743 induced a locomotor pattern with a period of 1.9±0.3 s (figure 3*c*), still slower than the one induced by NMDA, but faster than its control without the antagonist. Pooled data (figure 3*e*) show that the deceleration induced by 20 μM 5-HT in the presence of GR 125,743 was significantly less effective (181±25% compared with 149±22%, $p<0.001$) than the one observed in control solution. Unlike NAN-190 or PIP, GR 125,743 had no effect on NMDA-induced rhythms.

In four preparations previously tested with GR125,743 we also co-applied NAN-190 (1 μM) to investigate whether simultaneous blocking of 5-HT_{1A} and 5-$HT_{1B/D}$ receptors might result in a further decrease in the inhibitory action of 5-HT. As shown in figure 3*d*, application of 3 μM NMDA and 20 μM 5-HT in the presence of both antagonists induced a pattern comparatively faster than those observed in control solution (figure 3*b*) or in the presence of GR125,743 alone (figure 3*c*), although still slower than the NMDA-control one. Pooled data (figure 3*e*) show that the period induced by 20 μM 5-HT was significantly shorter in the presence of both antagonists (130±14%, $p<0.001$), even though it remained significantly ($p<0.01$) longer than the control.

4. DISCUSSION

The principal finding of the present study is the demonstration that the complex effects of 5-HT on the CPG operation were pharmacologically dissected into distinct components. Although it is known that the excitatory action of 5-HT in the spinal cord is largely mediated through 5-HT_2 receptors (Elliott & Wallis 1992; Bracci *et al.* 1998), the present experiments clearly indicated that the rhythm-inhibitory effect of 5-HT was mediated, at least in part, by 5-HT_1 receptors. In fact, specific agonists for this receptor subclass mimicked the decelerating action of 5-HT on the NMDA-induced locomotor pattern. Likewise, the 5-HT-induced increase in cycle period was strongly reduced after blocking of the 5-HT_{1A} and 5-$HT_{1B/D}$ receptor subclasses.

A considerable limitation of the present study is represented by the fact that responses were observed as changes in fictive locomotor patterns. These effects were obviously nonlinearly related to drug receptor occupancy and were detected within a restricted range of concentrations: below-threshold activity and tonic excitation constituted the lower and upper limits of responses to concentration changes. These considerations thus prevented construction of dose–response curves and any quantitative analysis of the drugs' properties in terms of agonism and antagonism. Nonetheless, by using receptor-saturating concentrations of selective agonists and antagonists (Hoyer *et al.* 1994; Audinot *et al.* 1997), the measured variable of the system became the speed at which a complex functional network operated, therefore yielding useful data on how efficiently the locomotor programme was translated into motor output whenever a certain population of receptors was occupied.

The reduction in rhythm periodicity observed after applying 5-HT (or related receptor agonists) presumably developed at the level of the CPG network as a slower rhythm could be associated with higher spiking activity recorded from ventral roots, indicating that motoneurons still generated action potentials at a high rate. In this sense the 'inhibitory' action of 5-HT could not be considered as mainly caused by enhanced synaptic inhibition but by a combination of various factors (synaptic and nonsynaptic ones) which all together led to a slower operation of the CPG. How could 5-HT depress the locomotor rhythm? This may result from several distinct processes all concurring to impair the CPG activity. In particular, the 5-HT_1 receptors (located mainly at pre- and partly at postsynaptic levels (Hoyer *et al.* 1994)) may decrease excitatory synaptic transmission via their calcium-channel block, which reduces calcium entry into the presynaptic terminals (Bayliss *et al.* 1995) and leads to a reduction in transmitter release (Schmitz *et al.* 1998). Postsynaptic 5-HT_1 receptors are found to depress responses of dorsal horn neurons to NMDA (Lopez-Garcia 1998). Note that the deceleration in the locomotor rhythm induced by 5-HT is similar to the effect of blocking a distinct class of receptors for glutamate, the main transmitter governing the operation of the locomotor network (Beato *et al.* 1997). Any reduction in the efficiency of glutamatergic transmission either directly via excitatory receptor antagonism or indirectly, presumably via activation of 5-HT_1 receptors, resulted in an increased cycle period.

Because the inhibitory action of 5-HT on fictive locomotion was readily mimicked by 5-HT_1 receptor agonists (CGS 12066A or 5-CT), these receptors were regarded as important for the expression of 5-HT-induced inhibition.

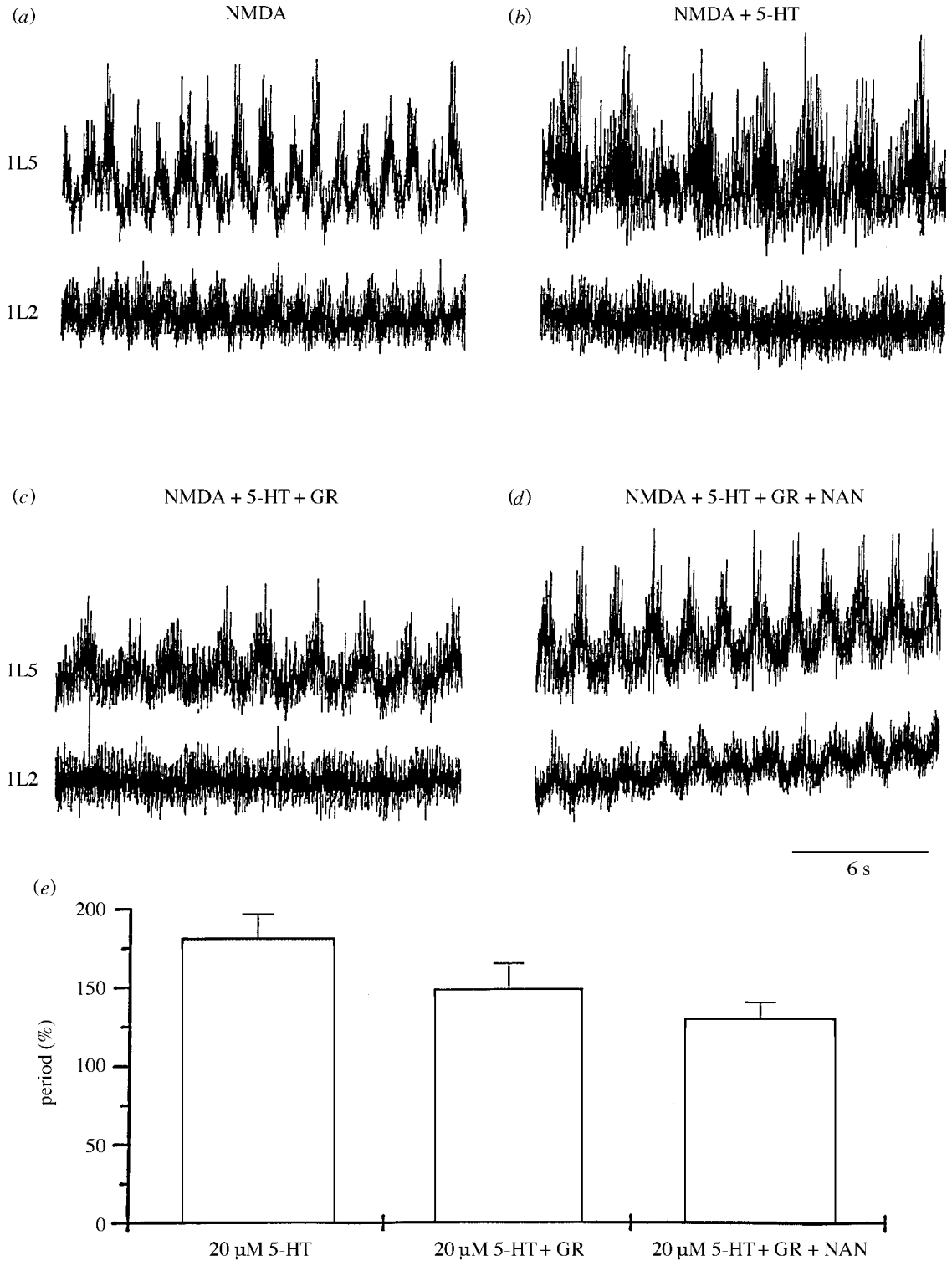

Figure 3. Inhibitory action of 5-HT is decreased by the 5-$HT_{1B/D}$ receptor antagonist GR 125,743. Control locomotor pattern induced by 3 μM NMDA (*a*), is depressed by 20 μM 5-HT (*b*), an effect partly prevented by 1 μM GR 125,743 (*c*). Simultaneous application of 1 μM GR 125,743 and 1 μM NAN-190 largely attenuates the inhibition evoked by 5-HT (20 μM). (*e*) Histogram of period increase evoked by 20 μM 5-HT ($n=7$ preparations), 5-HT plus 1 μM GR 125,743 ($n=7$), and 5-HT plus GR 125,743 and 1 μM NAN-190 ($n=4$). For statistical analysis see text.

One might expect that, even if this receptor family is heterogeneous (Hoyer *et al.* 1994), application of blockers of the 5-HT_{1A} (NAN-190 and PIP) or 5-$HT_{1B/D}$ (GR 125,743) group, especially in combination, should have disclosed a purely excitatory action of 5-HT. Nonetheless, although any 5-HT-induced depression of rhythmic patterns was largely and significantly reduced, these antagonists failed to prevent it completely. One possibility is that other, yet unidentified, receptors were also involved (Manuel *et al.* 1995). Another possible explanation is that 5-HT merely acted through other receptors insensitive to the antagonists used in the present study.

In particular, although receptors belonging to the 5-HT_3 and 5-HT_4 category do not seem to be responsible for the rhythm-inhibitory action of 5-HT (Crick *et al.* 1994), receptors belonging to the newly described families of 5-HT_{1E}, 5-HT_{1F} and 5-HT_7 share common targets with 5-HT_{1A} and 5-$HT_{1B/D}$ receptors (Hoyer *et al.* 1994); the lack of availability of selective antagonists against these receptors makes it difficult to test this hypothesis. In summary, it appears that the rhythm-inhibitory action of 5-HT was predominantly but not exclusively mediated by 5-HT_{1A} and 5-$HT_{1B/D}$ receptors. At the present time the latter category cannot be further resolved with subclass-specific compounds.

It is also interesting to consider another explanation for the lack of complete antagonism of 5-HT inhibition, namely that the 5-HT_1 antagonists currently available (including the ones tested in the present study) have a partial agonist effect (Fletcher *et al.* 1993), which, to a certain degree, imitates the action of 5-HT. A partial agonist would bind receptors with higher affinity (thus preventing the action of a full agonist) but would activate receptors with lower efficacy (yielding a smaller maximum response). This possibility is partly supported by our data, which indicate a slight increase in the cycle period of NMDA-induced patterns with NAN-190, an effect that was enhanced when the concentration of this antagonist was increased tenfold. Nevertheless, no evidence of partial agonism was detected when using GR 125,743, thus restricting this possibility to data on 5-HT_{1A} receptors only.

In conclusion, the present results indicate that the pleiotropy of 5-HT actions on spinal rhythmogenesis comprises a major blocking component via activation of the 5-HT_1 receptor subclass. It has therefore been possible to identify at least one mechanism responsible for the comparatively slow periodicity of fictive locomotion induced by 5-HT against other excitatory agents such as NMDA or high potassium solution. One might suggest that facilitating locomotor rhythms or depressing network hyperexcitability might be effected by pharmacological blocking or activation, respectively, of 5-HT_1 receptors.

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