Contribution of NMDA and non-NMDA glutamate receptors to locomotor pattern generation in the neonatal rat spinal cord

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SUMMARY

The motor programme executed by the spinal cord to generate locomotion involves glutamate-mediated excitatory synaptic transmission. Using the neonatal rat spinal cord as an *in vitro* model in which the locomotor pattern was evoked by 5-hydroxytryptamine (5-HT), we investigated the role of N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors in the generation of locomotor patterns recorded electrophysiologically from pairs of ventral roots. In a control solution, 5-HT (2.5–30 μM) elicited persistent alternating activity in left and right lumbar ventral roots. Increasing 5-HT concentration within this range resulted in increased cycle frequency (on average from 8 to 20 cycles min^{-1}). In the presence of NMDA receptor antagonism, persistent alternating activity was still observed as long as 5-HT doses were increased (range 20–40 μM), even if locomotor pattern frequency was lower than in the control solution. In the presence of non-NMDA receptor antagonism, stable locomotor activity (with lower cycle frequency) was also elicited by 5-HT, albeit with doses larger than in the control solution (15–40 μM). When NMDA and non-NMDA receptors were simultaneously blocked, 5-HT (5–120 μM) always failed to elicit locomotor activity.

These data show that the operation of one glutamate receptor class was sufficient to express locomotor activity. As locomotor activity developed at a lower frequency than in the control solution after pharmacological block of either NMDA or non-NMDA receptors, it is suggested that both receptor classes were involved in locomotor pattern generation.

1. INTRODUCTION

One fundamental property of the spinal cord is the generation of a motor programme that allows locomotion through coordination of limb muscle activity. This complex phenomenon is based on a locomotor rhythm that comprises alternating motor signals generated by the spinal cord through a local network (referred to as central pattern generator, CPG) even in the absence of sensory and supraspinal inputs (Grillner *et al*. 1986). The neuronal mechanisms underlying this activity remain uncertain. A suitable model to clarify these phenomena is the neonatal rat spinal cord *in vitro*, as application of neurotransmitter agonists such as 5-hydroxytryptamine (5-HT) and N-methyl-D-aspartate (NMDA) to this preparation produces patterns of activity ('fictive locomotion') closely resembling those observed during *in vivo* locomotion (reviewed by Rossignol & Dubuc (1994)). These rhythmic patterns, which can be recorded from ventral roots (VRs) or peripheral motor nerves, are characterized by left–right alternation (Kudo & Yamada 1987). In particular, the 5-HT-evoked patterns are very similar to those found during locomotion because they include finely tuned extensor–flexor motoneurone phasing for a large range of hindlimb muscles (Cowley & Schmidt 1994; Kiehn & Kjaerulff 1996).

Although the effects of exogenously applied agonists have been extensively studied in this system (Rossignol & Dubuc 1994), little is known about endogenous neurotransmitters that are synaptically released during the operation of the CPG. As far as the synaptic drive from the CPG to motoneurones is concerned, phasic activation of motoneuronal glutamate and glycine receptors has been shown to occur during rhythmic activity (Cazalets *et al*. 1996). With regard to the operation of the CPG itself, chloride-mediated inhibition has been reported to be necessary for pattern alternation but not for rhythmogenesis (Cowley & Schmidt 1995; Bracci *et al*. 1996*a*), which is suggested to require glutamatergic transmission via NMDA and non-NMDA receptors (Smith *et al*. 1988; Cazalets *et al*. 1992; Douglas *et al*. 1993). However, the demonstration that activation of NMDA and non-NMDA receptors by synaptically released glutamate is necessary for the operation of the CPG during locomotor activity is still lacking. The role of NMDA receptors in spinal pattern generation is of special relevance, as it has been proposed that NMDA-dependent pacemaker neurones could play a role in spinal rhythmogenesis (Hochman *et al*. 1994).

The present experiments were performed to clarify to what degree locomotor patterns depend on the synaptic activation of NMDA and non-NMDA receptors, which are known to mediate kinetically distinct responses (Jonas & Spruston 1994). This aim

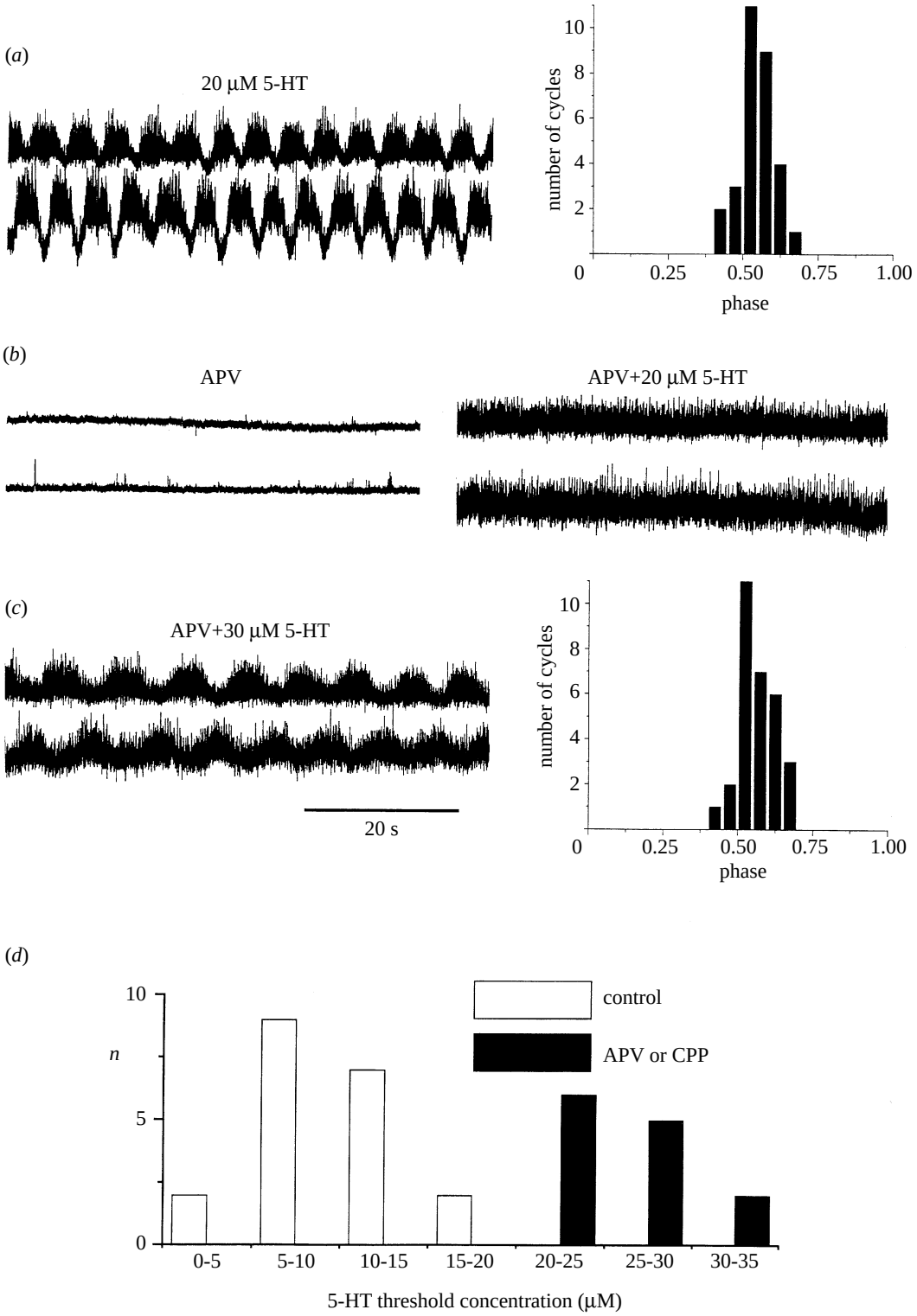

Figure 1. Effect of NMDA receptor block on fictive locomotion. Each pair of tracings illustrates recording from left (upper) and right (lower) L2 Vrs. (*a*) In control solution, 20 μM 5-HT elicited a persistent alternating pattern. The histogram of figure 1 *a* shows the distribution of the relative phase of the right VR with respect to the cycle developed in the left VR. (*b*) Left, spontaneous activity in the presence of APV (after 5-HT washout). Subsequent application of 20 μM 5-HT did not elicit alternating activity but only tonic firing (right). (*c*) 30 μM 5-HT evoked a persistent alternating pattern characterized by reduced amplitude and frequency (left), but normal phase relation (right).Time calibration applies to all tracings. (*d*) Pooled data of the distribution of the threshold concentration of 5-HT able to elicit stable alternating activity in control solution or in the presence of NMDA receptor antagonists. n = Number of preparations.

was pursued with a protocol that avoided glutamate receptor activation by exogenously applied agonists. For this purpose, the CPG was activated by 5-HT in all experiments; NMDA or non-NMDA receptor antagonists (in concentrations which fully blocked their receptors) were then applied to investigate their

ability to inhibit the rhythm. If any antagonism of 5-HT-induced locomotor activity was partial and/or surmountable with increasing concentrations of 5-HT, this observation would demonstrate that the function of the CPG does not critically depend on a particular receptor class activated by synaptically released glutamate. Consequently, it might be implied that the wiring properties of the network more than the characteristics of the excitatory signal are relevant for the genesis of locomotor activity.

2. METHODS

Experiments were performed on spinal cord preparations from neonatal Wistar rats (0–2 days old) as previously described (Bracci *et al*. 1996*a*). The isolated spinal cord (from the mid-thoracic region to the *cauda equina*) was fixed to the bottom of a recording chamber and continuously superfused (7.5 ml min^{-1}) with Krebs solution (in mM: NaCl 113, KCl 4.5, $MgCl_2 7H_2O$ 1, $CaCl_2$ 2, NaH_2PO_4 1, $NaHCO_3$ 25 and glucose 11, gassed with 95% O_2–5% CO_2, pH 7.4) at room temperature. Recording was performed simultaneously from pairs of VRs (usually at L2 or L5 segment level; Kjaerulff & Kiehn 1996) with miniature suction electrodes. VR responses were always recorded with a DC-coupled amplifier (WPI DAM50), band-passed (0.1 Hz–30 KHz), displayed on-line on a chart recorder (Gould RS3400) and stored on video tape or computer hard disk. Dorsal root stimulations were delivered via a miniature bipolar suction electrode. Stimulus duration was fixed at 0.1 ms and intensity was two times threshold (xT). Threshold was defined as the minimum stimulation intensity that elicited a detectable signal in the homolateral VR (on average $T = 2.0 \pm 0.5$ V, $n = 4$). All drugs were bath-applied via the superfusing solution at the concentrations mentioned in the text. Only those applications of 5-HT that elicited persistent alternating patterns were considered for analysis. All applications of 5-HT were maintained for > 10 min. Threshold concentration for 5-HT, both in the control and in the presence of glutamate antagonists, was defined in each preparation as the minimum concentration that could evoke a persistent alternating pattern. Period and relative phase measurements were performed over 30 consecutive cycles. Period was defined as the time between the onset of two cycles of locomotor activity, and phase was defined as the latency for the onset of a cycle in one root during the cycle of the contralateral root, divided by the period (Kjaerulff & Kiehn 1996). All data are presented as means $\pm$ s.d. Statistical significance was assessed by Student's *t*-test. 5-HT was purchased from Sigma; *R*(-)-2-amino-phosphonovaleric acid (APV), 3-((*R*)-2-carboxy-piperazin-4-yl)-propyl-1-phosphonic acid (CPP), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) were purchased from Tocris.

3. RESULTS

Simultaneous recordings were obtained from left and right L2 ($n = 12$) or L5 ($n = 13$) VRs in 25 spinal cord preparations. These recordings (acquired with a DC amplifier) were typically displayed at a slow speed to monitor locomotor activity triggered by 5-HT. In this case periodic widening of the traces due to increased motoneurone activity with superimposed fast biphasic changes (predominantly made up by patterned firing of segmental motoneurones) was clearly detected (cf. example in figure 1*a*). The slower changes in root polarization level mainly corresponded to slowly summating depolarization within the motoneurone population (Ho & O'Donovan 1993; Bracci *et al*. 1996*b*).

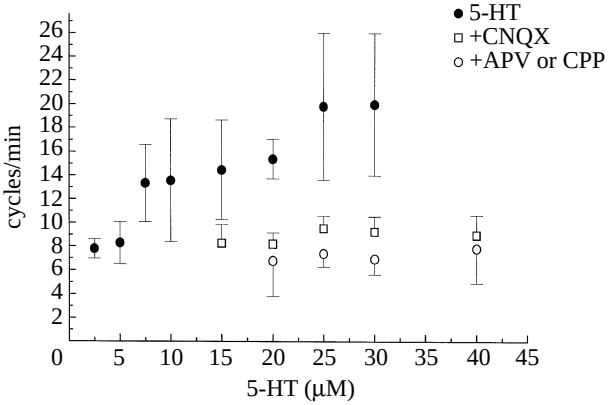

Figure 2. Frequency of locomotor activity (ordinate) versus 5-HT concentration (abscissa) before and after block of NMDA or non-NMDA receptors. Only concentrations of 5-HT that elicited persistent locomotor activity are plotted. Data are from 22 preparations.

Bath application of 5-HT evoked persistent alternating patterns in 22 out of 25 preparations. The threshold concentration of 5-HT was determined in 20 preparations by sequential applications with 2.5 or 5 μM increments interposed with > 15 min washouts. On average, threshold concentration was 9.4 ± 4.1 μM (see open columns in figure 1*d*); doses below this value, although ineffective in inducing fictive locomotion, were often capable of eliciting sustained firing recorded from VRs. The range of doses of 5-HT effective in evoking persistent alternating patterns was relatively narrow because concentrations higher than 20–30 μM elicited only early and transient episodes of alternating activity followed by tonic firing, presumably as a result of a gradual increase in the tissue concentration of 5-HT (Cazalets *et al*. 1992; Kjaerulff & Kiehn 1996). Within the 2.5–30 μM range the action of 5-HT on the frequency of fictive locomotion was dose-dependent and characterized by a relatively shallow growth with increasing doses (see filled circles in figure 2).

(*a*) *Fictive locomotion in the presence of NMDA receptor antagonists*

The effects of pharmacological block of NMDA receptors on the ability to generate 5-HT-induced alternating patterns were studied in 13 preparations. NMDA receptors were blocked by bath application of 20 μM APV ($n = 7$) or 10 μM CPP ($n = 6$). Although these concentrations are known to result in complete blocking of NMDA receptors (Corradetti *et al*. 1985; Jiang *et al*. 1990; Pinco & Lev-Tov 1993; Trueblood *et al*. 1996), we performed direct tests to confirm such a block in the present experimental conditions. To this end we examined the effects of bath application of 10 μM NMDA ($n = 6$) before, during, and after application of these antagonists. In the control solution, NMDA always elicited a very strong increase in VR

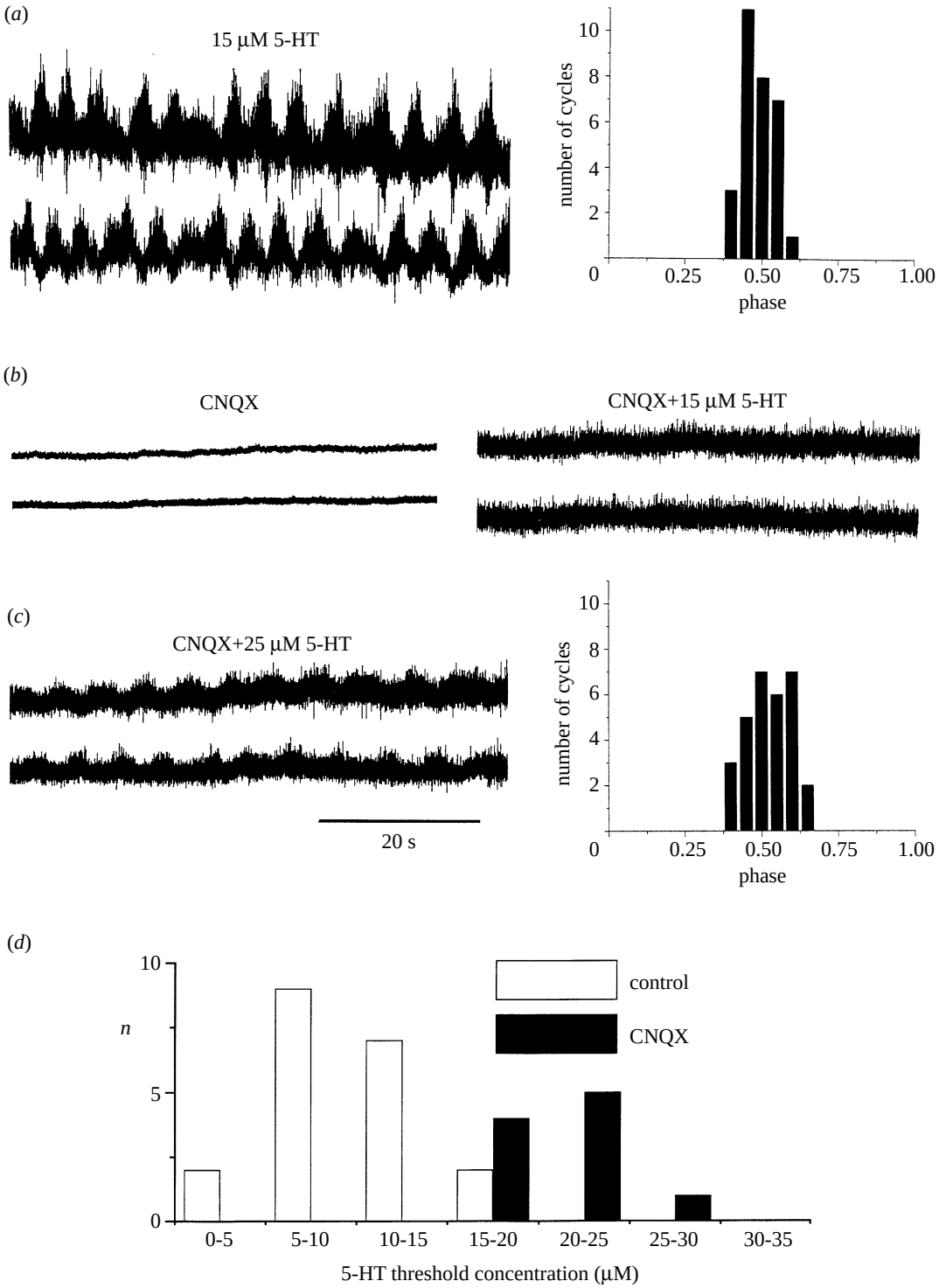

Figure 3. Effect of non-NMDA receptor block on fictive locomotion. (*a*) In control solution, 15 μM 5-HT elicited a persistent alternating activity in left (upper trace) and right L5 (lower trace) VRs. Histogram on the right illustrates the distribution of the relative phase of the right VR during the cycle of the left VR. (*b*) Left, baseline activity in the presence of CNQX (after 5-HT washout). Right, subsequent application of 15 μM 5-HT failed to elicit alternating activity, while tonic firing was present. (*c*) 25 μM 5-HT evoked persistent alternating activity with reduced amplitude and frequency (left), but normal phase relation (right). (*d*) Distribution of the threshold concentration of 5-HT for inducing stable alternating activity in control solution or in the presence of non-NMDA receptor antagonists. n = Number of preparations.

firing accompanied by transient or persistent alternating activity (not shown), as previously reported (Kudo & Yamada 1987). In the presence of APV or CPP, NMDA failed to produce any detectable effect on VR activity (not shown). Washout (> 20 min) after APV (but not CPP) application restored NMDA responses. Furthermore, we examined the effects of NMDA antagonists on the VR reflex induced by dorsal root stimulation at the same segmental level ($n = 2$). In the presence of APV, amplitude and duration of the

reflex were strongly decreased with a corresponding reduction in area by $78 \pm 14\,\%$ (not shown) consistent with previous observations on the NMDA component of this event (Jiang *et al.* 1990). These experiments, although not designed to investigate quantitatively NMDA receptor antagonism, indicate that at the antagonist doses used in the present study NMDA receptors were effectively blocked.

These observations allowed testing of the action of NMDA receptor antagonists on fictive locomotion evoked by different doses of 5-HT (figure 1). In this example, the 5-HT concentration threshold for alternating activity in control solution was 10 μM (not shown). Application of 20 μM of 5-HT elicited a persistent alternating pattern that was recorded from left and right L5 VRs (figure 1*a*, left). Concentrations of 5-HT $\geqslant 30$ μM elicited only transient alternating activity (not shown). The average relative phase of this pattern was 0.55 ± 0.06 (see histogram of phase distribution in figure 1*a*, right). In the presence of 20 μM of APV, baseline activity was very small and random (figure 1*b*, left); subsequent application of 20 μM of 5-HT evoked a strong increase in VR tonic firing without any detectable alternating pattern (figure 1*b*, right). However, a larger dose of 5-HT (30 μM) elicited a very regular persistent pattern of alternating bursts (figure 1*c*, left). Although burst amplitude and frequency were reduced with respect to the ones observed in control solution (compare traces in figure 1*a* with those in 1*c*), the phase relation between right and left VRs was not significantly changed (0.56 ± 0.07; see histogram of phase distribution in figure 1*c*, right). Similar results were obtained in 13 preparations. Pooled data indicated that in the presence of APV or CPP alternating patterns could still be elicited by 5-HT, even if the threshold concentration was always larger than that of the control solution (on average 24.8 ± 3.2 μM; see filled columns in the distribution histogram of figure 1*d*). It is worth noting that high doses (30–40 μM) of 5-HT, typically unable to induce persistent fictive locomotion in control solution, acquired the ability to evoke a sustained pattern in the presence of one of these antagonists. While these phenomena were not associated with a significative change in phase relation, figure 2 shows that the alternating pattern frequency (expressed as cycles min^{-1}) was significantly lower ($p < 0.005$) than in control solution over the narrow concentration range (20–30 μM) in which persistent fictive locomotion was present in either condition (compare filled with open circles in figure 2). Curiously, in the presence of NMDA receptor antagonism the fictive locomotor response to 5-HT displayed no apparent dose dependence (figure 2), and further dose increments up to 50 μM generated only transient episodes of locomotor activity.

(*b*) *Fictive locomotion in the presence of CNQX*

To test the role of non-NMDA receptors we used the non-NMDA antagonist CNQX (10 μM). Initially, the effectiveness of CNQX antagonism was tested on responses induced by the selective non-NMDA receptor agonist AMPA (1 μM; $n = 5$). In control solution AMPA elicited a strong increase in VR tonic firing that in two preparations was accompanied by transient episodes of alternating activity; in the presence of CNQX, AMPA had no effect on VRs (not shown). In two preparations DR-evoked reflex was monitored before and during application of CNQX; in the presence of this antagonist the early component of the reflex was strongly depressed (with a corresponding reduction in area by 84 ± 8 %; not shown) confirming blocking of non-NMDA receptors (Pinco & Lev-Tov 1993). The effects of CNQX were poorly reversible on washout.

Figure 3 shows typical results of 5-HT application before and after blocking of non-NMDA receptors. In this preparation 5-HT concentration threshold for alternating activity (recorded from left and right L2 VRs) was 10 μM (not shown). With 15 μM of 5-HT a persistent alternating pattern was observed (figure 3*a*, left), with an average relative phase of 0.52 ± 0.09 (phase distribution is shown in figure 3*a*, right). In the presence of CNQX there was minimal, random, spontaneous activity (figure 3*b*, left), and application of 15 μM of 5-HT elicited VR tonic firing without alternation (figure 3*b*, right). Subsequent application of 25 μM of 5-HT, however, revealed a persistent alternating pattern (figure 3*c*, left). Even though the amplitude and frequency of this pattern were markedly reduced with respect to that observed before application of the antagonist (compare traces in figures 3*a* and 3*c*), left–right coordination was apparently unchanged, as shown in the histogram of figure 3*c* (right; relative phase in CNQX was 0.47 ± 0.07). In all preparations ($n = 10$) 5-HT was effective in evoking alternating patterns in the presence of CNQX, even if the concentration threshold was larger than that of the control solution (on average 21.4 ± 3.6 μM; see distribution histograms in figure 3*d*). Cycle frequency was lower than in the absence of this antagonist and poorly dependent on 5-HT concentration (see open squares in figure 2). Pooled data show that, by analogy with the results with NMDA receptor antagonism, there was a narrow window of 5-HT doses (15–30 μM) at which persistent patterns could be evoked in the control as well as in the CNQX solution (figure 2). Like the case of NMDA antagonists, in the presence of CNQX, burst amplitude was lower than in control solution and the phase relation was preserved (not shown). Higher doses of 5-HT were unable to generate persistent rhythmic activity. When APV and CNQX were simultaneously applied ($n = 4$), 5-HT (5–120 μM) always failed to elicit alternating patterns.

Collectively, these results show that highly coordinated alternating patterns were induced by 5-HT even when the NMDA or non-NMDA component of glutamatergic transmission was pharmacologically blocked; in this case activation of these patterns required higher doses of 5-HT and displayed lower burst frequency and amplitude.

(*c*) *Fictive locomotion following sequential application of APV and CNQX*

The observation that a narrow range of 5-HT doses

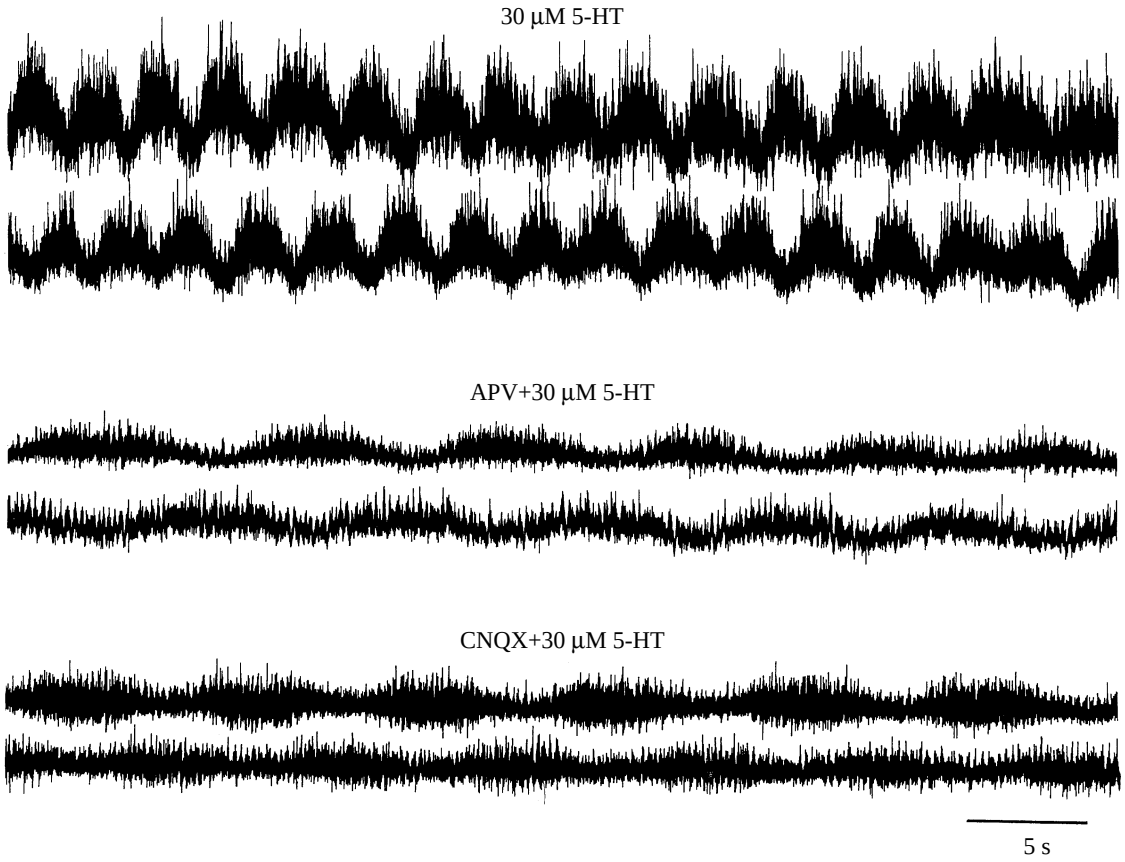

Figure 4. Comparison of locomotor activity before and after block of NMDA or non-NMDA receptors in the same preparation. Locomotor activity induced by 30 μM 5-HT was recorded from left (upper trace) and right (lower trace) L5 VRs in control solution (top), in the presence of 20 μM APV (middle) or in the presence of 10 μM CNQX (bottom; after APV washout). Note that in this example in the presence of CNQX there was lack of slow changes in root polarization level, although this was not a consistent observation (see for instance alternating baseline shifts in figure 3*c*).

(20–30 μM) could elicit a stable pattern in control solution as well as in the presence of NMDA or non-NMDA receptor antagonism prompted further studies to compare the characteristics of the rhythm in the same preparations under these three experimental conditions. This goal was difficult because in individual preparations there was little or no overlap between effective 5-HT concentrations under the three conditions. In fact, only in two out of six preparations was it possible with the same concentration of 5-HT (20 or 30 μM) to induce a stable pattern first in control solution and subsequently in APV or CNQX solution. For this purpose the same dose of 5-HT was retested in APV solution and then in CNQX solution after APV washout (> 30 min). Figure 4 shows one such example in which 30 μM of 5-HT induced alternating patterns in control solution (upper traces), in the presence of APV (middle traces) and CNQX (lower traces). Although bursts observed in the presence of APV or CNQX were clearly reduced in amplitude, phase relation was not significantly different in these three cases. Burst frequency was much larger in control solution (20 cycles min^{-1}) than in the presence of APV (7.2 cycles min^{-1}) or CNQX (7.1 cycles min^{-1}). Similar slowing down of rhythmic activity (while phase was preserved) was found in the other preparation in which such a comparison was possible.

4. DISCUSSION

The principal finding of the present study is the novel demonstration that the spinal network producing locomotor-like patterns remained operational despite blocking of either NMDA or non-NMDA receptors, because normally alternating patterns of activity took place in left and right lumbar VRs in the presence of antagonists for one class of glutamate receptors.

(*a*) *Fictive locomotion persists after blocking of either NMDA or non-NMDA receptors*

In the rat spinal cord, *in vitro* 5-HT produces fictive locomotion by activating a CPG, and it is considered to be the agent that elicits rhythmic patterns most closely similar to those found during locomotion *in vivo* (Cowley & Schmidt 1994; Kiehn & Kjaerulff 1996). After application of NMDA or non-NMDA receptor antagonists, 5-HT induced an alternating pattern of activity, which, although smaller in amplitude and cycle frequency, retained identical phase shift between left and right VRs, indicating that this rhythm could still be considered as fictive locomotion. These observations were strengthened by results obtained from preparations in which the same dose of 5-HT could be used to evoke rhythmic activity before and after

application of each antagonist. Hence, it can be inferred that in the presence of APV (CPP) or CNQX the operation of the the locomotor network was maintained, even though with a distinctly slower output and a reduced sensitivity to the triggering action of 5-HT. This view is compatible with the observation that after blocking of one class of glutamate receptors the dose–response relation for 5-HT became almost flat.

(*b*) *Role of glutamate receptors in the operation of the CGP*

The excitatory input from the CPG to motoneurones has been suggested to be mediated both by NMDA and non-NMDA receptors at motoneuronal level (Cazalets *et al*. 1996). In line with this proposal, the observed reduction in burst amplitude by APV (CPP) or CNQX might be explained by depression of motoneuronal sensitivity to the glutamatergic drive. It is, however, difficult to use the same explanation (which merely implies a decrease in the number of active synapses on motoneurones) for the decreased frequency of fictive locomotion in the presence of either antagonist, because this finding implies an operational change at the level of the rhythm-generating network. It is therefore apparent that both NMDA and non-NMDA synaptic receptors were involved in the intrinsic operation of the CPG, as well as in the synaptic drive from the CPG to motoneurones.

NMDA and non-NMDA receptors are thought to be colocalized at excitatory synapses, although they mediate membrane responses with very different kinetics (Jonas & Spruston 1994) and voltage-dependence (Mayer & Westbrook 1987). Curiously, fictive locomotion was expressed by activation of either class of receptors; in this case the frequency, although slower than in control solution, was remarkably similar in APV (CPP) or CNQX solution (see figure 4). The mechanism that enables the motor network to express rhythmic activity at a similar frequency regardless of the absence of a particular glutamate receptor class remains to be clarified, but confers a peculiar property to the spinal CPG that will have to be taken into account when modelling spinal rhythmogenesis. It is necessary that at least one class of glutamate receptors is available for activation as simultaneous blocking of NMDA and non-NMDA receptors prevented the onset of alternating patterns even with very high doses of 5-HT (up to 120 μM). This condition thus differs from the one recently reported for the chick embryo spinal cord in which some coordinated motor activity can take place even after simultaneous blocking of NMDA and non-NMDA receptors (Chub & O'Donovan 1996). It remains to be established whether in the rat spinal cord simultaneous blocking of NMDA and non-NMDA receptors abolished fictive locomotion by either suppressing the operation of the CPG or simply preventing transmission of rhythmic output to motoneurones.

Although the cellular mechanisms of spinal pattern generation in mammals are poorly understood, it has been proposed that intrinsic pacemaker neurones may drive this kind of rhythmicity (see review by Katz (1996)). Pacemaker neurones have been described in the lamprey spinal cord (Grillner *et al*. 1991) as well as in the area sorrounding the central canal of the rat spinal cord (Hochman *et al*. 1994). NMDA receptors, which activate with unusual voltage dependence (Mayer & Westbrook 1987), are thought to determine the oscillatory firing behaviour of these cells. The present finding, that alternating activity developed even in the presence of NMDA receptor antagonists, shows that intact function of NMDA receptor-dependent pacemaker neurones is not a prerequisite for spinal rhythmogenesis.

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