Is NMDA Receptor Activation Essential for the Production of Locomotor-Like Activity in the Neonatal Rat Spinal Cord?

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Submitted 7 January 2005; accepted in final form 19 August 2005

INTRODUCTION

An important role for N-methyl-D-aspartate (NMDA) receptors in locomotor network operation is suggested by the results of numerous studies using a variety of vertebrate spinal cord preparations. In vitro bath application of NMDA promotes locomotor activity in the neonatal rat (e.g., Cazalets et al. 1990, 1992; Kudo and Yamada 1987; Smith and Feldman 1987; Smith et al. 1988), mouse (Sillar and Simmers 1994), turtle (Guertin and Hounsgaard 1998), and neonatal rat (Hochman and Schmidt 1998). Not surprisingly, the NMDA receptor antagonist d-2-amino-5-phosphono-valeric acid (AP5) blocks locomotion induced by bath application of NMDA (Cazalets et al. 1992; Kudo and Yamada 1987). However, to determine whether NMDA receptors have an essential role in locomotor networks, it is necessary to test whether NMDA antagonists block locomotion elicited by methods other than NMDA application. Such evidence was reported in one early study of the in vitro neonatal rat preparation (Smith et al. 1988). NMDA receptor antagonists also suppress locomotion in decerebrate (Douglas et al. 1993) and spinalized (Giroux et al. 2003) cats, and l-DOPA–induced locomotion in the spinalized rabbit (Fenaux et al. 1991). However, more recent studies, involving the in vitro neonatal rat preparation, reported that locomotor-like activity induced by 5-hydroxytryptamine (5-HT) or increased extracellular K+ concentrations persists in the presence of AP5 (Beato et al. 1997; Bracci et al. 1998). In addition, NMDA receptor activation does not seem to be essential for locomotor activity in the neonatal mouse cord (Nishimaru et al. 2000; Whelan et al. 2000). These observations, which challenge the idea that NMDA receptor activation is critical for motor rhythogenesis, stimulated the present re-examination of the role of NMDA receptors in the in vitro neonatal rat.

Our interest in exploring the role of NMDA receptors in locomotor networks is also prompted by the fact that nonlinear membrane voltage behavior is associated with NMDA receptor activation. A voltage-sensitive conductance, caused by voltage-dependent Mg2+ blockade of the NMDA receptor channel, confers a region of negative slope conductance (RNSC) in the current-voltage (I-V) relationship (Flatman et al. 1983; MacDonald et al. 1982; Mayer and Westbrook 1987; Mayer et al. 1984; Nowak et al. 1984). This property underlies the production of rhythmic voltage oscillations in synaptically isolated spinal neurons in the lamprey (Sigvardt et al. 1985; Wallen and Grillner 1987), frog (Sillar and Simmers 1994), turtle (Guertin and Hounsgaard 1998), and neonatal rat (Hochman et al. 1994a,b; MacLean and Schmidt 2001; MacLean et al. 1997, 1998). As expected, the RNSC and intrinsic voltage oscillations are abolished by Mg2+ removal (MacLean and Schmidt 2001; Reith and Sillar 1998; Sillar and Simmers 1994; Wallen and Grillner 1987). We and others have speculated that NMDA receptor–mediated active membrane properties may be particularly well suited for neurons participating in an oscillatory network such as the one generating locomotion (e.g., Alford and Sigvardt 1989; Grillner et al. 2001; Guertin and Hounsgaard 1998; Kiehn et al. 2000; Schmidt et al. 1998; Sillar and
Simmers 1994; Wallen and Grillner 1987), and this property has been incorporated into computer-simulated models of the locomotor network (Brodin et al. 1991; Hellgren et al. 1992; Roberts et al. 1995). In addition, 5-HT enhances the NMDA receptor–mediated RNSC and promotes intrinsic membrane voltage oscillations (MacLean and Schmidt 2001; MacLean et al. 1998). This modulatory interaction may be one mechanism underlying the fact that combined 5-HT/NMDA application induces a locomotor-like pattern more reliably than administration of NMDA or 5-HT alone (Cowley and Schmidt 1994a; Kjaerulf et al. 1994; Ssquilli-Houssaini et al. 1993) and further implicates NMDA receptor–mediated active membrane properties in the operation of the locomotor network.

In these experiments, we blocked NMDA receptors in the neonatal rat spinal cord during locomotion induced by bath application of 5-HT or acetylcholine (ACh) or elicited by electrical stimulation of the brain stem. The results indicate that, although NMDA receptors make an important contribution to locomotor network activation using some experimental protocols, the spinal cord nevertheless retains an inherent capacity to generate locomotor output if appropriate levels of network excitation are achieved, even in the presence of NMDA receptor blockade. Consistent with this conclusion, the data also show that nonlinear membrane properties associated with NMDA receptor activation are unessential for locomotor rhythm generation. Some of the following data has been presented previously in abstract form (MacLean and Schmidt 1997).

METHODS

Experiments were performed on 113 Sprague-Dawley rats (1–5 days old). The technique for isolation of the spinal cord, extracellular recording, and neurochemical induction of rhythmic activity has been described previously (e.g., Cowley and Schmidt 1995). In brief, animals were anesthetized with ether, decerebrated at the mid-collicular level, eviscerated, and placed in a bath chamber containing artificial cerebrospinal fluid (ACSF) composed as follows (in mM): 128 NaCl, 3.0 KCl, 0.5 NaH2PO4, 1.5 CaCl2, 21 NaHCO3, and 30 glucose, equilibrated to pH 7.4 with 95% O2-5% CO2. Unless otherwise indicated, the MgSO4 concentration was 1.0 mM. In some experiments, the K+ concentration was manipulated, as indicated in RESULTS. The spinal cord was transected at C1, isolated bilaterally intact, and positioned in the bath ventral side up. In some preparations, the spinal cord was transected at T3, or the brain stem was left intact (transected at the mid-collicular level) for purposes of electrical stimulation of the brain stem. Experiments were conducted at room temperature (ACSF ~ 22°C). When left intact, the brain stem was separated from the spinal cord bath using a partition at C1 (see Cowley and Schmidt 1997).

Ventral root activity was recorded using glass suction electrodes applied to L2 and L5, which monitor flexor and extensor phases of the step cycle, respectively (Cowley and Schmidt 1994a; Kieln and Kjaerulf 1996). The recordings were band-pass filtered (30–3,000 Hz), digitized, and captured using AxoScope (v 8.1, Axon Instruments) software. In six experiments, wideband filtering at 0.1–3,000 Hz was used to allow monitoring of locomotor-related ventral root electrotonic potentials (as described by Beato et al. 1997; Kremer and Lev-Tov 1997). AxxoScope files were converted to an appropriate binary format for further analysis using special purpose software (developed by the Spinal Cord Research Centre, University of Manitoba).

Neurochemicals were applied from concentrated stock solutions (10 mM) to a static bath that was continuously oxygenated and agitated. All concentrations refer to final bath concentrations, which ranged as follows: NMDA, 0.3–10 μM; 5-HT, 6–80 μM; dihydrokainate (DHK), 50–100 μM; edrophonium, 20 μM; ACh, 10–30 μM; D-AP5, 2–20 μM. NMDA and 5-HT were purchased from Sigma; DHK and D-AP5 were purchased from Tocris. Recordings were obtained after a stable response to the applied neurochemical was obtained (usually this required 5–15 min).

In some experiments, the brain stem was left intact (transected at the mid-collicular level); locomotion was induced by electrical stimulation of the brain stem using a silver wire in an ACSF-filled glass electrode with an internal tip diameter of 200–300 μm, as described previously (Zaporozhets et al. 2004). The tip of the glass electrode was placed in contact with the ventral surface of the brain stem, and monophasic rectangular current pulses (4–20 ms, 0.5–10 mA, 0.8–2.0 Hz) were delivered using bipolar stimulation.

Whole cell patch electrode recordings were obtained from two motoneurons to show that Mg2+ ion removal in this preparation effectively abolished the NMDA-induced RNSC. The cells were identified as motoneurons by the presence of an antidromic response to ventral root electrical stimulation. The whole cell patch recording method is described in our earlier work (MacLean and Schmidt 2001).

Data analysis

To determine whether Mg2+ ion removal disrupted rhythmic activity, two forms of analysis were performed. The coefficient of variance (CV) of successive cycle periods in a given episode was determined (i.e., SD of cycle period/mean cycle period). This allowed analysis of the regularity of the interburst intervals (cycle period) for a given episode. Polar plots of left and right L2 ventral root recordings allowed analysis of side-to-side coordination (Batschelet 1981; Kjaerulf and Kiehn 1996; Zar 1974). The cycle period of L2 ventral root discharge was measured for each cycle in a given episode, and the latency to onset of L2 discharge on the contralateral side was determined for each corresponding cycle. A phase value for each step cycle was calculated using the formula

$$\text{phase lag} = \frac{\text{left L2 latency (ms)} - \text{right L2 latency (ms)}}{\text{left L2 cycle period (ms)}}$$

The phase values were displayed graphically as data points on a polar plot (Figs. 4–6). The mean phase value for each episode was displayed as a vector with length r (Zar 1974). The length of the vector ranged from 0 to 1 and is a measure of the concentration of phase lags around the mean phase value for the episode. Rayleigh’s circular statistical test (Batschelet 1981; Zar 1974) was applied to determine whether the phase lags in a given episode were significantly dispersed, indicating no phase relationship, or concentrated, suggesting coupling of left-right discharge activity. If r was greater than the critical Rayleigh’s value (cr) for a given P value, the left-right relationship was considered phase-related.

RESULTS

NMDA receptor blockade

The NMDA receptor antagonist AP5 was used to examine whether NMDA receptor activation is essential for motor rhythm generation. According to previous work, an AP5 concentration of 20 μM completely blocks NMDA receptors in the in vitro neonatal rat spinal cord (e.g., Beato et al. 1997; Pinco and Lev-Tov 1993). Consistent with these reports, AP5 (20 μM) completely blocked all forms of RNMDA (10 μM) induced ventral root discharge, both tonic and phasic, in 17 of 17 preparations tested in this study (Fig. 2D).

Several methods were used to induce locomotor-like activity. Under some experimental conditions, AP5 consistently
blocked locomotor-like activity, and under other conditions, it did not.

In 20 preparations, 20 μM 5-HT was applied to spinal cord, transected at either C1 (n = 15) or T6 (n = 5). After a stable locomotor-like pattern was established in the lumbar region (Fig. 1A1), AP5 (20 μM) abolished rhythmic ventral root discharge within 1–3 min in all 20 preparations (Fig. 1A2). 5-HT–enhanced tonic background discharge persisted in the presence of AP5. After 5-HT–induced rhythmic activity was abolished by AP5, no recovery of locomotor-like ventral root activity was observed in the presence of AP5 during monitoring for up to 3 h. AP5 washout successfully restored locomotor-like activity in the presence of 5-HT after 5–15 min (Fig. 1A3).

In 5 of the 20 experiments, we examined whether additional increases in 5-HT concentration promoted recovery of rhythmic ventral root activity (in the presence of 20 μM AP5) as reported by Beato et al. (1997). In keeping with the experiments described by Beato et al., these preparations were transected at the mid-thoracic level (T6). After initial 20 μM 5-HT–induced rhythmic activity was blocked by AP5 (20 μM), the 5-HT concentration was slowly increased in 10-μM increments up to a total of 80 μM. In all five preparations, tonic activity persisted, and rhythmic activity failed to reappear, regardless of the 5-HT concentration, as long as AP5 (20 μM) remained in the bath.

In another set of experiments, electrical stimulation of the brain stem (Zaporozhets et al. 2004) was used to induce locomotor-like activity (n = 9). A barrier was established at the C1 level to separate the brain stem and spinal cord bath compartments. After activation of locomotor-like activity (Fig. 1B1), AP5 application to the spinal cord portion of the bath abolished rhythmic activity in all nine preparations (Fig. 1B2). Effective AP5 concentrations in these particular experiments ranged from 5 to 20 μM.

To determine whether AP5 also blocked locomotor-related activity in motoneurons that was subthreshold for action potential generation, wideband (0.1–3,000 Hz) ventral root recordings were examined in three 5-HT–induced and three 5-HT/NMDA–induced preparations. The third channel in Fig. 1C1 (right L2) uses the wideband filter setting and shows an underlying locomotor phase-related oscillation of the ventral root potential. This ventral root potential is caused by electrotonically conducted voltage shifts in the motoneuron population. After application of AP5 (20 μM), ventral root fast (spike-related) and slow (electrotonic) activity is suppressed (Fig. 1C2), suggesting that NMDA receptor activation mediates an important component of the rhythmogenic excitation of motoneurons during 5-HT–induced locomotor-like activity.

ACh combined with the acetylcholinesterase inhibitor edrophonium (Edro, 200 μM) usually elicits sustained rhythmic activity characterized by left-right alternation and nonlocomotor-like co-activation of ipsilateral flexor and extensor discharge (Cowley and Schmidt 1994b). However, more recent experiments have shown that if low levels of cholinergic stimulation are used, transient episodes of rhythmic activity with a locomotor-like pattern can be evoked (Jordan and McVagh 2004). Thus in 18 C1-transected preparations, a low concentration of edrophonium (20 μM) was combined with ACh (20 μM), and transient locomotor-like patterns were obtained (Figs. 2A1 and B1, and 7A1). AP5 (20 μM) abolished the rhythmic activity in 14/18 preparations (Fig. 2A2). However, in 4/18 preparations, the locomotor-like rhythm persisted despite AP5 (Fig. 2B2). Adequate blockade of NMDA receptors in these experiments was confirmed by the lack of effect on ventral root discharge of subsequently adding a high concentration of NMDA (10 μM; Fig. 2B3).

In five preparations, an attempt was made to restore rhythmic activity by increasing the ACSF K+ concentration, in 1-mM increments, after AP5 had terminated 5-HT–induced

FIG. 1. Effect of the N-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonovaleric acid (AP5) on locomotor-like activity induced by 5-hydroxytryptamine (5-HT) or electrical stimulation of the brain stem. A1: 5-HT (20 μM) induced ventral root discharge that alternated between flexors (L2) and extensors (L5), and between left (L) and right (R) sides at the same segmental level of the cord, consistent with a locomotor-like pattern. A2: AP5 (20 μM) abolished the 5-HT–induced rhythm. A3: subsequent washout of AP5 was associated with restoration of the locomotor-like activity. B1: brain stem electrical stimulation (BES) at 1.4 Hz induced a locomotor-like pattern of ventral root discharge. B2: AP5 (20 μM) abolished the rhythm. B3: locomotor-like pattern was restored after washout of AP5. C1: wideband (0.1–3,000 Hz) filtering was used to record subthreshold (for spike generation) slow activity on the R-L2 channel. Voltage oscillations caused by electrotonically conducted motoneuron population activity on R-L2 alternated with R-L5 ventral root discharge. L-L2, L-L5, and R-L5 were recorded at 30–3,000 Hz. C2: AP5 (20 μM) suppressed phasic spike discharge on all channels as well as the slow voltage fluctuations in the wideband filtered R-L2 channel.
locomotor-like rhythm (Fig. 2C). Bracci et al. (1998) showed that excitation of spinal cord neurons by increased K+ concentration could elicit locomotor-like activity in the in vitro neonatal rat preparation. In this series, elevation of the K+ level to 10 mM successfully restored locomotor-like activity in two of the five 5-HT/AP5 preparations (Fig. 2C). In addition, increased K+ concentration alone (i.e., without 5-HT added to the bath) in the range of 10–11 mM, was able to elicit locomotor-like activity in the presence of AP5 blockade in five of seven preparations, consistent with previous observations by Bracci et al. (1998).

Suppression of NMDA receptor–mediated nonlinear membrane voltage response

To examine the role of NMDA receptor–mediated nonlinearity on locomotor network function, we selectively abolished the RNSC, while otherwise preserving the capacity for NMDA receptor activation. This was accomplished by removing Mg2+ from the bath. The current-voltage plots obtained from two lumbar motoneurons, subjected to whole cell voltage clamp, show that NMDA application induced a RNSC (Fig. 3A), which can be effectively abolished by Mg2+ ion removal (Fig. 3B) as reported previously in the in vitro neonatal rat preparation (MacLean and Schmidt 2001) and as predicted by earlier studies of NMDA receptors (Fitzman et al. 1983; MacDonald et al. 1982; Mayer and Westbrook 1987; Mayer et al. 1984; Nowak et al. 1984).

In the absence of bath-applied neurochemicals, exposure of the spinal cord to Mg2+–free ACSF promoted arrhythmic phasic ventral root discharge, and sometimes synchronous bursts, in 17/17 C1-transected preparations (Fig. 4, A1 and B1). This activity developed after a delay of 5–15 min and never evolved to a locomotor-like pattern during observations lasting ≤90 min. The discharge was abolished by application of AP5 (n = 10; Fig. 4A2), consistent with its origin from enhanced NMDA receptor activity in the absence of Mg2+ ion blockade. Addition of 5-HT (6–30 μM) to Mg2+–free ACSF transformed disorganized ventral root discharge (Fig. 4B1) into a locomotor-like pattern in 12/16 preparations tested (Fig. 4B2). The other four preparations continued to display an arrhythmic pattern despite the presence of 5-HT.

In six preparations, NMDA (3–10 μM) was co-applied with 5-HT (10–40 μM). This combination promotes a stable locomotor-like pattern in the neonatal rat spinal cord (Fig. 5A1) (Kjaerulff et al. 1994; Sqalli-Houssaini et al. 1993). One to 8 min after removal of Mg2+ ions, 5-HT/NMDA–induced rhythm frequency increased (Fig. 5A2). Five to 20 min after Mg2+ ion removal, the rhythm became disorganized and eventually transformed to tonic discharge (n = 6/6; Fig. 5, A3 and A4). Restoration of normal Mg2+ ion concentration in the ACSF converted disorganized rhythms, or tonic discharge, in the presence of NMDA/5-HT to a locomotor-like pattern (n = 5/5; Fig. 5A5). If 5-HT alone was used to induce locomotor-like activity in Mg2+–free ACSF (Fig. 5B1), application of NMDA,
even at very low concentrations (e.g., 0.3 μM), provoked a disorganized pattern (n = 2, Fig. 5B2). These results suggest that NMDA receptor-mediated nonlinear membrane properties are not essential for locomotor rhythm generation. However, excessive NMDA receptor channel activity in the absence of Mg2+ ions can interfere with the ability of the network to organize a locomotor-like pattern.

In 12 other experiments, a locomotor-like pattern was established using a combination of DHK (50–100 μM) and 5-HT in normal ACSF (Fig. 6A1) (Cowley and Schmidt 1994a). DHK is an excitatory amino acid uptake inhibitor that enhances excitatory amino acid action at sites of endogenous glutamatergic transmission, in contrast to the widespread and less discriminating excitation produced by whole cord (bath) application of NMDA. Unlike the disruption of 5-HT/NMDA–induced rhythms consistently observed in Mg2+–free ACSF (e.g., Fig. 5, A3 and A4), Mg2+ removal failed to disrupt the locomotor-like pattern induced by DHK/SHT in 7 of 12 preparations tested. The rhythm frequency increased in Mg2+–free ACSF (Fig. 6A2) and decreased after normal Mg2+ concentration (1.0 mM) was restored (n = 3; Fig. 6A3) or AP5 (2–6 μM) was added. The increase in rhythm frequency caused by Mg2+ ion removal for all 12 preparations is shown in Fig. 6B. In 5 of the 12 preparations exposed to DHK/SHT, locomotor-like coordination was initially preserved, at an increased frequency, in Mg2+–free ACSF (Fig. 6C1), but 5–10 min later, the ventral root activity became disorganized (Fig. 6C2). Subsequent partial blockade of NMDA receptors using a relatively low concentration of AP5 (9 μM) restored a locomotor-like pattern in Mg2+–free ACSF (Fig. 6C3). This observation again suggests that excessive NMDA receptor channel activity, in the absence of Mg2+ blockade, interfered with the development of a locomotor-like pattern of network activity.

Similar to the results using 5-HT, locomotor-like rhythms induced by ACh/Edro (Fig. 7A1) could still be elicited in 5/12 preparations after Mg2+ ion removal (Fig. 7A2).

The effect of Mg2+ ion removal on locomotor-like activity induced by electrical stimulation of the brain stem was examined in 13 brain stem spinal cord preparations (Fig. 7B1). In each example, in the absence of electrical stimulation of the brain stem, removal of Mg2+ ions produced spontaneous disorganized ventral root discharge (Fig. 7B2), similar to the spontaneous Mg2+–free ventral root activity noted above (Fig. 4A1). Electrical stimulation of the brain stem, in the absence of Mg2+, evoked a well-organized locomotor-like pattern similar to the pattern observed before Mg2+ ion removal (Fig. 7B1), but faster in frequency (Fig. 7B2).

**DISCUSSION**

The main finding of this study is that NMDA receptor activation and its associated nonlinear membrane voltage property are not universally essential for locomotor rhythm generation in the in vitro neonatal rat spinal.

One limitation of using ventral root recordings to monitor locomotor-like activity during NMDA receptor antagonist ap-
plication is that information is provided only about the final output of the locomotor network. That is, the precise elements within the spinal cord where NMDA receptor actions take place is not determined by these experiments. However, locomotor rhythm frequency has been shown to increase in relation to NMDA concentration (Cazalets et al. 1992; Kudo and Yamada 1987) and decrease in relation to AP5 concentration (Beato et al. 1997; Smith et al. 1988), which implies that at least some NMDA receptors supporting locomotor network operation are located on interneuronal components, in addition to any NMDA receptors activated on motoneurons.

Role of NMDA receptors in rhythm generation

As outlined in the Introduction, NMDA receptors are known to contribute to locomotor rhythm generation in a variety of vertebrate preparations. However, examples of spinal motor rhythm generation occurring independent of NMDA receptor activation have also been documented. Of particular relevance is the study by Beato et al. (1997), who reported that 5-HT–induced locomotor rhythms, initially blocked by AP5 (20 μM), re-emerged if higher 5-HT concentrations (25–40 μM) were applied (Beato et al. 1997). In our experiments, however, AP5 consistently blocked 5-HT–induced rhythms. The reason for the discrepancy between our results and those of Beato et al. is unclear. We specifically included preparations with mid-thoracic transection in an attempt to replicate as closely as possible the in vitro preparation used by Beato et al. We used 5-HT and AP5 concentrations similar to those reported in their paper, including application of increasing 5-HT concentrations in an attempt to overcome AP5 blockade. Nevertheless, locomotor-like activity induced by 5-HT was always blocked by AP5 (20 μM) in our experiments, regardless of 5-HT concentration. Of note, we used a lower ACSF K⁺ concentration (4.5 mM) than Beato et al. (4.5 mM). In view of the effect of high K⁺ concentration on eliciting locomotor-like activity in this preparation (Bracci et al. 1998), the K⁺ level deserves consideration. However, we were unable to elicit locomotor-like activity in the presence of AP5 until the K⁺ level reached 10–11 mM. K⁺ levels in the range of 4–9 mM failed to elicit rhythmic activity. We also observed that locomotor-like activity induced by electrical stimulation of the brain stem was always abolished by AP5.

Although our initial observations, using 5-HT and brain stem electrical stimulation, showed consistent AP5-mediated blockade of locomotor-like activity, further experiments revealed this was not an invariable rule. In particular, increased K⁺ levels (in the range of 10–11 mM) were able to elicit locomotor-like activity despite AP5 blockade of NMDA receptors, consistent with the study by Bracci et al. (1998). In addition, although AP5 pretreatment usually blocked rhythmic activity induced by ACh/Edro in this series, locomotor-like activity could still be evoked in 22% of the preparations.

The literature contains other examples of locomotor network operation occurring independent of NMDA receptor activation. In the lamprey spinal cord, slow low-level locomotor activity is NMDA receptor-dependent, whereas intense fast rhythmic activity is unperturbed by NMDA receptor antagonists (Brodin and Grillner 1985; Brodin et al. 1985). In the neonatal rat sacrococcygeal cord, a “fast” NMDA receptor–independent rhythm evoked by alpha-1 adrenergic agonists was recently
described (Gabbay and Lev-Tov 2004). This caudally located rhythm generator recruited flexor but not extensor motoneurons in the lumbar cord and was postulated to facilitate the appearance of rhythmic tail and locomotor movements in response to sacrocaudal stimulation (Gabbay and Lev-Tov 2004). Spontaneous hindlimb rhythmic activity in the chick was suppressed by NMDA receptor blockade, but rhythmic activity re-emerged 30–90 min later, despite continued exposure to AP5 (Barry and O'Donovan 1987; Chub and O'Donovan 1998). In contrast, we found no evidence of locomotor rhythm escape from NMDA receptor blockade while monitoring the effect of 5-HT in the presence of AP5 for up to 3 h. Locomotion in in vivo spinalized rabbits, evoked by L-DOPA, was abolished by the noncompetitive NMDA receptor antagonist MK-801 (Fenaux et al. 1991). However, the effect of MK-801 on stepping elicited by electrical stimulation of the sural nerve or brain stem in the decerebrate rabbit was more complex. Burst amplitude decreased, but rhythm enhancement occurred in the form of increased rhythm frequency and prolongation of rhythmic discharge after stopping the stimulation (Fenaux et al. 1991).

Role of NMDA receptor–mediated membrane voltage nonlinearity

Mg\(^{2+}\) ion removal was associated with disruption of 5-HT/NMDA–induced locomotor rhythms. Originally, this particular observation led us to hypothesize (MacLean and Schmidt 1997; Schmidt et al. 1998), as had others (see Introduction), that the RNSC may be important for the development or stabilization of a locomotor-like pattern. This more comprehensive series of experiments indicates that this is not the case. Pattern disruption in Mg\(^{2+}\)/H\(^{11001}\)-free ACSF under certain experimental conditions, using bath-applied NMDA or DHK in particular, was likely caused by excess NMDA receptor channel conductance rather than the result of abolishing the RNSC. Otherwise, the main effect of Mg\(^{2+}\) removal in this series was an increase in rhythm frequency, without interfering with the capacity for rhythm production or coordination. Mg\(^{2+}\) ion removal has a similar effect on the frequency of rhythm generated by cultures of dissociated cells from fetal rat spinal cord (Legrand et al. 2004). Mg\(^{2+}\) removal has a destabilizing influence on NMDA-induced swimming in the lamprey (Brodin and Grillner 1986).
and *Xenopus* (Soffe and Roberts 1989). More specifically, Mg\(^{2+}\) ion removal is associated with faster rhythms at low (or threshold) NMDA concentrations, which become irregular and disappear as the NMDA concentration is increased over a narrow range. However, if instead of using NMDA application, swimming is induced in the lamprey by bath application of kainate or in the *Xenopus* by natural skin stimulation, Mg\(^{2+}\) removal has no effect on swimming pattern (Brodin and Grillner 1986; Soffe and Roberts 1989). In this series, NMDA receptor activation in Mg\(^{2+}\)-free ACSF, using even very low concentrations NMDA (Fig. 5B), caused the rhythm to increase in frequency and become disorganized. On the other hand, the excitatory amino acid uptake inhibitor DHK enhances NMDA receptor activation only at sites of endogenous glutamatergic transmission. Compared with NMDA application, DHK should produce less nonspecific excitation of spinal neurons uninvolved in locomotor network operation. Thus a locomotor-like pattern was often preserved using DHK (with 5-HT) in Mg\(^{2+}\)-free ACSF. In preparations where DHK/5-HT–induced rhythms became unstable, subsequent application of submaximal concentrations of AP5, which decreased excessive NMDA receptor stimulation, restored a locomotor-like pattern. Locomotor-like patterns were unperturbed by Mg\(^{2+}\) removal when exogenous application of NMDA or DHK was avoided completely (i.e., using 5-HT alone, ACh/Edro, or brain stem electrical stimulation). In combination, these observations suggest the locomotor network can operate in the absence of NMDA receptor–mediated nonlinear membrane voltage properties, consistent with the conclusion that NMDA receptor activation in general is not universally essential for locomotor activity. Destabilization of rhythm activity, if it occurs, is likely caused by the susceptibility of unblocked NMDA receptor channels to excessive conductance in the Mg\(^{2+}\)-free bath condition.

Although previous reports, including our own, speculated that an NMDA receptor–mediated nonlinear voltage property may be important for locomotor network operation, these observations are more compatible with other evidence suggesting that this particular active membrane property is not essential. Atsuta et al. (1990) found that removal of Mg\(^{2+}\) ions had no effect on rhythmic left-right hip flexor alternation in the in vitro neonatal rat, induced by electrical stimulation of the brain stem. Bertrand and Cazalets (1999) reported that in vitro neonatal rat locomotor activity persists in Mg\(^{2+}\)-free ACSF. Bonnot et al. (1998) showed normal locomotor-like activity in vitro postnatal day 0–2 mouse spinal cord perfused with Mg\(^{2+}\)-free ACSF; in postnatal day 3–5 mice, unstable patterns occurred, with or without Mg\(^{2+}\) in the bath.

In summary, these results suggest that NMDA receptor activation, and its associated nonlinear voltage-sensitive channel behavior, is not a universal requirement for locomotor rhythm generation in the in vitro neonatal rat. The main function of NMDA receptors during spinal motor rhythogenesis is likely general support of network excitation, as suggested by Nistri and colleagues (Beato et al. 1997; Bracci et al. 1998) and Legrand et al. 2004. Under some experimental conditions (such as 5-HT–induced or brain stem stimulation–induced stepping) NMDA receptor–mediated excitation may seem critical. However, the ability to evoke locomotor-like activity in the presence of NMDA receptor blockade, through nonspecific spinal cord excitation (e.g., increased K\(^{+}\) concentration), suggests that locomotor pattern generation is simply the default consequence of exposing spinal cord circuitry to appropriate levels of excitation. A corollary of this concept is that considerable redundancy likely exists among the endogenous neural strategies available to activate locomotor output. This is not surprising given the fundamental importance of locomotor behavior.

**Acknowledgments**

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**Grants**

This study was supported by the Canadian Institutes of Health Research and National Institute of Neurological Disorders and Stroke Grant NS-40903-02). K. C. Cowley was supported by the Will-to-Win Scholar Fund.
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