THE ROLE AND MODULATION OF NMDA RECEPTOR-MEDIATED MEMBRANE PROPERTIES IN MOTOR PATTERN GENERATION IN THE MAMMALIAN SPINAL CORD

By

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ABSTRACT

Nonlinear membrane properties are nearly ubiquitous in motor pattern generating neural systems and can result in rhythmic oscillations of membrane voltage. N-methyl-D-aspartate (NMDA) receptor activation is capable of producing rhythmic motor activity, a nonlinear current voltage relationship and voltage oscillations in mammalian spinal neurons (Smith and Feldman 1987; Douglas et al 1993; Hochman et al 1994a,b). However, it is unclear what contribution NMDA receptor-dependent nonlinearity plays in mammalian locomotor pattern generation.

The first section of the thesis tests the hypothesis that NMDA-induced voltage oscillations produced by intrinsic membrane properties can be recruited by synaptic events. I report here that the ability of spinal cord lumbar motoneurons to generate NMDA receptor-dependent nonlinear behaviors (plateau potentials and voltage oscillations) is not limited to motoneurons which are synaptically isolated, as the same properties can be elicited in motoneurons under the influence of functionally intact circuitry. These oscillations were terminated by afferent input indicating that synaptic inputs were capable of influencing this activity.

The second section of this thesis tests the hypothesis that NMDA receptor-dependent nonlinear membrane properties are modulated by serotonin. The production of rhythmic

voltage oscillations in synaptically isolated spinal motoneurons requires both NMDA and 5-HT receptor activation. 5-HT induced oscillatory activity in neurons which initially expressed only tonic depolarization of their membrane potential or nonlinear membrane behavior in the presence of NMDA. Conversely, 5-HT_2 receptor antagonists abolished NMDA receptor mediated membrane voltage oscillations and the rhythmic motor output of intact spinal motor networks.

The third section of this thesis tests the hypothesis that serotonin promotes NMDA receptor-dependent voltage oscillations by decreasing the efficacy of the voltage-dependent Mg^{2+} blockade of the NMDA channel. In addition, whether the nonlinear membrane property imparted by the voltage-dependent Mg^{2+} blockade of the NMDA channel is necessary for locomotion, was examined. Voltage clamp recordings demonstrated that 5-HT shifts the voltage for activation of the negative slope conductance to more hyperpolarized potentials. The effect of 5-HT on the NMDA receptor-dependent negative slope conductance was mimicked by decreasing the concentration of extracellular Mg^{2+} suggesting that 5-HT reduces Mg^{2+} blockade of the NMDA channel. When the Mg^{2+} ion was removed from the bath during neurochemically-induced locomotor behavior (in the absence of exogenous NMDA), this activity was replaced by poorly organized patterns of phasic activity.

These experiments have provided evidence that the rhythm-generating spinal network may call upon NMDA-dependent nonlinear membrane properties. In addition, an endogenous transmitter (5-HT) is capable of modulating NMDA-dependent nonlinearity

and promoting oscillatory behavior. Because of the deleterious effect on pattern generation produced by abolishment of the region of negative slope conductance, via removal of Mg^{2+}, enhancement of the region of negative slope conductance by 5-HT application is expected to help promote stable locomotor network activity as has been reported (Cowley and Schmidt 1994; Kjaerulff et al 1994). The data suggest that one important mechanism through which 5-HT may modulate NMDA receptor-mediated effect is through regulation of the voltage-dependent Mg^{2+} blockade and thereby promote rhythm generation in the mammalian spinal cord.

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--Jason

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General Introduction

"the characterization of [a motor pattern generating system] *as either a burster-driven or a network based central pattern generator would be equally naive"* Miller and Selverston, 1982

Overview

The N-methyl-D-aspartate (NMDA) receptor has emerged as a critical component of neural circuits producing rhythmic motor activity in all vertebrate spinal cord preparations used thus far to examine locomotor activity (Grillner 1981; Dale and Roberts 1984; Kudo and Yamada 1987; Smith and Feldman 1987; Fenaux et al 1991; Hernandez et al 1991; Wheatley et al 1992; Douglas et al 1993; Sernagor et al 1995). The NMDA receptor channel is unique in that it exhibits a voltage dependency for activation (MacDonald et al 1982), which gives rise to a nonlinear current-voltage (I-V) relationship. As outlined below, nonlinear membrane properties are virtually ubiquitous in motor pattern generating systems and can result in rhythmic oscillations of membrane voltage. Thus, the potential role of NMDA receptor-mediated membrane properties in mammalian motor pattern generating circuitry is a topic of utmost importance and the subject of this thesis.

Until recently, the prevailing theory regarding the operation of the mammalian neuron was based primarily on the conductance of sodium and potassium ions as recorded in the squid giant axon (Hodgkin and Huxley 1952) and by intracellular recordings of cat spinal motoneurons (Coombs et al 1955). The neuron was viewed as a passive follower which summed synaptic inputs linearly, termed the 'platonic neuron' by Llinas (1988). If threshold for action potential generation was achieved in the initial segment, then spiking of the neuron would occur. As a result, it was thought that activity generated by a neuronal network was determined by the synaptic interconnections between simple homogeneous neuronal elements. Thus, connectivity determined function. However, "knowledge of connectivity alone is not sufficient to account for the operation of neural networks" (Getting 1989). Although understanding a neural network requires knowledge of its anatomical components and the connections between the neurons, there is a "cooperative interaction among multiple network, synaptic and cellular properties, many of which are inherently nonlinear... neurons not only sum synaptic inputs but are endowed with a diverse set of intrinsic properties that allow them to generate complex activity patterns" (Getting 1989). A complex interaction occurs between the components of the pattern generating network, the connections between them, and the cellular and synaptic properties imparted by the particular neurochemical environment in which the network finds itself .

This section is intended to introduce the reader to early neuronal models used to examine nonlinear membrane properties. Nonlinear membrane properties can result in oscillations of membrane potential, termed 'bursting', when a train of action potentials is

superimposed on the oscillations, or prolonged depolarizations referred to as plateau potentials. Because of the breadth of the introduction, from *Aplysia californica* to the cat spinal cord, no topic is dealt with exhaustively. Rather, key references and major discoveries will be highlighted in order to familiarize the reader with nonlinearity and the theory behind it.

Early evidence of pacemaker properties in neurons.

Early examination of nonlinear membrane properties, and in particular of endogenous fluctuations of membrane potential (i.e. the neuron continues to burst when completely isolated from all phasic or tonic input), focused on molluscan neuronal systems (for list see Rapp, 1979). Investigation of invertebrate neuronal networks quickly revealed that the view of a neuron as a passive summation device should be abandoned. Although there had been numerous examples of rhythmic activity within isolated ganglia, as recorded by electroneurograms, the first convincing intracellular recordings of intrinsically generated slow fluctuations of membrane potential in a neuron were recorded in the isolated heart ganglia of stomatopod *Squilla oratoria* by Watanabe et al 1967. These slow fluctuations of membrane potential were considered to be the result of an intrinsic conductance, as injection of hyperpolarizing current abolished the slow potentials without the emergence of post-synaptic potentials. Definitive evidence of pacemaker properties in a neuron came a year later (Alving, 1968). Intracellular recordings were made of neurons contained within the abdominal ganglia of the marine

mollusc *Aplysia californica*. It is possible to separate the somata of these neurons from synaptic input by separating the soma from the proximal axon on which all synaptic inputs terminate. Intracellular recordings were made from neurons while still connected to the rest of the ganglia and identified as potential pacemaker or non-pacemaker cells based on the presence or absence of burst activity. The axon was then separated from the somata by tightening a noose of silk around the axon hillock. Following this procedure pacemaker neurons continued to demonstrate rhythmic bursting while non-pacemaker neurons failed to show fluctuation of membrane potential. The method of isolating neurons from synaptic influences was refined by Chen et al 1971 who used the protease trypsin to acutely dissociate the neurons of the abdominal ganglia of *Aplysia californica*. Pacemaker neurons of the abdominal ganglia were capable of generating autorhythmic activity for more than 24hrs following isolation. The discovery that tetrodotoxin (TTX) blocks synaptic transmission by selectively blocking fast sodium conductances (Narahashi et al 1964) allowed Mathieu and Roberge (1971) to demonstrate that slow membrane potential oscillations underlie the burst activity of *Aplysia* pacemaker neurons. It was also determined that both amplitude and period of the oscillations decreased with depolarization and increased with hyperpolarization indicating that the oscillations of membrane voltage arose from endogenous conductances.

Other models used to examine neuronal endogenous burst activity included cell 11, a neurosecretory cell in the cardiac ganglia of *Otala lactea* (Gainer 1972; Barker and Gainer 1975a,b), the nine neurons which comprise the cardiac ganglia of the crab *Portunnus sunguinoletus* (Tazaki and Cooke 1979a,b; Benson and Cooke 1984), and

burst generating neurons of the *Helix pomatia* (Eckert and Lux 1975, 1976). Three criteria were established from these recordings of isolated neurons to define the properties necessary for a neuron to be considered an endogenous pacemaker: 1) The neuron must exhibit slow gradual regenerative depolarization in the absence of phasic input, 2) the pacemaker potential must be blocked by hyperpolarizing current (if sufficient hyperpolarizing current is injected into the neuron, eventually the neuron will be forced out of the region of negative slope conductance, abolishing oscillations; see below), and 3) the pacemaker potential must be reset by intracellular current pulses.

Conductances underlying invertebrate nonlinearity.

Although many different conductances generate pacemaker activity in the various invertebrate models, in all cases there is a regenerative depolarizing conductance followed by a slow onset repolarizing conductance which generates the membrane oscillations. Voltage clamp recordings of oscillating neurons permitted an examination of the conductances which produce pacemaker potentials. The *Aplysia* R15 neuron (which innervates the heart and plays a role in osmoregulation) and cell 11 (found in the cardiac ganglia of *Otala lactea*) were two models used for these voltage studies. In both cells the I-V relationship forms an 'N'-shaped trajectory and the net steady state current is always inward at potentials more negative than -30 mV. In abdominal ganglia of *Aplysia,* L2-L6 neurons also demonstrate pacemaker potentials. This activity can be reversibly blocked by cooling the perfusate from 22 to 10 °C. Examination of the steady state I-V relationship of the warm neuron revealed a region of negative slope conductance (Wilson

and Watchel 1974; see also Barker and Gainer 1975a; Eckert and Lux 1975). In the unclamped cell, the region of negative slope precludes the maintenance of a stable potential and produces a regenerative depolarization beyond the threshold for action potential generation, resulting in burst activity. This region of negative conductance was not present in the cooled cell which also failed to exhibit oscillations of membrane potential. It was concluded that while there is an outward current which is responsible for the repolarization of the membrane (Junge et al 1973), the negative slope conductance is the most critical determinant of whether a cell is capable of demonstrating oscillations. Ionic manipulation of the bath medium, in conjunction with voltage clamp recordings, permitted an examination of the ionic conductances which underlie this region of negative slope conductance (Smith et al 1974; Barker and Gainer 1975 a,b; see also Meech 1979; Berridge and Rapp 1979). In both the *Aplysia* R15 neuron and cell 11 of *Otala lactea*, replacement of Na^+ with $Tris^+$ produced a significant reduction in the negative slope conductance, suggesting that a voltage dependent, partly non-inactivating Na^+ conductance generated the region of negative slope conductance (Smith et al 1974). Other work revealed that this conductance was actually a mixed Na^+ and Ca^{2+} conductance explaining why the region of negative slope was not completely abolished by removal of Na^+ from the bath (Carpenter and Gunn 1970). This conductance was insensitive to TTX.

The repolarization of the membrane voltage oscillations requires a separate conductance. Substitution of K^+ with Cs^+ blocked burst activity (Smith et al 1974).

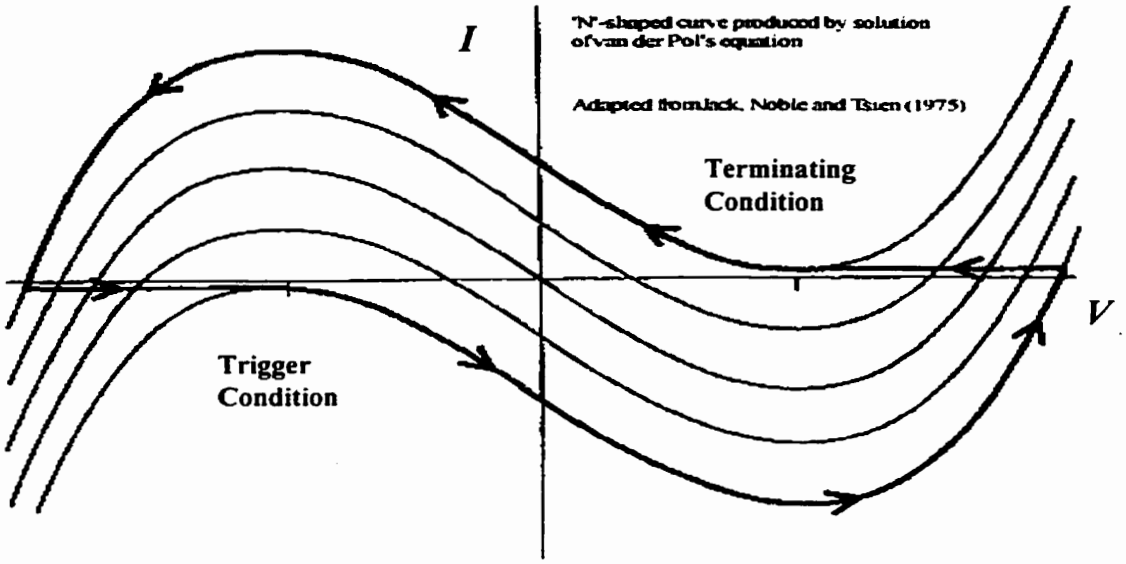
Work by Meech (1972, 1979) revealed that this was a Ca^{2+}-dependent K^+ conductance. Injection of EGTA prevented repolarization in the oscillating neuron. However, subsequent work suggested that the negative slope conductance was the product of a voltage-dependent Ca^{2+} conductance, and that the repolarization was due to Ca^{2+}-dependent inactivation of this current, combined with activation of K^+ conductances (Kramer and Zucker 1985; also see review Adams and Benson 1985).

Description of Nonlinearity.

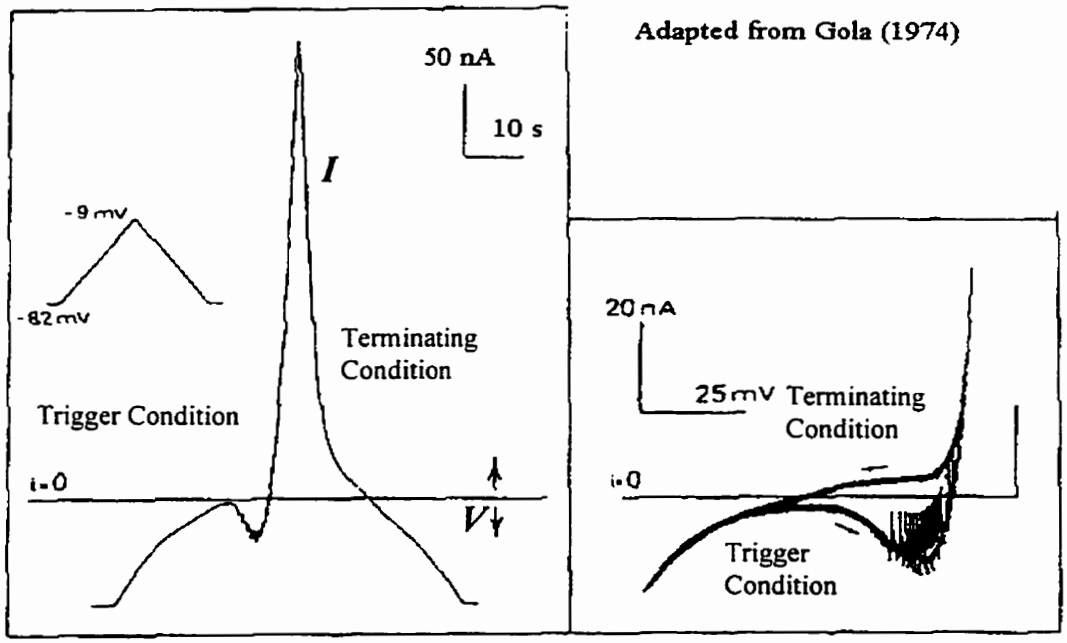
As described above all oscillating neurons demonstrate an 'N'-shaped I-V relationship characterized by a region of negative slope conductance. A negative slope conductance is inherently unstable, indicative that the conductance which generates the region of negative slope has overwhelmed the accommodative conductances of the neuron resulting in a regenerative depolarization (Jack, Noble and Tsien 1975). It is the relationship of the 'N'-shaped curve to the zero current axis which determines which membrane behavior will be expressed by the neuron. The negative slope conductance may cause the I-V curve of the neuron to cross the zero current axis with a positive slope at two distinct voltage levels, one at a more hyperpolarized potential and one at a more depolarized potential, resulting in two stable states of membrane voltage (i.e. bistability). The presence of membrane voltage bistability alone is insufficient for the generation of spontaneous oscillatory activity. This is evidenced as a plateau potential requires external

input (i.e. intracellular current injection or synaptic input) to shift it between the two points of stability.

In 1926 van der Pol generated a differential mathematical formula describing the function of an oscillator valve (for derivation see Jack, Noble and Tsien, 1975). This formula generated an 'N'-shaped I-V curve. The formula indicates that as the voltage moved out of the region of nonlinearity on the I-V curve in the depolarizing direction the ordinate (current axis) shifts downward resulting in a loss of stability at the most depolarized potential and leaving a potential of stability at the hyperpolarized potential. As a result repolarization occurs. This has been designated the 'terminating condition' (Meech 1972; Hartline and Graubard 1992). Conversely, as the voltage shifts in the hyperpolarized direction, out of the region of nonlinearity of the I-V curve, the ordinate shifts upward. The point of stability at the most hyperpolarized direction is lost and only the depolarized point of stability remains, therefore the membrane depolarizes. This is the 'trigger condition' (Meech 1972; Hartline and Graubard 1992). Cyclic fluctuation between these two conditions results in rhythmic oscillatory behavior.



Amazingly, the equation generated to study a valve oscillator comes very close to describing the I-V relationship of an oscillating neuron. Injection of an ascending and descending voltage ramp into Aplysia R15 and Otala lactea cell 11 neurons revealed that current which corresponded to the depolarizing arm of the ramp followed a different path than the current producing the repolarizing arm, indicating different conductances are activated by the depolarizing and repolarizing voltages (Gola 1974).



These data were instrumental in reinforcing the concept that although the 'N'-shaped I-V curve is critical for generation of rhythmic voltage oscillations, repolarizing conductances are also necessary. Unlike van der Pol's equation, which shifted the ordinate about a static 'N'-shaped I-V curve, in the neuron there is a shift from an 'N'-shaped curve (the trigger condition) to a separate I-V curve, governed by the repolarizing conductances (the terminating condition). In effect, the steady state I-V relationship shifts from one relationship to another. Because each I-V relationship has its own unique point of stability, where the zero current axis is crossed with positive slope, the neuron effectively has, at its extremes, two points of stability. We (Brian Schmidt and I) refer to this phenomena as dynamic bistability (see part 3). A common model for the I-V relation of oscillatory neurons emerges in which a region of negative slope conductance produces a regenerative depolarization followed by a slow conductance change which repolarizes the neuron. The cyclic alternation between these two main conductances results in the generation of membrane potential oscillations.

NMDA receptor-dependent nonlinearity.

The NMDA-receptor is the most prevalent excitatory amino acid receptor type in the vertebrate CNS (Mayer and Westbrook 1987). Invertebrates, on the other hand, appear to lack them (Mayer and Westbrook 1987; although see Dale and Kandell 1993). Activation of NMDA receptors produce a region of negative slope conductance in the I-V relationship of a cell (MacDonald et al 1982; Flatman et al 1983). The result is an 'N'-

shaped I-V plot. This I-V relationship is the product of the voltage dependent block of the NMDA receptor-channel by Mg^{2+} (Nowak et al 1984; Mayer et al 1984). At the single channel level, Mg^{2+} decreases the open probability of the NMDA channel (slow channel block) and produces a flickering of channel conductance (fast channel block) (Nowak et al 1984). Removal of Mg^{2+} from the perfusate results in maximal conductance through the NMDA channel (Mayer et al 1984). The region of negative slope conductance produced by the NMDA receptor may have an important role in vertebrate rhythm generation, as does the production of an 'N'-shaped I-V curve via other conductance mechanisms in invertebrates (Grundfest 1972; Wilson and Watchel 1974; Smith et al 1975; Gola 1978; also see above).

NMDA receptor activation has been shown to produce inherent (i.e. TTX-resistant) voltage oscillations throughout the mammalian central nervous system including the neocortex (Flatman et al 1983, 1986), nucleus basalis (Khateb et al 1995; Pape et al 1998), thalamus (Leresche et al 1990, 1991), supraoptic nucleus (Hu and Bourque 1992), substantia nigra (Johnson et al 1992), abducens nuclei (Durand 1991), nucleus tractus solitarii (Tell and Jean 1991, 1993), trigeminal motor nucleus (Kim and Chandler 1995), and medial vestibular nuclei (Serafin et al 1992). Of course NMDA oscillations are not truly endogenous in nature. Rather, these oscillations should be considered conditional, that is, the ionic mechanisms that underlie the NMDA oscillations persist in the absence of phasic synaptic input, but require tonic activation of NMDA receptors.

The lamprey spinal cord has figured prominently as a vertebrate preparation in which the role of NMDA receptors in the generation of a fictive locomotion can be examined. Although there had been suggestive evidence that excitatory amino acids could produce swimming in the lamprey (Cohen and Wallen 1980), it was not until 1981 that NMDA was shown to elicit a fictive swim pattern in the lamprey spinal cord (Grillner et al 1981). Soon after it was determined that NMDA played an important role in the generation of locomotion in other vertebrate preparations used for the examination of motor patterning including *Xenopus* (Dale and Roberts 1984), mudpuppy (Wheatley et al 1992), chick (Sernagor et al 1995) and mammalian preparations such as rat (Kudo and Yamada 1987; Smith and Feldman 1987) mouse (Hernandez et al 1991), rabbit (Fenaux et al 1991), and cat (Douglas et al 1993). Intracellular recordings of lamprey ventral horn neurons revealed that in some cases oscillations of membrane potential persisted in TTX (Sigvardt et al 1985; Grillner 1985; Grillner and Wallen 1985). Injection of hyperpolarizing current increased the amplitude and period of the oscillations providing further evidence that this membrane behavior was generated intrinsically. The ionic basis for these oscillations was determined through the use of specific antagonists and through substitution of specific ions in the bathing medium (Grillner and Wallen 1985, Wallen and Grillner 1987). It was determined that the slow rise in potential (i.e. to threshold for removal of Mg^{2+} block) was produced by a mixed cation conductance, and that the subsequent rapid rise in potential was dependent upon the removal of Mg^{2+} from the NMDA ionophore. Repolarization of the membrane depended on activation of Ca^{2+}-dependent K^{+} conductances until the Mg^{2+} block of the NMDA channel was re-established leading to rapid repolarization of the membrane potential starting the cycle over again. Examination

of the ionic basis of oscillations in mammals revealed similar underlying conductances in neurons of the nucleus tractus solitarii (Tell and Jean 1993), the supraoptic nucleus (Hu and Bourque, 1992) and trigeminal motor nucleus (Kim and Chandler 1995). Thus, just as in the invertebrate, oscillations of membrane voltage in vertebrate neurons require both a region of negative slope conductance and a separate repolarizing conductance.

NMDA induces oscillatory activity in mammalian spinal neurons.

Application of NMDA to the neonatal rat spinal cord slice preparation elicits TTX-resistant voltage oscillations in interneurons located near the central canal (Hochman et al 1994a). Oscillations that are low in frequency (0.26 Hz) were either rhythmic or arrhythmic (Hochman et al 1994a). NMDA also induced TTX-resistant high frequency (2-26 Hz) voltage fluctuations in other spinal interneurons (Hochman et al 1994a). The frequency of interneuronal oscillations was related to membrane holding potential (Hochman et al 1994a, Kiehn et al 1996), suggesting the intrinsic nature (albeit conditional on NMDA receptor activation) of the oscillations.

TTX-resistant NMDA receptor-dependent membrane voltage oscillations, as well as NMDA receptor-dependent bistable membrane behavior, were recorded in 4 of 6 lumbar motoneurons of the neonatal rat spinal cord (Hochman et al 1994b). In the case where CsF was included in the pipette filling solution instead of KGluconate, the motoneuron was incapable of oscillations, suggesting that an outward potassium current may be

necessary for the repolarizing phase of the oscillation. It was also reported that in two cases, despite the persistence of bistability, the cell was incapable of generating voltage oscillations. This provided further evidence that bistability alone is not synonymous with oscillatory behavior, as described above. NMDA, applied to cat spinal motoneurons by microelectrophoresis, produces a few transient oscillations and then the membrane potential becomes locked at a depolarized level (Engberg et al 1984). The threshold for the activation of this membrane behavior was -50 mV (Engberg et al 1984).

Two studies using a partial hemisected in vitro neonatal rat spinal cord preparation, have examined the membrane behavior of interneurons that were rhythmically active during neurochemically evoked locomotor-like activity, in the presence of NMDA and TTX (MacLean et al 1995; Kiehn et al 1996). In the study by MacLean and colleagues the two interneurons tested, that were rhythmically active in phase with ventral root activity in the presence of NMDA/5-hydroxytryptamine (5-HT), failed to display oscillatory behavior following application TTX to the bath (MacLean et al 1995). In the study conducted by Kiehn and colleagues, 12% of the interneurons that were rhythmically active following application of neurochemicals to the bath displayed a nonlinear increase in the amplitude of the rhythmic membrane activity, suggestive of nonlinear membrane properties (Kiehn et al 1996). Following application of TTX to two interneurons (in the presence of NMDA/5-HT), voltage oscillations were observed in one neuron but not in the other (Kiehn et al 1996). Both studies are best considered preliminary because of the very small number of interneurons tested in TTX and NMDA. Thus, it remains to be demonstrated whether the interneurons which comprise the central

pattern generator for locomotion demonstrate conditional oscillations of membrane potential.

Analysis of the post-synaptic currents (PSCs) occurring in spinal interneurons located in the vicinity of the central canal revealed that a surprisingly small number of synaptic events (8-300) generates the rhythmic activity seen in these neurons (Raastad et al 1996, 1997). Perhaps so few synaptic inputs are capable of generating rhythm in these interneurons because of their nonlinear membrane properties.

Nonlinearity in spinal motoneurons and its possible contribution to locomotion.

The possibility of pacemaker potentials in mammalian motoneurons was considered in a review of the neural generation of respiration (Wyman 1977), that cited slow potentials recorded in intercostal motoneurons (Sears 1964) as possible evidence of autorhythmicity.

Early evidence that intrinsic properties of motoneurons may help shape motor output was presented following examination of motoneurons which innervate superficial muscles of the crayfish abdomen (Gillary and Kennedy 1969a). These motoneurons fire in repetitive bursts when stimulated by tonic input from a single command interneuron, suggesting an intrinsic property converted this tonic input to phasic output. Recordings

of endplate potentials and tension in the target muscles revealed that this burst activity of the motoneurons was more effective in generating contraction than unpatterned discharge of the same mean frequency (Gillary and Kennedy 1969b, see also Kernell 1986).

Voltage clamp analysis of lamprey ventral spinal neurons during NMDA-evoked fictive swimming demonstrated excitatory and inhibitory synaptic currents in phase with ventral root phasic activity, the excitatory currents showed a voltage dependence suggesting a potential role for the NMDA channel in generation of locomotion (Moore et al 1987). Following application of TTX, a region of negative slope conductance was observed in these same motoneurons which was then abolished by removing Mg^{2+} (Moore et al 1987). Analysis of locomotor drive currents (LDCs, the voltage-clamp counterpart to locomotor drive potentials (LDPs)) in rat spinal motoneurons has provided evidence that NMDA receptor activation does contribute to LDP generation in mammalian motoneurons (Hochman and Schmidt 1998). LDCs were larger in amplitude at holding potentials of -40 mV compared to -80 mV, this voltage dependent increase in amplitude is compatible with a voltage-dependent NMDA receptor-mediated effect (Hochman and Schmidt 1998).

Functional roles for nonlinearity in cat spinal motoneurons have been suggested following examination of motoneurons using intracellular recording methods. Recordings of motoneurons in which the fast sodium conductance had been inactivated by the lidocaine derivative QX314 revealed a voltage dependent component to the excitatory phase of the LDP that was not mimicked by current injection in the quiescent

preparation (Brownstone et al 1994). The range of potentials for the nonlinear jump in amplitude of the LDP ranged from -45 to -50 mV suggesting that there is an endogenous activation of the NMDA receptor during brainstem-evoked locomotion. Analysis of EPSPs produced by stimulation of the flexor reflex and Ia afferents in the L-DOPA treated cat demonstrated a similar voltage dependency, increasing in amplitude with depolarization. Microiontophoresis of DL-amino-phospho-novaleric acid (AP5, an NMDA antagonist) reduced the excitatory phase of the LDP (Brownstone et al 1991) providing further evidence of an NMDA component in the excitatory phase of the LDP. The voltage-dependent extension enhancement observed during fictive locomotion and following afferent stimulation in the MLR cat (McCrea et al. 1997) also has a threshold for activation around -50 mV. Again this finding suggests that the nonlinear membrane property is mediated by the voltage-dependent removal of Mg^{2+} blockade from the NMDA channel.

Contribution of the voltage-sensitive Mg^{2+} block of NMDA channels to locomotor activity.

The effect of selectively abolishing NMDA receptor-mediated nonlinear membrane responsiveness, while otherwise preserving the capacity for NMDA receptor activation, can be examined by removing Mg^{2+} from the bath in locomotor experiments (Brodin and Grillner 1986; Soffe and Roberts 1989; Reith and Sillar 1998). As predicted, TTX-

resistant NMDA-induced oscillations were abolished by the removal of Mg^{2+} (Grillner and Wallen 1985; Wallen and Grillner 1987; Moore et al 1987). In the lamprey, the frequency of ventral root activity increased and was more variable from cycle to cycle after removal of Mg^{2+} (Brodin and Grillner 1986). Similar effects of Mg^{2+} removal on the stability of NMDA-induced locomotion have been observed in *Xenopus* (Soffe and Roberts 1989). Removal of Mg^{2+} shortened the cycle period, increased the incidence of synchronous bursts between contralateral ventral roots, and increased the variability of cycle period in *Xenopus* (Soffe and Roberts 1989). The presence of Mg^{2+} in the bath allowed a larger range of NMDA concentrations to produce fictive swimming as opposed to Mg^{2+}-free solution. Modeling studies of the *Xenopus* spinal pattern generator suggest that voltage-dependent Mg^{2+} blockade of NMDA receptors stabilizes swimming activity through enhancement of postinhibitory rebound at the single cell level (Roberts et al 1995). NMDA receptor voltage dependency allows a given inhibitory synaptic conductance to produce a larger hyperpolarization and therefore a larger rebound response of soma membrane. Their model also indicated that the Mg^{2+} block could lead to a better control over swim frequency (Roberts et al 1995). A mathematical model (developed by Brodin et al 1991) of lamprey spinal neurons capable of NMDA-dependent nonlinear membrane properties indicated that the voltage-dependent block by Mg^{2+} of the NMDA receptor channel stabilizes the swim pattern (Traven et al 1993).

Modulation of Nonlinearity.

During early investigations of pacemaker cells, it became clear that nonlinear membrane properties could be modified by external inputs. Examination of *Otala lactea* cell 11 in dormant snails revealed that these neurons were electrically inactive and exhibited a linear I-V relationship (Barker and Gainer, 1974). Bath application of vasopressin and oxytocin converted this linear membrane response to one that exhibited a region of negative slope conductance and burst potentials in the unclamped cell (Barker and Gainer, 1974, Barker et al 1975c).

5-HT and other neuromodulators are capable of functionally reconfiguring invertebrate rhythmogenic circuits by regulating the strength of synaptic interactions and the intrinsic membrane properties of neuronal elements distributed throughout the network (e.g. Harris-Warrick et al 1992; Katz et al. 1994; Johnson et al. 1995). In the R15 neuron, 5-HT can inhibit bursting by producing a slow onset, long duration hyperpolarization (Drummond et al 1980). 5-HT does this by increasing a K^+ conductance (Benson and Levitan 1983). Through amplification of this outward conductance the I-V curve is shifted so that a previous point of instability at more hyperpolarized potentials now crosses the zero current axis with a positive slope, creating a new point of stability at a potential that is hyperpolarized relative to the threshold for the region of negative slope conductance (terminating condition; Hartline and Graubard 1992). Lower concentrations of 5-HT (1 μM) applied to the R15 neuron can enhance the transition from beating (tonic

firing) to bursting in response to intracellular current injection (Lehner et al 1996). Dopamine decreases the negative slope conductance (Boisson and Gola 1976; Wilson and Watchel 1978) in the R15 neuron. This causes the I-V curve to cross the null current axis at a potential more hyperpolarized than the threshold for the region of negative slope conductance (terminating condition; Hartline and Graubard 1992).

The classic invertebrate model, used for the examination of modulation of nonlinearity, is the lobster *Pannulirus interruptus* stomatogastric ganglion. In the pyloric network all six major cell types which comprise the pyloric central pattern generator are conditional bursters (Miller and Selverston 1982a; Bal et al 1988). Early work suggested that only two of the 6 major types of neurons were bursters (active regenerative membrane properties, Selverston et al 1976). Russel and Hartline (1978, 1982, 1984; Hartline et al 1988, 1992) found that if the commissural ganglion was left connected to the stomatogastric ganglion, or if tonic stimulation was applied to the transected nerve from the commissural ganglion, then all neurons which comprised the pyloric central pattern generator were capable of generating plateau potentials. "Having plateau properties in several network neurons tends to blur the distinction between emergent and endogenous bursting mechanisms" (Hartline et al 1988). Providing that the input from the commissural nerve remained intact, this regenerative depolarization, observed in the pyloric neurons, persisted following isolation of the neurons from all other neurons in the stomatogastric ganglion (Russel and Hartline 1982). Applications of dopamine, 5-HT or octopamine (Flamm and Harris-Warrick 1986; Harris-Warrick and Flamm 1987) are all capable of producing burst activity in pyloric neurons, however, not

all by a common mechanism. Bursting induced by 5-HT or octopamine is largely generated by TTX-sensitive Na^+ conductances, while the burst activity produced by dopamine is resistant to TTX and blocked by small reductions in the Ca^{2+} concentration (Harris-Warrick and Flamm 1987). Therefore the neurochemical environment that the neuron is exposed to will critically determine the burst behavior of that neuron and thus the network. In the crab, exogenous application of 5-HT onto neurons of the stomatogastric ganglion produces a small depolarization in motoneurons which enables the cell to express plateau potentials when depolarized above a threshold (Kiehn and Harris-Warrick 1992a,b). Voltage clamp analysis of the effect of 5-HT revealed that 5-HT enhances I_h (hyperpolarization activated cation current) and decreases the Ca^{2+}-dependent K^+ conductance (Kiehn and Harris-Warrick 1992a,b). Subsequent examination has revealed that 5-HT also facilitates the production of plateau potentials through the potentiation of a voltage-dependent Ca^{2+} conductance (activated at -45 mV) (Zhang and Harris-Warrick, 1995a,b).

In the lamprey, application of 5-HT prolongs the plateau component of the NMDA receptor-mediated intrinsic voltage oscillations in spinal neurons (Wallen et al. 1989). It does this through depression of a Ca^{2+}-dependent K^+ conductance (Wallen et al. 1989).

In amphibian spinal neurons, 5-HT is required for the maturation of locomotor rhythms (Sillar et al. 1995) and the expression of intrinsic voltage oscillations (Sillar and Simmers 1994; Scrymgeour-Wedderburn et al. 1997). A recent study of embryonic and larval *Xenopus* spinal cord neurons suggests that $5\text{-}HT_{1A}$ receptor activation facilitates the

voltage-dependent blockade of NMDA channels by Mg^{2+} as evidenced by a greater apparent input resistance in the presence of 5-HT and NMDA as compared to NMDA alone; this interaction may explain why NMDA and 5-HT receptor co-activation is required for the production of voltage oscillations in these cells (Scrymgeour-Wedderburn et al. 1997). Examination of fictive locomotor patterns in the larval preparation, produced by NMDA, revealed a slow (0.5 Hz) rhythmic fluctuation of ventral root activity in which burst intensity, duration and frequency cyclically increased (Reith and Sillar 1998). Once spinalized the Xenopus larvae failed to demonstrate this slow modulation of ventral root activity until application of 5-HT to the bath. This activity was blocked by the $5\text{-}HT_{1A}$ receptor antagonist pindobind and by removal of Mg^{2+} from the bath indicating that it was dependent on 5-HT receptor activation as well as the voltage-dependent block of the NMDA ionophore. It is hypothesized that this slow fluctuation of ventral root activity may act as a boosting mechanism for motor output.

Intracellular recordings of cat neocortical neurons revealed that NMDA in the presence of TTX produced tonic depolarization of the cell membrane potential. Subsequent application of 5-HT produced oscillations of membrane potential (Nedergaard, Engberg and Flatman 1986, 1987). A preliminary report indicated that co-application 5-HT and NMDA resulted in the generation of oscillations of membrane potential in cat spinal motoneurons (Flatman and Engberg 1990). For a further description of the promotion of nonlinearity by amines in cat and turtle motoneurons see below.

Nonlinearity in cat spinal motoneurons: A case for NMDA.

The cat spinal cord has been the classic model for the investigation of mammalian locomotion. Examination of the cat spinal cord has demonstrated that mammalian motoneurons also possess conditional nonlinear membrane properties (see below). Early reports of mammalian motoneuronal nonlinearity suggested that this membrane property is the result of a voltage-dependent Ca^{2+} channel (e.g. see review by Kiehn 1991; see below). As a result the prevalent theory for mammalian motoneuronal nonlinear membrane properties is that they are mediated by a Ca^{2+} channel. Although this thesis does not directly address this issue I wish to challenge this theory by suggesting that the major underlying channel contributing to these nonlinear membrane properties is the NMDA channel, rather than a voltage-dependent Ca^{2+} channel. By questioning the assumption that a voltage-dependent Ca^{2+} channel mediates nonlinearity in cat spinal motoneurons I will attempt to place my work examining the effects of NMDA on rat spinal motoneurons in context with these early reports of nonlinear membrane properties, and I will suggest a common mechanism responsible for nonlinear membrane properties in mammalian motoneurons.

Initial reports of nonlinear membrane properties in cat spinal motoneurons did not appear until Schwindt and Crill's (1977) description of a 'persistent negative resistance' in the steady state I-V relationship of cat spinal motoneurons. Voltage clamp recordings of cat spinal motoneurons revealed a region of negative slope starting 10 mV depolarized

from resting membrane potential between -50 to -70 mV. In most cases, despite the region of negative slope, the net current was outward. However, in some cases there was a persistent net inward conductance which they named I_i. Having no correlate regenerative inward conductance in the mammal they looked to endogenous burst neurons in the mollusk *Helix pomatia* which demonstrated a regenerative inward current mainly mediated by a Ca^{2+} channel (Eckert and Lux 1975, see previous description). For this reason they hypothesized that the inward conductance which they observed in cat spinal motoneurons was mediated by a Ca^{2+} channel.

Intracellular injection of tetraethylammonium chloride (TEA, which blocks the delayed rectifier, A current, and the Ca^{2+}-dependent potassium currents) prolonged action potentials revealing a plateau potential (Schwindt and Crill 1980). When these motoneurons were examined in voltage clamp mode they demonstrated the same negative slope and a persistent inward conductance similar to that recorded in some control spinal motoneurons. They reported that in the control situation the negative slope is a transient phenomenon, and its expression was prolonged by application of TEA. During penicillin-induced spinal seizures, a positive correlation existed between the presence of a negative inward conductance and the prolonged burst activity recorded in motoneurons (Schwindt and Crill 1980b). Application of penicillin increased the likelihood of a net inward conductance, when a region of negative slope is observed, from 40% among control motoneurons to 60% of penicillin treated motoneurons. Iontophoretic application Ba^{2+} increased the likelihood that a negative slope would be observed in motoneurons, and in those cases where a negative slope was present it was enhanced by Ba^{2+} (Schwindt

and Crill 1980c). They also reported a depression of K^+ conductances by Ba^{2+}. It is now known that Ba^{2+} will depress the delayed rectifier K^+ conductance and the inward rectifier K^+ conductance when applied to the extracellular membrane of a neuron, and will depress Ca^{2+}-dependent potassium currents when injected into the neuron (Hille 1992). In a review of this work, published in the Handbook of the Spinal Cord, Schwindt and Crill conclude that I_i is mediated by a Ca^{2+} conductance, as it is present after intracellular application of a lidocaine derivative QX314 (which blocks the fast Na+ conductance responsible for action potential generation) and because the I_i is enhanced by iontophoresis of Ba^{2+} (Schwindt and Crill, 1984). These data are consistent with I_i being mediated by a Ca^{2+} conductance; however, Ca^{2+} conductance and Ca^{2+} channel are not synonymous. It should be emphasized here that the NMDA channel had not yet been characterized when the original experiments were performed by Schwindt and Crill. Curiously, however, in subsequent work by these researchers and by Flatman (1983, 1986), in which they characterized the region of negative slope conductance produced by NMDA receptor activation in cat neocortical cells, no mention is made of their earlier work on cat motoneurons.

Selective activation of Ia afferents in the decerebrate cat produced a long duration increase in the excitability of motoneurons of homonymous and synergist muscles and an increase in the monosynaptic reflex response. It was originally postulated that this increase in excitability may reflect a maintained excitatory synaptic input to the motoneurons, the product of a reverberating loop of interneurons (Hultborn et al 1975). However, intracellular recordings of motoneurons revealed that the prolonged level of

excitation recorded in extensor motoneurons was not the result of a reverberating loop of interneurons but rather the product of intrinsic membrane properties of spinal motoneurons (Hounsgaard et al 1984, 1988). Plateau potentials could be elicited in motoneurons with intracellular current injection or following afferent stimulation in the decerebrate cat. In order to examine the conductance which generated that plateau potential without it being obscured by action potentials they inactivated the fast Na+ conductance by injecting large amplitude positive current (up to 100 nA) for 2 minutes. The sodium spike remained inactivated for a short time even when membrane potential was returned to the control level (Hounsgaard et al 1988). Plateau potentials, approximately 7-10 mV in amplitude, could then be initiated with an intracellular depolarizing current pulse and inactivated by a hyperpolarizing current pulse, or initiated and terminated by stimulating afferent inputs from synergist or antagonist muscles, respectively. Following spinalization, plateau potentials could no longer be induced in the motoneurons. Subsequent intravenous application of 5-hydroxytryptophan (a 5-HT precursor) in the spinalized cat enabled the induction of plateau potentials indicating a serotonergic dependence of this nonlinear membrane property. Further examination in the cat demonstrated that plateau potentials could also be elicited following intravenous injection of L-DOPA (a noradrenergic precursor) or clonidine (an alpha adrenergic-receptor agonist, Conway et al 1988). Hounsgaard and colleagues concluded that much like I_i, these plateau potentials were mediated by Ca^{2+} conductances.

In order to examine the mechanisms which underlie plateau potentials, Hounsgaard and colleagues turned to the turtle spinal cord slice preparation. Plateau potentials in

turtle spinal motoneurons were also found to be 5-HT dependent (Hounsgaard and Kiehn 1985). These plateau potentials in turtle spinal motoneurons were TTX-resistant and abolished by Mn^{2+} suggesting that they are an intrinsic Ca^{2+}-dependent phenomenon (Hounsgaard and Kiehn 1985). Threshold for activation of the plateau potentials was approximately -60 mV. Subsequent experiments revealed that these serotonergic plateau potentials were abolished by nifedipine, an L-type Ca^{2+} channel antagonist (Hounsgaard and Kiehn 1989). Hounsgaard and colleagues concluded that plateau potentials in cat spinal motoneurons are also mediated by an L-type Ca^{2+} channel, referring to the data from turtle motoneurons and the work by Schwindt and Crill who demonstrated a persistent inward current in cat motoneurons which appeared to be mediated by a Ca^{2+} conductance although they provided no evidence of a voltage dependent Ca^{2+} channel. Certainly, on first inspection, this seems like an unreasonable conclusion. However, I think the issue deserves further consideration.

The conductances which generate nonlinear membrane properties vary from preparation to preparation, and in some cases within a preparation (e.g. Harris-Warrick et al 1992). I propose that the negative slope conductance which is necessary for all of these descriptions of nonlinearity in cat spinal motoneurons, unlike turtle motoneurons, is much more likely to be mediated by the voltage-dependent block of the NMDA channel by Mg^{2+} than by a voltage-dependent Ca^{2+} channel. A potential role for the NMDA channel in cat spinal motoneuronal nonlinearity has also been proposed by Brownstone (1992).

The major argument against a Ca^{2+} channel as the mediator of the negative slope conductance observed in mammalian motoneurons is the potentials at which the negative slope is initiated. I_i, as reported by Schwindt and Crill, is activated 10 mV positive to rest, placing the activation somewhere between -60 and -40 mV. In addition, cat motoneurons are difficult to space clamp (Clements and Redman 1989). If the channel which mediates the inward conductance is mainly distributed in the dendrites, the voltage of activation for the region of negative slope conductance is likely even less depolarized than that recorded in the soma. Although Hounsgaard and co-authors fail to give any indication of threshold for the activation of the plateau potentials, the plateau is 7-10 mV in amplitude following inactivation of the fast Na^+ conductance. It is unlikely that this potential exceeds -30 mV or the threshold for action potential generation. The voltage-dependent amplitude increase of both the LDP and extension enhancement following afferent stimulation occur at potentials ranging from -45 to -50 mV (see above). Whole cell recordings of rat spinal motoneurons indicate that high voltage activated (HVA) calcium channel conductances are activated at potentials more depolarized than -20 mV and that low voltage activated (LVA) calcium channel conductances are activated at -67 mV (Berger and Takahashi 1990). Application of 5-HT to these motoneurons potentiates the amplitude of the LVA conductances but does not shift the I-V relationship to more negative potentials ruling out the possibility that 5-HT has shifted the activation of the HVA to more negative potentials (Berger and Takahashi 1990). The plateau potential in the turtle is mediated by a large, persistent Ca^{2+} conductance through a nifedipine sensitive Ca^{2+} channel. In the mammalian CNS there has been a single demonstration of

a persistent dihydropyridine sensitive calcium channel which is activated at very hyperpolarized potentials (-50 mV) (Avery and Johnston 1996). However, even in the presence of multiple K^+ channel blockers the maximal whole cell current which passes through these Ca^{2+} channels ranges from 5-10 pA. Such small currents make these channels unlikely mediators of the plateau potentials observed in cat spinal motoneurons. The threshold for the region of negative slope conductance of the NMDA channel (although dependent upon the concentration of Mg^{2+}) in 1mM Mg^{2+}(i.e. physiological levels; Ault et al 1980) occurs at -70 mV (Mayer et al 1984) in cultured spinal neurons. In all cases of reported nonlinearity in cat motoneurons, the threshold for activation falls much more convincingly in the negative slope region of the NMDA channel than it does for activation of HVA calcium channel conductances.

Evidence that Ba^{2+} enhances I_i does not weaken the assertion that this conductance is mediated by the NMDA channel. Although Ba^{2+} is highly permeable through Ca^{2+} channels, it is also highly permeable through NMDA channels (Mayer and Westbrook 1987; Ascher and Nowak 1988). As reported earlier Ba^{2+} antagonizes various K^+ conductances. By decreasing the competing outward currents there will be an apparent increase of the inward conductances. As a result it is difficult to conclude based on the data presented by Schwindt and Crill that the increase of I_i is the result of a increased conductance through Ca^{2+} channels, as opposed to an apparent increase in the amplitude of the NMDA receptor-dependent negative slope conductance due to Ba^{2+} inactivation of competing outward K^+ conductances.

As previously reported microelectrophoresis of NMDA onto cat lumbar motoneurons produces a few transient oscillations before the membrane potential becomes locked in a plateau at approximately -35 mV (Engberg et al 1984). The threshold for activation of this plateau potential is -50 mV. This result indicates that NMDA can induce plateau potentials in cat spinal motoneurons which stabilize at membrane potential levels similar to those observed in studies using intracellular current injection or afferent stimulation to induce plateau potentials (Hounsgaard et al 1988). Further, AP5, when applied to a single cat motoneuron, depresses the excitatory phase of the LDP (Brownstone et al 1991).

Thus, while plateau potentials appear to be mediated by a high voltage-activated calcium channel in turtle motoneurons, the data reported thus far in cat motoneurons is more consistent with activation of the NMDA channel. This is not to suggest that Ca^{2+} channels play no role in the generation of plateau potentials in cat spinal motoneurons, but rather that the major underlying conductance which produces a regenerative depolarization of membrane potential is the result of removal of Mg^{2+} blockade from the NMDA channel.

Objectives.

The intact *in vitro* neonatal spinal cord has proven to be an excellent mammalian model to examine the role of neuronal membrane properties in generating rhythmic motor patterns, permitting direct neurochemical access to the networks which control locomotion and allowing sophisticated intracellular recording methods to be used. Our experiments focus on motoneurons. Electrophysiological identification of motoneurons can be easily accomplished through antidromic firing in response to ventral root stimulation. This is in stark contrast to the difficult task of functionally identifying interneurons. Thus by focusing on motoneurons, we have been able to examine the membrane properties of a relatively homogenous and functionally identified population of spinal neurons. It is reasonable to speculate that at least some of the observations made in motoneurons may apply to other types of spinal cord neurons, although direct evidence will require further experiments beyond the scope of this thesis.

The general hypothesis of this thesis is ***NMDA receptor-mediated nonlinearity is promoted by serotonin and required for locomotion***. This thesis is divided into three sections, the first two representing published manuscripts and the third manuscript that will be submitted in the summer of 1998. As the thesis title indicates, this project examines NMDA receptor-mediated nonlinear membrane properties of spinal motoneurons, the contribution of these properties to locomotor pattern generation, and the modulation of these properties by 5-HT.

The first section tests the hypothesis that ***NMDA-induced voltage oscillations produced by intrinsic membrane properties in motoneurons can be recruited by synaptic events***. Previously Brian Schmidt's laboratory demonstrated that lumbar motoneurons in the *in vitro* neonatal rat spinal cord undergo TTX-resistant NMDA receptor-dependent oscillations of membrane voltage (Hochman et al 1994b). However, it is important to determine whether these motoneuronal oscillations occur only under specific experimental conditions or whether these membrane properties can be recruited by an intact network (i.e. synaptic connections maintained) before further consideration of their possible role in shaping the final common output of intact spinal networks can be made. These data are published in the *European Journal of Neuroscience* 9: 2702-2711, 1997.

The second part of this thesis tests the hypothesis that ***NMDA receptor-dependent membrane nonlinear properties are modulated by 5-HT, which has a permissive effect on the generation of voltage oscillations***. We have observed that not all motoneurons exposed to NMDA and TTX undergo voltage oscillations. Previous reports in the *Xenopus* spinal cord, in cat neocortex and in the cat spinal cord (see above) indicate that 5-HT promotes oscillations of membrane voltage in the presence of NMDA. Therefore, it is of interest to determine whether 5-HT, an endogenous neurotransmitter in the mammalian spinal cord, modulates NMDA-receptor nonlinear membrane properties and promotes motoneuronal oscillations of membrane voltage. These data are published in the *Journal of Neurophysiology* 79: 2804-2808, 1998.

The third section of this thesis tests the two part hypothesis that a) ***5-HT promotes NMDA receptor-dependent voltage oscillations by decreasing the efficacy of the voltage dependent Mg^{2+} blockade of the NMDA channel*** and b) ***nonlinear membrane property imparted by the voltage dependent Mg^{2+} blockade of the NMDA channel is necessary for locomotion***. Recent publications have indicated that protein kinase C (PKC) is capable of reducing the Mg^{2+} blockade of the NMDA channel (Chen and Huang 1992; Blank et al 1996). 5-HT_2 receptors are known to activate the PKC pathway (see Martin and Humphrey 1994). It is reasonable then to examine whether 5-HT exerts its modulatory action by reducing Mg^{2+} blockade of the NMDA channel. In order to examine specifically the role of NMDA receptor-dependent oscillations in rhythmogenesis it is necessary to abolish NMDA dependent nonlinearity and examine the effects on rhythmic network activity. This we achieve in a series of experiments examining the effect of Mg^{2+} removal on locomotor pattern generation.

SECTION I

NMDA RECEPTOR ACTIVATION TRIGGERS VOLTAGE OSCILLATIONS, PLATEAU POTENTIALS AND BURSTING IN NEONATAL RAT LUMBAR MOTONEURONS *IN VITRO*

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ABSTRACT

Whole-cell recordings of lumbar motoneurons in the intact neonatal rat spinal cord *in vitro* were undertaken to examine the effects of N-methyl-D-aspartate (NMDA) receptor activation on membrane behavior. Bath application of NMDA induced rhythmic voltage oscillations of 5.9 ± 2.1 mV (S.D.) at a frequency of 4.4 ± 1.5 Hz. Amplitude, but not frequency, of the voltage oscillations was membrane potential-dependent. Voltage oscillations could recruit action potentials and/or plateau potentials with or without superimposed bursting. Blockade of synaptic transmission with tetrodotoxin (TTX) sometimes resulted in a loss of oscillatory activity which could then be restored by increasing the NMDA concentration. After application of TTX, the trajectory of NMDA-induced oscillations was similar to the trajectory induced in the presence of intact synaptic networks, although the mean oscillation duration was longer and the oscillation frequency was slower (1.8 ± 1.1 Hz). Current ramps delivered after bath application of NMDA demonstrated bistable membrane properties which may underlie the plateau potentials. Injection of intracellular current pulses could initiate, entrain and terminate individual plateau potentials. The results suggest that membrane depolarization produced by oscillations may activate other intrinsic conductances which generate plateau potentials thereby providing the neuron with enhanced voltage sensitivity, compared to that produced by NMDA receptor activation alone. These oscillatory events may have a role in the regulation of motor output in a variety of rhythmic behaviors including locomotion.

INTRODUCTION

A region of negative slope conductance in the current-membrane voltage (I-V) relationship of a cell results in an 'N'-shaped I-V curve. Depending on the relation of this curve to the zero current axis, spontaneous repetitive firing or bistable oscillations of membrane potential may occur (Wilson and Wachtel 1974; Smith et al 1975; Schwindt and Crill 1977). Hence, N-methyl-D-aspartate (NMDA) receptor activation, which confers this type of non-linear I-V relationship (MacDonald et al., 1982; Flatman et al., 1983, 1986; Mayer and Westbrook, 1984), induces intrinsic voltage oscillations and rhythmic bursting in a variety of neurons (e.g. Flatman et al., 1983, 1986) including spinal neurons of lower vertebrates (Wallen and Grillner 1987) and mammals (Hochman et al 1994a; 1994b).

In a preliminary report, we described NMDA receptor-mediated bistable membrane properties and intrinsic voltage oscillations (0.5-2.0 Hz) in synaptically isolated mammalian lumbar motoneurons (Hochman et al., 1994b). Two other studies showed that membrane potential oscillations can be induced in mammalian spinal cord motoneurons by afferent nerve or root stimulation in the presence of synaptically intact networks, and in the absence of exogenously applied excitatory amino acids (Conway et al. 1988; Baranauskas and Nistri 1995). It was concluded that afferent evoked oscillations observed in the cat preparation, (in the presence of L-DOPA or clonidine), reflected intrinsic membrane properties (Conway et al 1988). In contrast, it was proposed that the afferent-induced oscillations observed in the neonatal rat model were most likely generated by network activity (Baranauskas and Nistri 1995). Thus, it appears that

membrane voltage oscillations in lumbar motoneurons may arise under a variety of conditions and possibly through several mechanisms. The details of these mechanisms, however, as well as the functional role of oscillations in motor control remains to be delineated.

There is considerable data supporting an essential requirement for NMDA receptor activation in the production of rhythmic motor behavior in a variety of higher and lower animals (see Daw et al 1993), including locomotion in mammals (e.g. Kudo and Yamada 1987; Smith and Feldman 1987; Douglas et al. 1993). In the lamprey, NMDA receptor-induced oscillations in synaptically isolated spinal cord motoneurons and interneurons were linked to the production of locomotor activity by the intact network (Wallen and Grillner 1987). Intrinsic oscillatory properties which require an interaction between NMDA and 5-HT receptors were recently described in amphibian embryo motoneurons (Sillar and Simmers 1994). Microelectrophoretic application of NMDA to cat spinal motoneurons, *in vivo*, produces a transient phase of voltage oscillation as the cell membrane depolarizes from -50 to -35 mV (Engberg et al. 1984; Flatman and Engberg 1990). However, it remains to be shown whether the sustained NMDA receptor-mediated oscillations we described in tetrodotoxin (TTX)-treated preparations (Hochman et al. 1994b) also occur in non-synaptically isolated motoneurons. This information is critical to any further consideration of the potential role of NMDA-induced oscillatory properties in shaping the final common output of intact spinal motor networks. Therefore, in addition to extending our analysis of NMDA receptor-mediated oscillations in synaptically isolated lumbar motoneurons, the present study examines whether

motoneurons can produce similar membrane behavior in the presence of intact circuitry and whether these oscillations are capable of recruiting action potentials and thereby influencing motor output.

A preliminary report of some of this work has appeared in abstract form (Hochman et al 1994c).

METHODS

Sprague-Dawley rats, ranging from 2 to 4 days of age, were decapitated, eviscerated, and placed in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 128, KCl 3.0, NaH_2PO_4 0.5, $CaCl_2$ 1.5, $MgSO_4$ 1.0, $NaHCO_3$ 21, and glucose 30, equilibrated with 95% O_2/ 5% CO_2, pH 7.4. The intact spinal cord was isolated in ACSF at 4 ^oC, while recordings were obtained at room temperature. The spinal cord was stabilized with insect pins on the bottom of a recording chamber that had been coated with Sylgard (Dow Corning Corporation). The spinal cord was oriented with the ventral surface upward and ventral roots were reflected over the cord to the contralateral side. The pia mater was removed in areas where the lumbar (L3-L5) motor nuclei were presumed to be subjacent.

Whole-cell recording electrodes contained (in mM): K-gluconate 140; ethylene glycol-bis(-amino ethyl ether) N,N,N',N',-tetra-acetic acid (EGTA) 11; KOH 35; N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10; and $CaCl_2$ 1. In some experiments lidocaine N-ethyl bromide (QX-314, 5mM) was included in the electrodes to

block voltage-dependent Na^+ channels. Electrodes were made from borosilicate glass (WPI) pulled on a vertical puller (Narishige PP-83). Internal tip diameters ranged from 2-4 μm, and resistances measured in ACSF ranged from 3-6 MΩ. Whole-cell recordings (Hamill et al., 1981) were obtained using a "blind" approach (Blanton et al., 1989). A piezomanipulator (PM 500-20) controlled electrode movement in 1.5 μm step increments. Positive pressure was continuously applied to the patch electrode by injecting 1-2 ml of air using a syringe and tubing attachment, while tracking for motoneurons. Voltage steps were also applied to monitor resistance during tracking, which was performed in voltage-clamp mode. When the monitored resistance increased by at least 20%, positive pressure was removed and slight (1 ml) negative pressure was applied. Once the seal resistance reached the GΩ range slight additional negative pressure was applied to rupture the cell membrane. Cells were identified as motoneurons by their antidromic response to ventral root stimulation via glass suction electrodes. After achieving a stable whole-cell patch configuration, data was subsequently acquired in current-clamp mode. The whole-cell recordings were obtained in conjunction with an Axopatch 1D amplifier (Axon Instruments), filtered at 2 kHz (4-pole low-pass Bessel). Series resistance (mean 28 MΩ) was continuously monitored and compensated during the recordings. The liquid junction potential between electrode and ACSF was measured at 10 mV and corrected for all data presented.

A switch box enabled the same ventral root suction electrode used for antidromic stimulation to be used for recording motor unit activity. In several experiments, an

additional suction electrode was applied to a dorsal root (L4 or L5) to assess the effects of afferent stimulation. Ventral and dorsal root stimuli were applied using an electrically-isolated stimulator (Eide, 1972). Data was acquired and analyzed on a 486-based computer using pCLAMP software (v6.0 Axon Instruments). Records were also analyzed by special purpose software on a Masscomp 5400 computer.

NMDA was applied as stock solution (1 mM) to a static bath (volume = 20 ml) that was continuously oxygenated. Because recent reports suggest that 5-hydroxytryptamine (5-HT) may have a facilitatory effect on the development of NMDA receptor-mediated oscillations in amphibian spinal cord neurons (Sillar and Simmers 1994) as well as cat neocortical neurons (Nedergaard et al. 1986; 1987), 5-HT was co-applied as stock solution (10 mM) in some experiments. All concentrations reported in this paper refer to final bath concentrations, which ranged from 2.5 to 20 μM for NMDA, and 10 to 100 μM for 5-HT. In some experiments DL-2-amino-5-phosphonovaleric acid (AP5; 10-20 μM) was added to block NMDA receptors. Recordings were obtained after a stable response to the applied neurochemical was obtained; this generally required 5-15 minutes.

RESULTS

For the purposes of this report, the term "oscillation" refers to a fluctuation of membrane potential characterized by an unsustained peak depolarization that is brief in duration compared to the rising and falling phases (e.g. Fig. 1B); "plateau" describes a voltage shift with a relatively persistent phase of depolarization compared to abrupt rising and falling phases (e.g. Fig. 7). These definitions are independent of whether or not action potentials are recruited. The term "bursting" is used in reference to paroxysmal trains of high frequency firing (e.g. Fig 7).

Thirty-one motoneurons from 26 newborn rats were examined. Mean biophysical measurements of these cells were as follows: resting membrane potential, -70 $\pm$9 mV; input resistance, 86 $\pm$84 MΩ; and membrane time constant 22 $\pm$11 ms. Motoneurons were examined in the presence (n=11), absence (n=17), or both presence and absence (n=3) of TTX. Oscillations induced by NMDA alone (n=10) behaved similar to those induced by NMDA and 5-HT combined (n=16), with respect to the response to TTX application, current injection, and capacity to recruit action potentials and plateau potentials. Sixteen motoneurons expressed plateau potentials with superimposed bursting.

Membrane voltage oscillations

- Figure 1 near here -

The initial effect of bath applied NMDA, with or without 5-HT, was membrane depolarization in all but three neurons. Continuous hyperpolarizing current injection was required to return the membrane potential to its original value after application of agonists (mean current injection required to return to original value = 230 ± 238 pA). The depolarization was accompanied by an increase in synaptic activity in those preparations not exposed to TTX. In some experiments (n=5), rhythmic ventral root discharge developed at relatively low concentrations of NMDA (mean 3 μM) and 5-HT (mean 30 μM), in association with phase-related membrane potential fluctuations, as shown in Figure 1A. As the concentration of NMDA increased, phasic ventral root activity was often superseded by intense tonic discharge. This intense tonic activity typically subsided after 5-20 minutes (e.g. Fig. 1B), possibly related to the development of depolarization block; subsequent washout of NMDA was associated with restoration of ventral root activity in response to applied NMDA. In the presence of increased NMDA concentration, motoneurons held near the original resting potential, via hyperpolarizing current injection, continued to display fluctuations of membrane potential despite the loss of rhythmic ventral root activity (Fig. 1B). The voltage oscillations recorded after increasing the NMDA concentration were typically faster (Fig. 1B) than the membrane potential fluctuations recorded at low concentrations (Fig. 1A). In two preparations, periods of phase-related ventral root activity were observed during high frequency membrane voltage oscillations. Application of the NMDA receptor antagonist, AP5 (10-20 μM), prevented the development of oscillations in 2/2 motoneurons (from 2 different preparations). Removal of the voltage-dependent blockade of NMDA channels, through

the use of Mg^{2+}-free bath solution, also prevented the induction of motoneuronal oscillations (n=2), consistent with observations of NMDA-mediated oscillatory activity in other systems (e.g. Wallen and Grillner 1987; Tell and Jean 1993; Kim and Chandler 1995).

- Figure 2 near here -

Twenty-six out of 31 motoneurons tested (10 in TTX, 14 without TTX, and 2 both before and after TTX) developed membrane voltage oscillations. The mean final bath concentrations of NMDA used alone to produce oscillations was 4.8 $\pm$ 2.2 μM. The mean final bath concentrations of NMDA and 5-HT when used in combination to produce oscillations was 6.3 $\pm$ 2.3 μM and 44 $\pm$ 31 μM, respectively. Oscillations were observed over the entire voltage range examined (-99 to -40 mV). Averaged oscillations normalized to cycle length and amplitude are presented in Figure 2 to compare the shapes of the oscillation between different motoneurons, both with (Fig. 2B) and without (Fig. 2A) TTX in the bath. Note that the oscillations were similar in shape under both conditions. Once NMDA-induced oscillations were established, the application of TTX resulted in a loss of oscillatory activity (Fig. 2C middle trace). However, a subsequent increase in NMDA concentration restored oscillations, that were of longer duration (Fig. 2C bottom trace) but otherwise displayed a similar trajectory to those observed before TTX was added (Fig. 2C top trace). The mean amplitude, frequency, and duration of oscillations recorded at holding potentials between -60 and -80 mV in 5 motoneurons, in

the presence of TTX, was 13.9 ± 6.4 mV, 1.8 ± 1.1 Hz, and 333 ± 228 ms, respectively. Duration was measured at a point on the oscillation trajectory corresponding to the half amplitude. In the absence of TTX, mean values for oscillation amplitude, frequency, and duration measured in 12 motoneurons at holding potentials between -60 and -80 mV were 5.9 ± 2.1 mV, 4.4 ± 1.5 Hz, and 93 ± 38 ms, respectively.

Oscillation amplitude increased during constant depolarizing current injection (Fig. 3A). In some cells an abrupt non-linear increase in amplitude occurred with slight membrane depolarization as a result of plateau potential recruitment, as shown in Fig. 3B and the last third of Fig. 3C(i). Plateau potentials were recruited in cells examined at holding potentials depolarized greater than -40 mV. The behavior of oscillation amplitude with respect to membrane potential was the same with or without TTX (Fig. 3C). In contrast to modulation of oscillation amplitude, frequency was not related in any predictable manner to membrane potential (Fig. 4).

- Figure 3 near here -

- Figure 4 near here -

Although membrane potential had no effect on oscillation frequency, either with or without TTX in the bath, there appeared to be a linear relationship between the concentration of NMDA and oscillation frequency, but only in the presence of intact neural circuitry ($n = 13$, $r = 0.84$, $p < 0.001$; Fig. 5A). When motoneurons were synaptically isolated with TTX, no clear relationship of NMDA concentration to oscillation frequency was observed (Fig. 5A).

Dorsal root stimulation evoked transient episodes of motoneuron membrane potential oscillation (Fig. 5B), similar to those recently described by Baranauskas and Nistri (1995). These oscillations were of relatively high frequency comparable to the frequency of oscillations induced by high concentrations of NMDA in the absence of TTX (Fig. 5A). Although afferent-induced oscillations have an NMDA receptor-mediated component (Baranauskas and Nistri 1995), the precise relationship of oscillations induced by the bath application of NMDA, to those induced by stimulating the dorsal root is uncertain.

- Figure 5 near here -

Recruitment of action potentials and plateau potentials.

In five motoneurons oscillations recruited action potentials (Fig. 6A(i)). Oscillations also recruited plateau potentials in five motoneurons, some of which displayed superimposed bursting (Figs. 6A(ii)). Membrane hyperpolarization could disengage plateau potentials and unmask membrane voltage oscillations occurring at the same frequency (e.g. Fig. 3B), suggesting that the low-amplitude voltage oscillations were capable of recruiting plateau potentials. Indeed plateau potentials appeared to be recruited during the peak of the voltage oscillations in some examples (e.g. Fig. 6A(ii)).

- Figure 6 near here -

Bistable membrane properties.

Figure 6B shows further evidence of the bistable membrane behavior developed by motoneurons in the presence of NMDA. Before adding NMDA to the bath, a ramp injection of depolarizing current produces a linear membrane voltage response with superimposed repetitive firing (Fig. 6B(i)). After NMDA application the same current ramp produces a non-linear response consisting of an initial steep rise in membrane voltage followed by a short burst of spikes which terminate in a plateau phase (Fig. 6B(ii)). A spontaneous burst occurring in the same motoneuron, also in the presence of NMDA, is presented in Figure 6B(iii) (on a faster time scale). Note the similarity in membrane voltage level of the plateau phase occurring during the spontaneous and ramp-induced depolarizations. The motoneuron in Figure 6C illustrates the induction of bistable membrane properties after bath application of NMDA (6 μM) while blocking action potential generation with intracellular QX-314. This nonlinear response of motoneurons to ramp current in the presence of intact neural circuitry is similar to that observed in TTX-treated preparations (Hochman et al 1994b).

Entrainment of plateau potentials.

The effect on plateau potentials of injecting short (5 ms) and long (333 ms) duration current pulses was examined. Plateau potentials were occasionally superimposed on low amplitude voltage oscillations, as shown in Figure 7A(i). Application of 100 pA hyperpolarizing current pulses (333 ms duration at 1 Hz) entrained plateau potentials on

the rebound from the hyperpolarizing pulse (Fig. 7A(ii)). Short duration (5 ms) depolarizing current pulses initiated plateau potentials (Fig. 7A(iii)), and brief (5 ms) hyperpolarizing current pulses terminated spontaneously occurring plateau potentials (Fig. 7A(iv)). The motoneuron shown in Figure 7B displayed irregular bursting in the presence of NMDA. Ten second periods were expanded in (Figs. 7B(ii) and (iii)). Dorsal root stimulation terminated these spontaneously arising bursts (Fig. 7B(iii)). Another 100 second record of NMDA-induced activity in the same motoneuron is shown in Figure 7C. Depolarizing current pulses (30 pA, 333 ms, 2 Hz) entrained these bursts (Fig. 7C(ii)).

- Figure 7 near here -

DISCUSSION

We previously showed that NMDA receptor activation can induce intrinsic membrane voltage oscillations in TTX-treated mammalian spinal motoneurons (Hochman et al 1994b). The present work demonstrates that the capacity to generate this bistable behavior is not limited to neurons studied in the artificial setting of synaptic isolation. The same NMDA receptor-mediated properties can be elicited in motoneurons under the influence of functionally intact spinal cord circuitry. In addition, the results show that motoneuron voltage oscillations and plateau potentials can recruit action potentials and bursts of cell firing, suggesting these properties could produce a major influence on motor output.

Source and frequency control of oscillations

Despite the presence of intact neural circuitry it appears that the oscillation trajectories may be governed, at least in part, by intrinsic membrane properties rather than network generated synaptic drive. This conclusion is supported by the similar oscillation trajectory before and after TTX application. The observation that motoneurons can display voltage oscillations when network activity is no longer rhythmic, as suggested by the development of tonic ventral root discharge in the presence of increased NMDA concentrations, is also consistent with an inherent oscillatory capacity of motoneurons. However, it should be noted that the occurrence of arrhythmic ventral root activity, does not in itself prove that pre-motoneuronal elements of the network are

depolarization block of motoneurons, while the network itself remains rhythmically active.

However, several observations suggest synaptic effects may be able to modulate these oscillatory events. First, the frequency of oscillations was often faster in the presence of functionally intact circuitry. Second, a higher concentration of NMDA was required to elicit oscillations following synaptic blockade. Third, a linear relationship of oscillation frequency to NMDA concentration was observed only in the absence of TTX, suggesting that a network effect, or a synaptic event other than NMDA receptor activation, can modulate frequency. Fourth, the occurrence in some experiments of oscillations synchronous with phasic ventral root activity is consistent with network-coordinated synaptic activation of motoneurons (although electrotonic coupling could give rise to a similar observation - see below). Finally, dorsal root stimulation is capable of promoting oscillatory activity and terminating plateau potentials. Therefore, the data suggest that NMDA receptor activation results in membrane voltage oscillations, which although self-sustained in the presence of synaptic blockade, may be recruited or modulated by synaptic events in the absence of TTX.

The precise mechanism controlling the frequency of the voltage oscillations described in this study is unknown. As discussed above, synaptic events may influence the recruitment of oscillations, which may account, at least in part, for the failure of intracellular current injection to perturb oscillation frequency in individual motoneurons studied under the influence of intact circuitry. Similarly, it has been proposed that the

lack of correlation between membrane potential and the frequency of oscillations produced by stimulation of afferent fibres favors a network, rather than intrinsic membrane, source of afferent-evoked oscillations (Baranauskas and Nistri 1995).

Interestingly, it appears that oscillation frequency can also be independent of membrane potential after synaptic effects are blocked with TTX (e.g. Fig. 3C, also see Hochman et al. 1994b). In contrast, voltage sensitivity of NMDA receptor-evoked oscillatory and plateau potential frequency has been observed in many types of isolated neurons (e.g. Durand, 1991; Tell and Jean, 1991; Wallén and Grillner, 1985; Kim and Chandler, 1995). An interaction of NMDA receptor effects with other intrinsic membrane conductances appears to determine the amplitude, shape, and frequency of voltage oscillations in these systems (e.g. Wallen and Grillner, 1985, 1987; Hu and Bourque, 1992; Johnson et al., 1992; Tell and Jean, 1993; De Waele et al., 1993; El Manira et al., 1994; Kim and Chandler 1995). The lack of effect of somal current injection on oscillation frequency in our experiments may be due to a predominantly distal location of the participating NMDA receptors. Thus, given the extensive dendritic arborization of motoneurons (Dekkers et al 1994), adequate space clamp may not be achieved by current injection at the soma, particularly during the increased membrane conductance that would accompany multiple channel activation. However, poor space clamp alone seems insufficient to explain the lack of effect of injected current on oscillation frequency, because oscillation amplitude was modulated in relation to holding potential, suggesting that sufficient current reached NMDA receptor sites to have at least some influence on channel conductance.

Alternatively, the existence of electrotonic coupling between motoneurons (Walton and Navarrete 1991) may explain why constant current injection failed to perturb oscillation frequency, as was suggested to account for the same observation in TTX-isolated embryonic amphibian motoneurons (Sillar and Simmers 1994). Thus, the oscillation frequency of a given motoneuron may become synchronized to the common periodicity of its electrotonically-coupled cells. In this setting, constant current injection in only one neuron may be insufficient to alter the synchronized frequency of the linked network. This hypothesis does not exclude an influence of constant current injection on the conductances of at least some coupled neurons, which may thereby contribute to the observed amplitude modulation.

In contrast to the present results, we previously demonstrated only a minimal shift in the amplitude of NMDA-induced "oscillations" during continuous current injection, in a motoneuron isolated in TTX (Fig. 2 in Hochman et al 1994b). We now distinguish between "oscillations" and "plateau" potentials. In retrospect, the motoneuron shown in our previous report displayed rhythmic plateau potentials, not oscillations as defined in the present paper. It seems then, that the amplitude of a plateau potentials, which is typically recruited as an all or none phenomenon (e.g. Figs. 6 and 7), is relatively less sensitive to injected current compared to oscillation potentials.

Plateau potentials, bursting, and action potentials.

Plateau potentials persist in TTX (Hochman et al., 1994a) and the present study showed that intracellular injection of current pulses can initiate, entrain, and reset plateau behavior, consistent with an intrinsic membrane property. The NMDA receptor-mediated plateau potentials were present within the range of membrane voltages that includes the negative slope conductance region (MacDonald et al., 1982; Flatman et al., 1983; Mayer and Westbrook, 1984). The generation of plateau potentials may be due to recruitment of additional conductances not otherwise activated by the subthreshold voltage oscillations. For instance, voltage-dependent Ca^{2+} conductances contribute to plateau potentials in cat and turtle spinal motoneurons (Hounsgaard and Mintz 1988; Hounsgaard et al. 1988; Kiehn, 1991). Such conductances may be triggered by the membrane depolarization initiated by NMDA receptor activation, providing the neuron with enhanced voltage sensitivity compared to that produced by NMDA receptor activation alone.

Functional relevance

L-glutamate, a putative endogenous ligand for excitatory synaptic transmission, activates both NMDA and non-NMDA receptors. Both receptor subtypes are usually co-localized postsynaptically (e.g. Bekkers and Stevens, 1989). However, there is also evidence of a discrete postsynaptic localization of NMDA and non-NMDA receptors (Mooney and Konishi, 1991; Sillar and Roberts, 1991), which may also be found in the mammalian spinal cord (see Davies, 1989). Although there seems little doubt that synaptic input can trigger oscillations in motoneurons (Durand et al. 1993; Baranauskas and Nistri 1995), direct evidence that combined endogenous activation of these excitatory

amino acid receptor subtypes can induce intrinsic oscillatory behavior of the type induced by bath applied NMDA, remains to be demonstrated.

During neurochemically induced rhythmic motor activity in the in vitro neonatal rat spinal cord, high frequency packets of discharge (4-8 Hz) have been observed within the main envelopes of alternating slow discharge recorded via electroneurograms or electromyograms (Cazalets et al 1990a; Cowley and Schmidt 1995). Similar high frequency packets of discharge have also been recorded in the in vitro neonatal mouse spinal cord (Hernandez et al 1991) and within hindlimb discharge episodes during rhythmic activity in the adult cat (Noga et al 1993). The role and exact mechanism of these high frequency events remains unknown (Cowley and Schmidt 1995). However, it is interesting to speculate that the voltage oscillations observed in the present series of experiments may contribute to the high frequency parceling of motor output observed in extracellular recordings. Furthermore, although pharmacologically-induced motor rhythms in the in vitro neonatal rat spinal cord are relatively slow (0.1 - 1 Hz, Smith et al 1988; Cazalets et al 1992; Ozaki et al. 1996), as is the frequency of locomotion (Stehouwer et al. 1994) and swimming (Cazalets et al. 1990b) in intact newborn rats (1-2 Hz), both types of motor rhythm occur in the 4-5 Hz range in older animals (Gruner and Altman 1980; Cazalets et al. 1990b; Stehhouwer et al. 1994). Therefore, although intact neonatal animals do not demonstrate high frequency hindlimb movements, the present observations suggest that components of the neural substrate required for generating the fast rhythms in adults may be present at birth.

The inherent oscillatory tendency of motoneurons may enable this final common output to limit the effect of temporal dispersion among phasic synaptic events generated by locomotor circuitry and other rhythmogenic networks.

ABBREVIATIONS

ACSF; artificial cerebrospinal fluid

AHP; afterhyperpolarization

NMDA; N-methyl-D-aspartate

5-HT; 5-hydroxytryptamine

TTX; tetrodotoxin

LDP; locomotor drive potential

LTP; long-term potentiation

QX-314; lidocaine N-ethyl bromide

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FIGURES AND FIGURE LEGENDS

FIGURES and FIGURE LEGENDS

Figure 1. Relationship of NMDA-induced motoneuronal activity to ventral root discharge in two different motoneurons (MN). The upper records in **A** and **B** show the intracellular events, recorded using a QX-314 filled electrode. The lower records show lumbar ventral root (VR) activity. **A**. Network driven rhythmic motoneuronal activity was out of phase with ventral root discharge in the presence of NMDA and 5-HT. **B**. Motoneuron voltage oscillations induced by higher concentrations of NMDA were observed in the absence of phasic ventral root discharge.

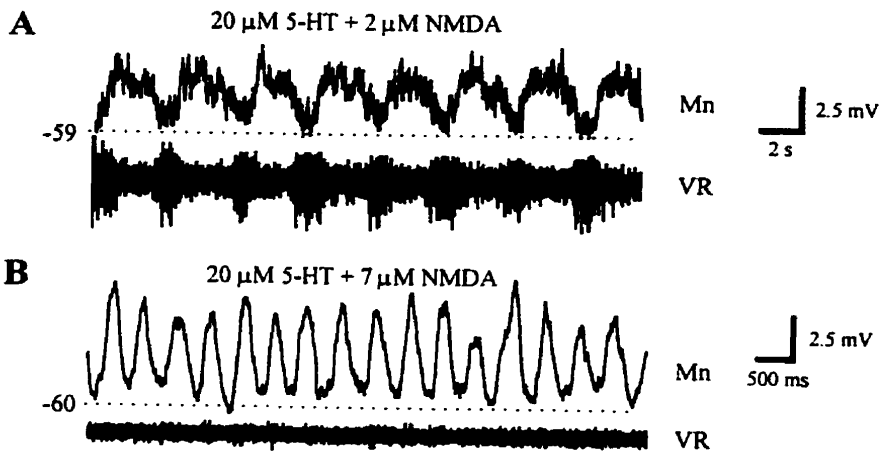

A
20 μM 5-HT + 2 μM NMDA
-59
Mn
VR
2.5 mV
2 s
B
20 μM 5-HT + 7 μM NMDA
-60
Mn
VR
2.5 mV
500 ms

Figure 2. Membrane voltage oscillations. **A.** Averaged voltage oscillations in 11 different motoneurons (amplitude and cycle normalized) presented in superimposed **(i)** and raster **(ii)** formats in the absence of TTX. **B.** Averaged voltage oscillations in 6 different motoneurons (amplitude and cycle normalized) presented in superimposed **(i)** and raster **(ii)** formats in the presence of TTX. **C.** NMDA-evoked voltage oscillations (top trace) were blocked by the addition of TTX (middle trace), but reappeared after increasing the concentration of NMDA (bottom trace).

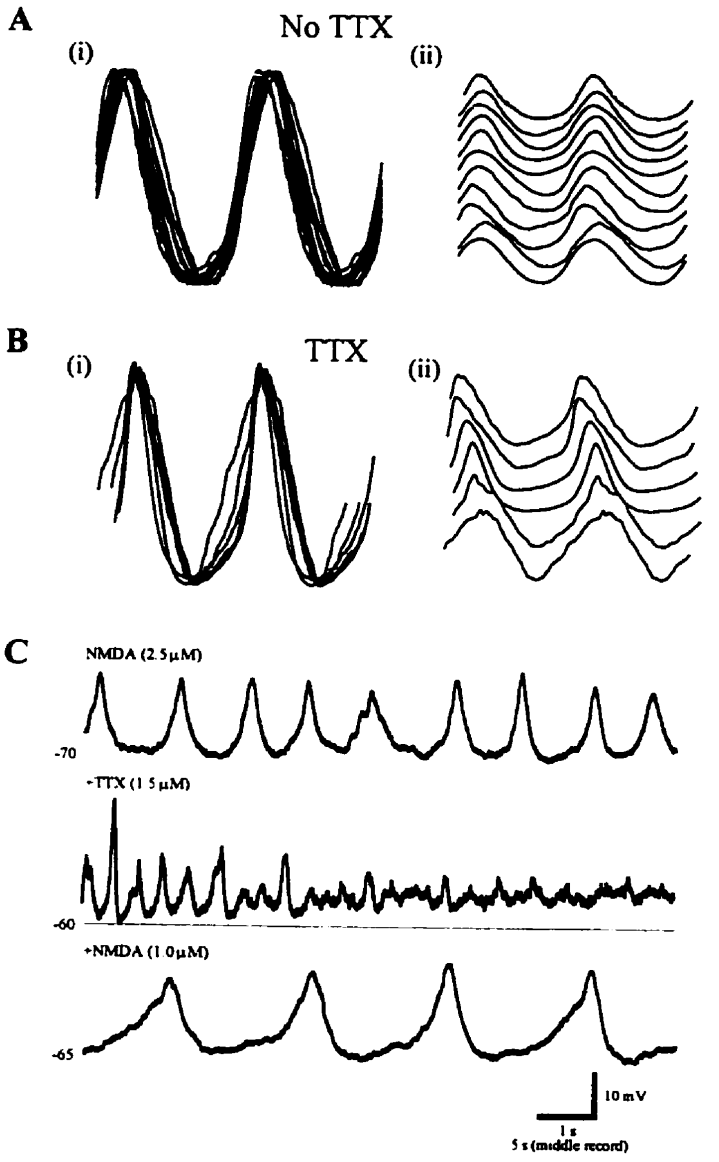

A
No TTX
(i)
(ii)
B
TTX
(i)
(ii)
C
NMDA (2.5μM)
-70
+TTX (1.5μM)
-60
+NMDA (1.0μM)
-65
10 mV
1 s
5 s (middle record)

Figure 3. Membrane voltage-dependency of oscillation amplitude. **A.** Voltage oscillations were observed at four different membrane potentials during current injection in a single motoneuron. QX-314 is included in the pipette to block the Na^+ spike. As the membrane is depolarized, oscillation amplitude increases. **B.** In another motoneuron, membrane behavior is shown at two different holding potentials. Membrane depolarization recruited plateau potentials, with superimposed bursting, at the same frequency as the membrane voltage oscillations observed at the more hyperpolarized potential. **C.** Membrane holding potential had the same effect on oscillation amplitude before **(i)** and after **(ii)** bath application of TTX to block synaptic transmission.

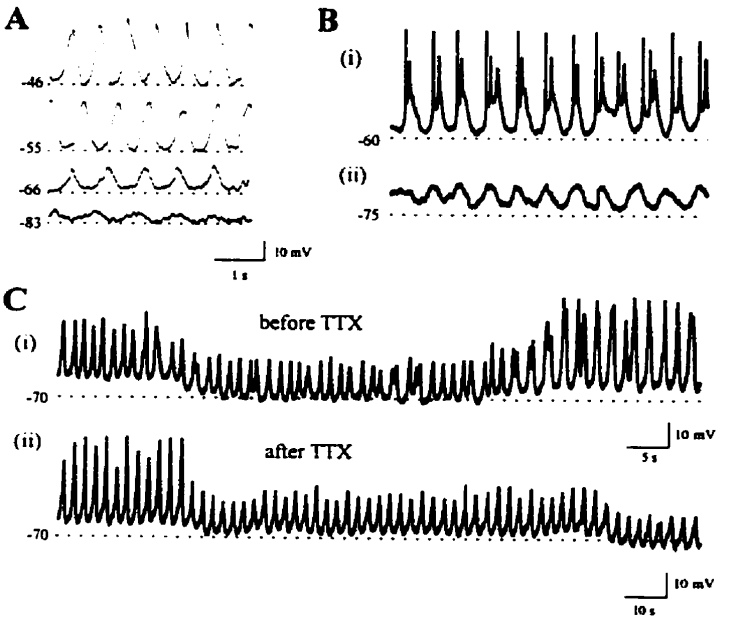

A
-46
-55
-66
-83
10 mV
1 s
B
(i)
-60
(ii)
-75
C
(i)
before TTX
-70
(ii)
after TTX
-70
10 mV
5 s
10 mV
10 s

Figure 4. Oscillation amplitude and frequency plotted with respect to membrane voltage in 8 motoneurons. Membrane holding potential was adjusted with intracellular current injection. Three electrodes contained QX-314 to block Na^+ spikes (filled square and circle, and open inverted triangle). **(i).** Membrane depolarization consistently produced an increase in oscillation amplitude. **(ii).** No consistent change in frequency occurred in response to adjustments of membrane voltage.

No TTX

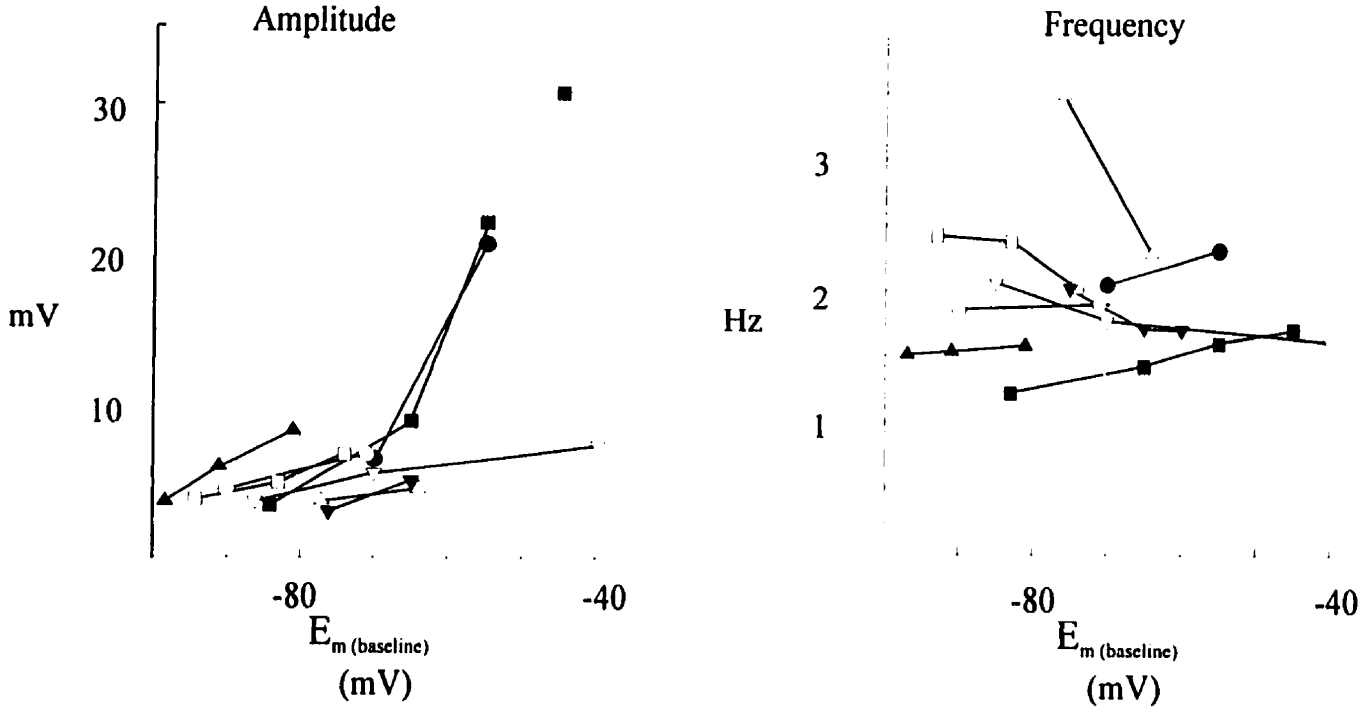
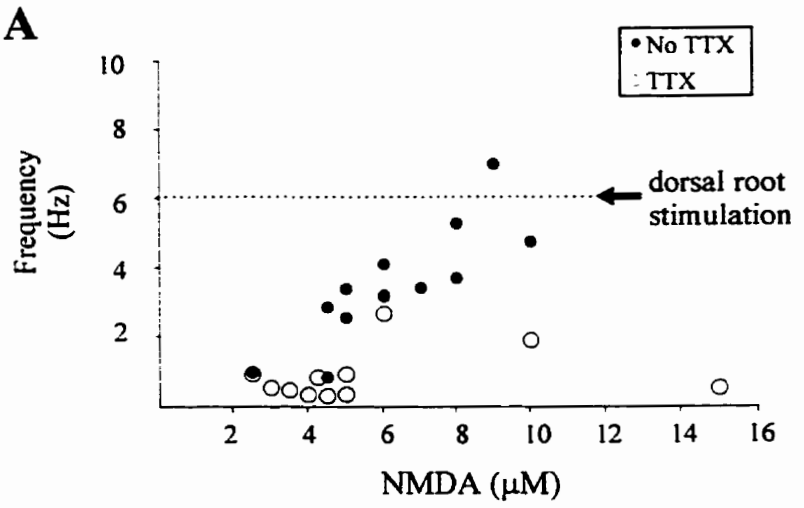

Amplitude
30
20
mV
10
-80
-40
$E_{m\ (baseline)}$
(mV)
Frequency
3
2
Hz
1
-80
-40
$E_{m\ (baseline)}$
(mV)

Figure 5. **A.** In contrast to oscillations observed in the absence of TTX (filled circles), synaptically-isolated motoneurons appeared to oscillate at frequencies unrelated to NMDA concentration (open circles). Voltage oscillations evoked by dorsal root stimulation (dotted horizontal line) supported higher oscillations frequencies. **B**. In the presence of 5-HT alone, low-threshold dorsal root stimulation evoked an early inhibition followed by membrane voltage oscillations. Spikes are truncated for illustrative purposes.


A
No TTX
TTX
dorsal root stimulation
Frequency (Hz)
10
8
6
4
2
2
4
6
8
10
12
14
16
NMDA (μM)

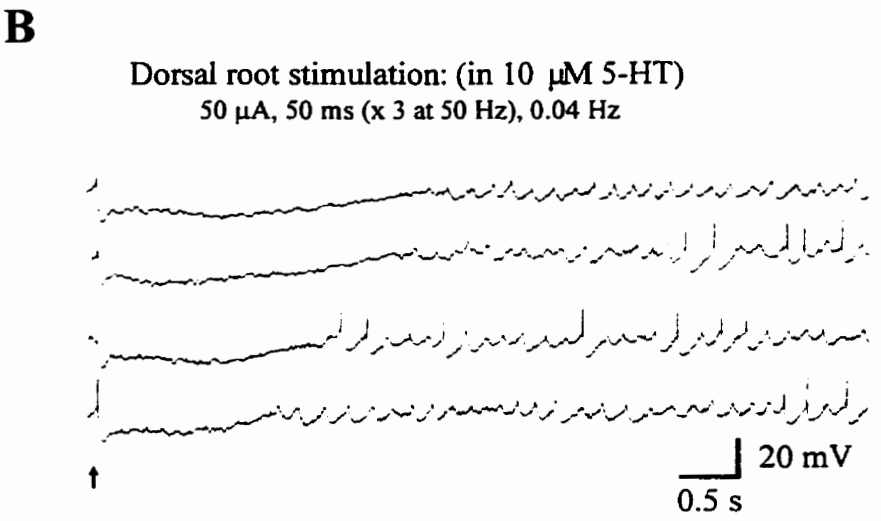

B
Dorsal root stimulation: (in 10 μM 5-HT)
50 μA, 50 ms (x 3 at 50 Hz), 0.04 Hz
20 mV
0.5 s

Figure 6. **A.** Voltage oscillations recruited action potentials in some motoneurons (**A(i)**) and plateau potentials with superimposed bursts in others (**A(ii)**). Activity in **6A(i)** was induced by 7 μM NMDA and 20 μM 5-HT, and in **6 A(ii)** by 8 μM NMDA. **B(i).** Current ramp injection (1500 pA) produced a linear rise in membrane voltage until repetitive firing started. **B(ii).** After bath application of 7.5 μM NMDA the same current ramp produced a non-linear increase in the rate of rise in membrane voltage until threshold for spike initiation was reached. The membrane voltage then plateaued concomitantly with depolarization blockade of spike generation. **B(iii).** In the same cell, a spontaneously arising burst displayed the same voltage plateau level as was evoked by current ramp (**Bii**). **C.** In another motoneuron, QX-314 was applied intracellularly to block Na^+ spikes. A current ramp (1500 pA) produced a linear I-V relation (**Ci**). After bath application of 10 μM 5-HT and 6.5 μM NMDA, a current ramp (± 1200 pA) produced a non-linear I-V relation characterized by rapid voltage jumps at the initiation and termination of the intervening plateau phase (**Cii**).

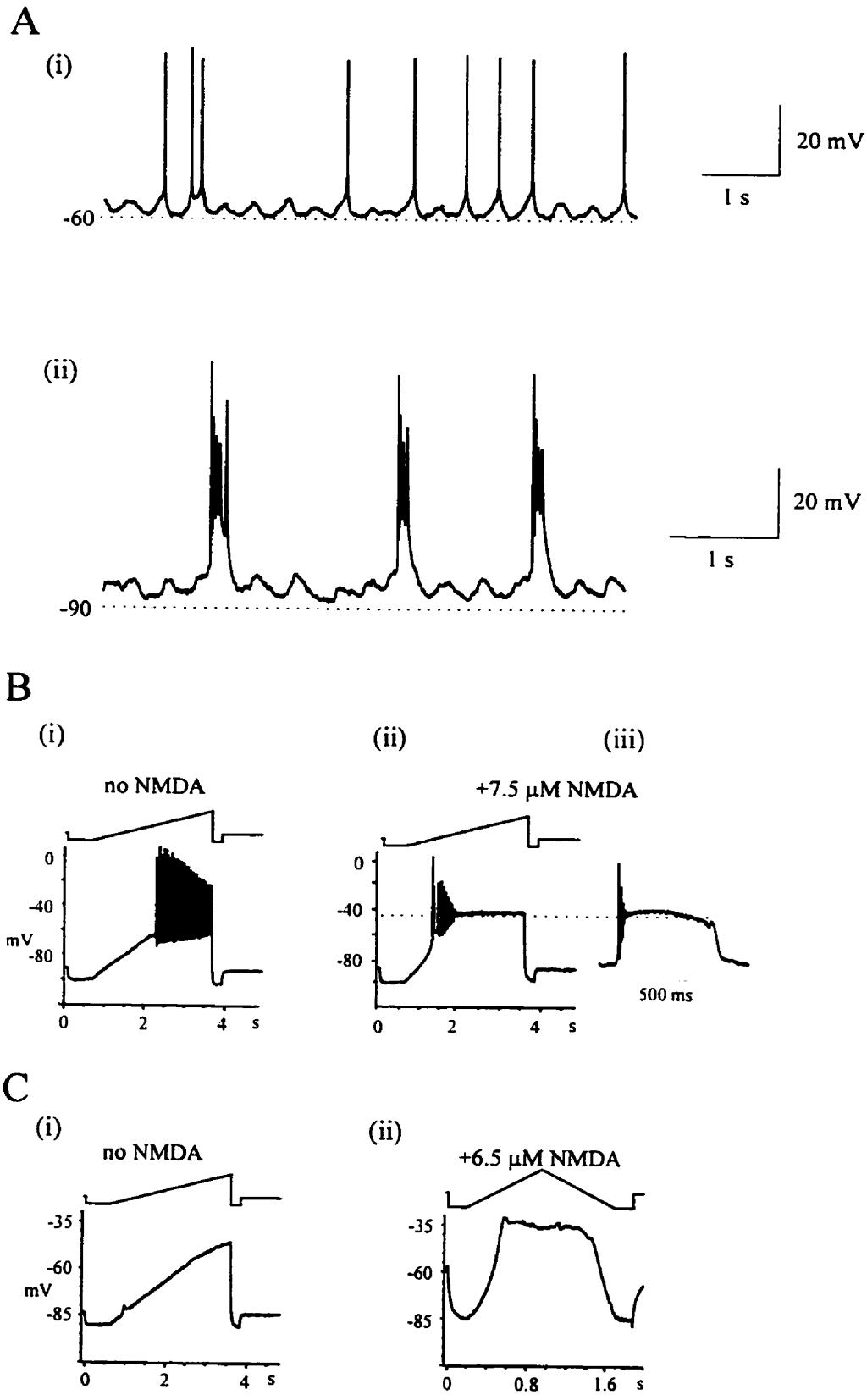
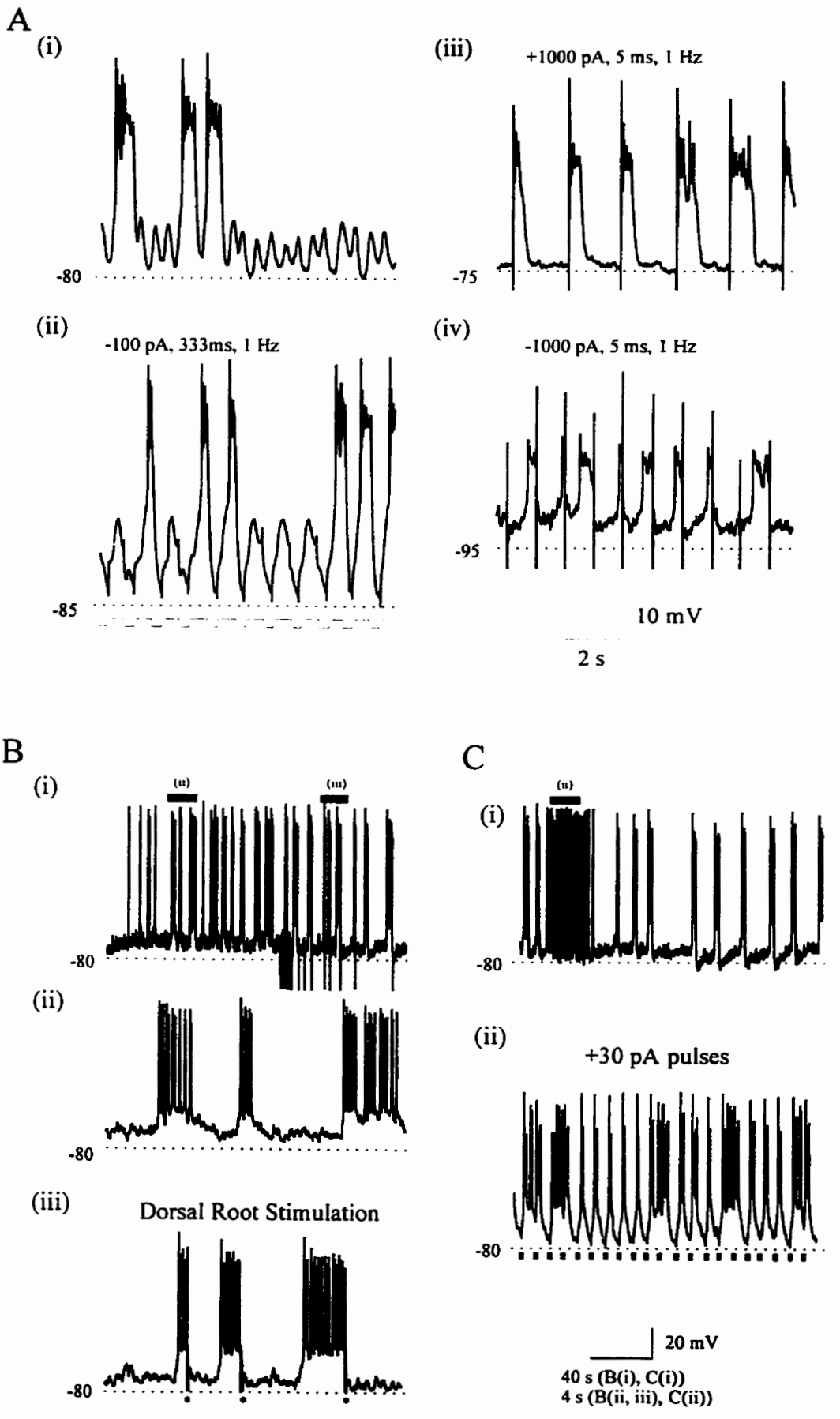

A
(i)
-60
20 mV
1 s
(ii)
-90
20 mV
1 s
B
(i)
no NMDA
0
-40
mV
-80
0
2
4
s
(ii)
+7.5 µM NMDA
0
-40
-80
0
2
4
s
(iii)
500 ms
C
(i)
no NMDA
-35
-60
mV
-85
0
2
4
s
(ii)
+6.5 µM NMDA
-35
-60
-85
0
0.8
1.6
s

Figure 7. **A.** Current pulses initiated, entrained, and terminated plateau potentials. The same motoneuron is shown in **A(i)** through **(iv)** (in 50 μM 5-HT and 10 μM NMDA). **A(i).** Membrane voltage oscillations and plateau potentials were interspersed. **A(ii).** Hyperpolarizing current pulses (-100 pA, 333 ms, 1 Hz) entrained plateau potentials on return from membrane hyperpolarization. Current pulses are schematically represented in the bottom trace. **A(iii).** Short-duration depolarizing current pulses (1000 pA, 5 ms, 1 Hz) initiated plateau potentials. **A(iv).** Hyperpolarizing current pulses (-1000 pA, 5 ms, 1 Hz) terminated spontaneously occuring plateau potentials. **B** and **C** show entrainment and resetting of bursting. Two epochs of bursting (**B(i)** and **C(i)**) from a single motoneuron (in 70 μM 5-HT and 6 μM NMDA) are shown. Sample bursts from segments indicated by horizontal bars in **B(i)** are shown on expanded time scales in **B(ii)** and **B(iii)**. Bursting was abruptly terminated by dorsal root stimulation (200 μA, 0.1 ms, 13 pulses at 50 Hz) as indicated by the asterisks in **B(iii)**. **C.** Depolarizing current pulse (30 pA, 333 ms, 2 Hz) injection (vertical bars) entrained burst frequency. Sample bursts from the segment in **C(i)** is shown on an expanded time scale in **C(ii)**.


A
(i)
-80
(ii)
-100 pA, 333ms, 1 Hz
-85
(iii)
+1000 pA, 5 ms, 1 Hz
-75
(iv)
-1000 pA, 5 ms, 1 Hz
-95
10 mV
2 s
B
(i)
(ii)
(iii)
-80
Dorsal Root Stimulation
C
(i)
(ii)
+30 pA pulses
-80
20 mV
40 s (B(i), C(i))
4 s (B(ii, iii), C(ii))

SECTION II

NMDA RECEPTOR-MEDIATED OSCILLATORY ACTIVITY IN THE NEONATAL RAT SPINAL CORD IS SEROTONIN-DEPENDENT

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Journal of Neurophysiology, 79: 2804-2808, (1998).

ABSTRACT

The effect of serotonin (5-HT) receptor blockade on rhythmic network activity and on NMDA receptor-induced membrane voltage oscillations was examined using an *in vitro* neonatal rat spinal cord preparation. Pharmacologically-induced rhythmic hindlimb activity, monitored via flexor and extensor electroneurograms or ventral root recordings, was abolished by 5-HT receptor antagonists. Intrinsic motoneuronal voltage oscillations, induced by NMDA in the presence of TTX, were either completely abolished or transformed to long-lasting voltage shifts by 5-HT receptor antagonists. Conversely, 5-HT application facilitated the expression of NMDA-receptor mediated rhythmic voltage oscillations. The results suggest that an interplay between 5-HT and NMDA receptor actions may be critical for the production of rhythmic motor behavior in the mammalian spinal cord, both at the network and single cell level.

INTRODUCTION

Rhythmic motor activity, including locomotion, can be induced in the *in vitro* neonatal rat spinal cord by bath application of a variety of neurochemicals, such as NMDA or 5-HT (Smith et al. 1988; Cazalets et al. 1992). The combined administration of NMDA and 5-HT may be more effective than either substance alone in promoting locomotor-like activity (Sqalli-Houssaini et al. 1993). However, elicitation of neural activity in response to whole-cord application of an excitatory substance does not prove that activation of the corresponding receptor system is critical for the production of the same behavior in the intact animal. Instead, the use of selective antagonists can help clarify which endogenous receptor types are important. For instance, both NMDA and non-NMDA excitatory amino acid (EAA) receptor antagonists suppress rhythmic motor activity induced by bath applied EAAs (Smith et al. 1988; Cazalets et al. 1992). In addition, EAA receptor antagonists suppress spinal motor rhythms induced by non-EAA neurochemical activators and brainstem electrical stimulation (Smith et al. 1988; Schmidt et al. 1989; Beato et al. 1997). These observations suggest a role for endogenous NMDA and non-NMDA EAA receptors in the generation of rhythmic motor activity in the mammalian spinal cord. NMDA receptor activation is of particular interest, because it elicits inherent oscillations of membrane potential in rat spinal interneurons and motoneurons (Hochman et al. 1994a,b), a property that may be well-suited for the production of rhythmic behaviors such as locomotion.

Although administration of 5-HT receptor antagonists to spinal cats reverses perturbations of the locomotor pattern produced by 5-HT agonists (Barbeau and Rossignol 1990), it remains to be shown whether endogenous 5-HT receptor activation is critical for network rhythmogenesis in the mammalian spinal cord. In the lamprey, application of 5-HT decreases swimming frequency (Harris-Warrick and Cohen 1985) and slows the repolarizing phase of NMDA receptor-mediated intrinsic voltage oscillations in spinal neurons (Wallen et al. 1989). In contrast, 5-HT is required for the maturation of locomotor rhythms (Sillar et al. 1995) and the expression of intrinsic voltage oscillations (Sillar and Simmers 1994; Scrymgeour-Wedderburn et al. 1997) in amphibian spinal neurons. The present study examines whether 5-HT is essential for network rhythmogenesis and/or modulation of NMDA-mediated oscillatory activity in the mammalian spinal cord.

Some of the following data has been presented previously in abstract form (MacLean and Schmidt 1995; MacLean et al. 1996).

METHODS

Experiments were performed on 37 Sprague-Dawley rats (aged 2-7 days). Techniques for isolation of the spinal cord, extracellular recording, and neurochemical induction of rhythmic activity have been described previously (e.g. Cowley and Schmidt 1995). In brief, animals were anesthetized with ether, decapitated, eviscerated, and placed in a bath chamber containing (in mM): NaCl 128; KCl 3.0; $Na_2H_2PO_4$ 0.5; $CaCl_2$ 1.5; $MgSO_4$ 1.0;

$NaHCO_3$ 21; and glucose 30 equilibrated to pH 7.4 with 95% O_2/5% CO_2. Bilaterally intact spinal cords, transected at C1, were then isolated. In some experiments, rhythmic network activity in the spinal cord was monitored via electroneurogram (ENG) recordings of ankle flexor (peroneal) and extensor (tibial) nerves or bilateral L_2 and L_5 ventral root recordings. In other experiments, the activity of synaptically isolated motoneurons in tetrodotoxin (TTX) was monitored using whole-cell patch recordings.

Whole-cell recordings of motoneurons were obtained as previously described (Hochman et al. 1994b). Recording pipettes contained (in mM): K-gluconate 140; ethylene glycol-bis(-amino ethyl ether) N,N,N',N',-tetra-acetic acid (EGTA) 11; KOH 35; N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10; and $CaCl_2$ 1. Cells were approached through a pial patch made over the ventrolateral surface of the spinal cord, and were identified as motoneurons by their antidromic response to ventral root stimulation. The recordings were obtained with an Axopatch 1D amplifier (Axon Instruments), filtered at 2 kHz. Series resistance was continuously monitored and compensated. The electrode-bath solution liquid junction potential (10 mV) was corrected for all data presented. Data was acquired and analyzed on a 486-based computer using pCLAMP software (v6.0 Axon instruments). Records were also analyzed using special purpose software on a Masscomp 5400 computer.

RESULTS

We first examined the effects of 5-HT receptor blockade on neurochemically induced network rhythmogenesis. Hindlimb rhythmic activity was induced by NMDA (6-10 μM, n=11), acetylcholine (ACh, 60 μM) in combination with edrophonium (EDRO, 200 μM, n=1), or NMDA (6-16 μM) and ACh/EDRO (60/200 μM) combined (n=2). Note that non-locomotor-like patterns of rhythmic activity, as for example the coactivation of flexor and extensor ENGs shown in Figure 1A, are not uncommon using NMDA alone and/or ACh in this preparation (Cowley and Schmidt 1994).

In 11/11 preparations, rhythmic activity was abolished after the bath application of 5-HT receptor antagonists (methysergide 20-100 μM, n=3, Fig. 1A; mianserin 70-200 μM, n=6; or cyproheptadine 60-80 μM, n=2). In 3/3 preparations, low concentrations of mianserin (1 μM) or ketanserin (2 μM) were also effective in terminating NMDA-induced rhythmic activity (Fig. 1B), although the latency to full blockade was longer (>60 minutes) than that required (15-30 minutes) using higher concentrations of the 5-HT antagonists. In 3 other experiments, NMDA was used to induce rhythmic activity, after which it was washed out of the bath. These cords were then exposed to low concentrations of mianserin (1 μM) for 90 minutes before re-application of NMDA in graded concentrations from 2-20 μM. Pre-incubation with mianserin prevented the elicitation of rhythmic activity.

Figure 1 near here.

Among the remaining 20 preparations, 20 motoneurons were examined using whole-cell recordings. Seventeen of these motoneurons were studied in the presence of N-methyl-D-aspartate (NMDA, 2-20 μM) and synaptic isolation with TTX (1.5 μM). Three types of motoneuron membrane behavior were observed. Some motoneurons (n=3) developed regular oscillations, characterized by peak depolarizations that were shorter in duration than the rise or falling phases of the voltage fluctuation (Fig. 2A(i)). The mean values for amplitude, frequency and duration of these oscillations were 20.4 ± 9.2 mV, 0.7 ± 0.2 Hz, and 427 ± 23 ms respectively. The second type of NMDA-induced behavior featured recurrent but long-lasting shifts in membrane voltage (n=5, Fig. 2A(ii)). The duration of the depolarized phase in these cells averaged 3.2 ± 2.0 s and the mean amplitude was similar to the amplitude of oscillations (21.6 ± 1.8 mV). The voltage shifts were rhythmic in 3 of the 5 motoneurons (mean frequency 0.26 ± 0.03 Hz), and arrhythmic in the other 2 cells. The third type of response consisted of sustained depolarization (10.8 ± 2.4 mV, n=9; Fig. 2B(ii)).

Figure 2 near here.

The effect of the 5-HT receptor antagonist mianserin (100-150 μM, n=7) or ketanserin (100 μM, n=1) on TTX-resistant oscillations induced by NMDA alone (2.5-4.5 μM, n=3), or by NMDA and 5-HT (15-100 μM) combined (n=5) was examined. Oscillations, induced by NMDA and 5-HT, were completely abolished in 3 motoneurons (e.g. Fig.

2B(i)). In 5 motoneurons, exposed to either NMDA alone (n=3) or both NMDA and 5-HT (n=2), oscillations and were transformed into long-lasting recurrent voltage shifts (Fig. 2A(i)). The voltage shifts were arrhythmic in 4/5 cells. Their amplitude (20.9 ± 5.9 mV) and duration (2.2 ± 0.8 s) were similar to the values obtained for long-lasting voltage shifts induced by NMDA alone. The emergence of fully developed long-lasting voltage shifts required up to 30 minutes or longer (e.g. Fig. 2A(i)) after adding the 5-HT receptor antagonist, and was accompanied by gradual membrane hyperpolarization. This transformation was not simply due to membrane hyperpolarization, since hyperpolarizing current injection alone, in the absence of a 5-HT antagonist, failed to convert oscillations into long-lasting voltage shifts (Fig. 2C). In the absence of 5-HT antagonists, oscillations persisted for over an hour, in those neurons which initially displayed oscillatory activity (n=8, e.g. Fig. 2D), suggesting that the development of long-lasting voltage shifts was unrelated to progressive dialysis of intracellular contents with the pipette filling solution, or 'run-down'.

The effect of adding 5-HT (15-100 μM) was examined in motoneurons displaying long-lasting membrane voltage shifts (n=3) or tonic depolarization (n=9) in response to NMDA alone. Seven of these motoneurons (3 with long-lasting voltage shifts and 4 with tonic depolarization) developed oscillations in response to 5-HT (Figs. 2A(ii) and 2B(ii), respectively). The mean amplitude, frequency and duration of these 5-HT facilitated oscillations were 11.8 ± 4.5 mV, 0.7 ± 0.3 Hz , and 755 ± 474 ms respectively. The transformation between long-lasting voltage shifts and oscillations always occured as a

slow transition rather than an abrupt threshold-like event (Fig. 2A). Five of the 9 motoneurons that initially displayed tonic depolarization in response to NMDA, failed to develop oscillations after 5-HT was added; the input resistance, time constant, resting membrane potential and spike height of these motoneurons was 60.3 ± 49.9 MΩ, 13.9 ± 9.7 ms, -76.6 ± 7.2 mV and 91.0 ± 10.1 mV, respectively. The corresponding values for motoneurons that were capable of developing oscillations (n=13) were 95.0 ± 57.0 MΩ, 22.5 ± 12.5 ms, -74.1 ± 5.8 mV and 68.1 ± 22.1 mV (n=13). Comparing the two groups, no significant difference in the mean values of these membrane properties was found (Student's t test, $p > 0.1$).

DISCUSSION.

Numerous studies have documented that 5-HT influences the excitability of motoneurons (e.g. Barasi and Roberts 1974; White and Neuman 1980; Takahashi and Berger 1990; Elliot and Wallis 1992; Ziskind-Conhaim et al 1993) and facilitates the development of plateau potentials (Hounsgaard et al. 1988, Hounsgaard and Kiehn 1989). Co-localization of glutamate and 5-HT in synaptic boutons surrounding motoneuron cell bodies (Nicholas et al. 1992), also suggests that EAA and 5-HT receptor systems may closely interact. 5-HT depresses NMDA receptor-mediated responses in the locus coeruleus (Charlety et al. 1993), dorsal horn (Murase et al 1990) and ventral horn (Chesnoy-Marchais and Barthe 1996) of the spinal cord. In contrast, 5-HT enhances the effects of NMDA application on neocortical cells (Nedergaard et al. 1986; 1987; Reynolds et al. 1988; Rahman and Neuman 1992), including the development of rhythmic bursting and TTX-resistant 'depolarization shifts' (Nedergaard et al. 1986; 1987). A preliminary report indicated that similar 5-HT modulatory actions may be present in cat motoneurons (Flatman and Engberg 1990). Certainly the present results are compatible with an important role for 5-HT in the control of rhythmic motor output in the mammalian spinal cord, both at the single cell and network level.

In the presence of TTX, most motoneurons failed to display oscillations in response to NMDA alone, consistent with a requirement for 5-HT receptor-mediated facilitation of intrinsic oscillatory activity. The few TTX-treated motoneurons (3/17) that did developed oscillations after adding NMDA alone may have been under the influence of

residual 5-HT receptor activation despite TTX. This possibility is supported by the observation that subsequent addition of 5-HT antagonists transformed oscillations in these cells into long-lasting voltage shifts.

In the absence of TTX, 5-HT antagonists always abolished rhythmic network activity. Thus, it appears that endogenous 5-HT receptor activation must persist, at least to some degree, in the isolated spinal cord, despite removal of the brainstem, and thus the source of spinal 5-HT (Dahlstrom and Fuxe 1965; although, see Newton and Hamill 1988).

Activation of either 5-HT_{1A} or 5-HT_2 receptors can modulate NMDA receptor-mediated currents via second messenger systems (Blank et al. 1996; Chen and Huang 1992). A recent study of embryonic and larval *Xenopus* spinal cord neurons suggests that 5-HT_{1A} receptor activation facilitates the voltage-dependent blockade of NMDA channels by Mg^{2+}; and this interaction may explain why NMDA and 5-HT receptor co-activation is required for the production of voltage oscillations in these cells (Scrymgeour-Wedderburn et al. 1997). In contrast, our observations, using mianserin and ketanserin, suggest that NMDA-induced oscillations in the neonatal rat spinal motoneurons may depend on 5-HT_2 receptor activation. However, confirmation of the specific receptor type(s) awaits further investigation. It is also possible that the interaction between 5-HT and NMDA in the production of motoneuronal oscillations may occur via 5-HT effects that are independent of direct modulation of NMDA receptors, or that 5-HT may act as an open channel voltage-dependent blocker of the NMDA ionophore (Chesnoy-Marchais and Barthe 1996) independent of 5-HT receptor activation.

5-HT and other neuromodulators have a critical role in functionally reconfiguring invertebrate rhythmogenic circuits; in these systems, modulators regulate the strength of synaptic interactions and intrinsic membrane properties of neuronal elements distributed throughout the network (e.g. Katz et al. 1994; Johnson et al. 1995). Although the present study demonstrates 5-HT receptor-mediated modulation of intrinsic membrane properties in motoneurons, it remains to be shown whether similar modulation occurs in other neural components of the network. It is quite possible that the blockade of rhythmogenic activity by 5-HT antagonists was related, at least in part, to the actions of the antagonists on network interneurons. Regardless of the specific sites of action, our results suggest that 5-HT is critical for the expression of rhythmic motor activity in the mammalian spinal cord.

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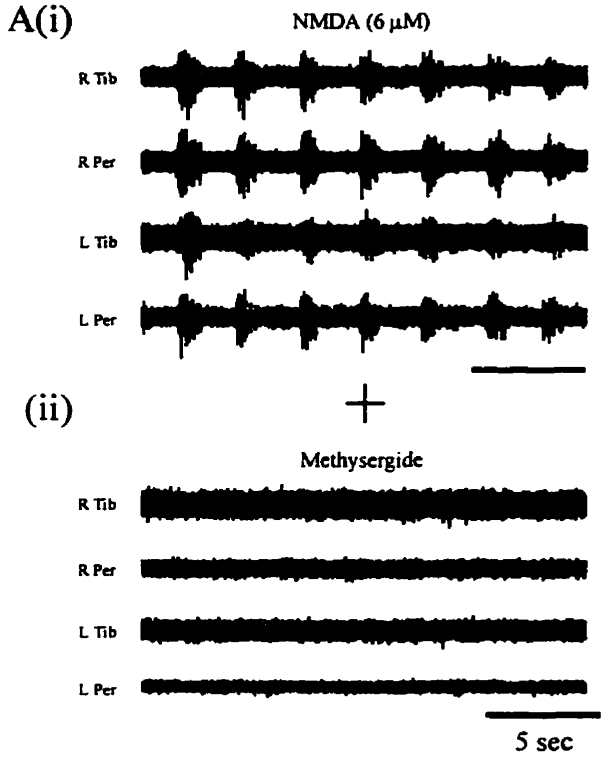
FIGURES and FIGURE LEGENDS

Figure 1. 5-HT receptor antagonists abolished network rhythmic activity. ***A*:** Peroneal (Per) and tibial (Tib) rhythmic activity was induced by NMDA (***Ai***) and abolished by methysergide (160 μM, ***Aii***). ***B*:** Rhythmic activity induced by NMDA (***Bi***) was blocked by a low concentration of mianserin (1 μM, ***Bii***).


A(i)
NMDA (6 μM)
R Tib
R Per
L Tib
L Per
(ii)
+
Methysergide
R Tib
R Per
L Tib
L Per
5 sec

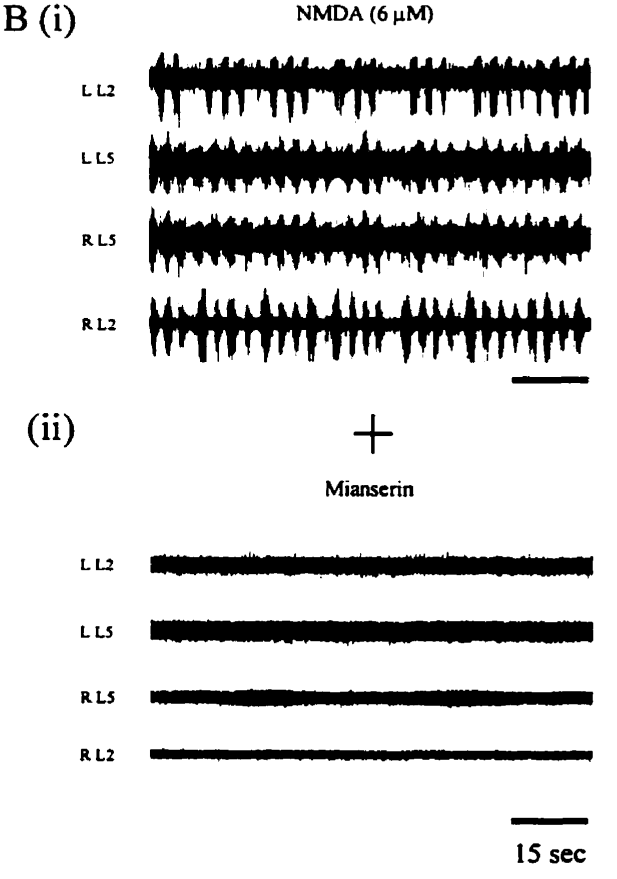
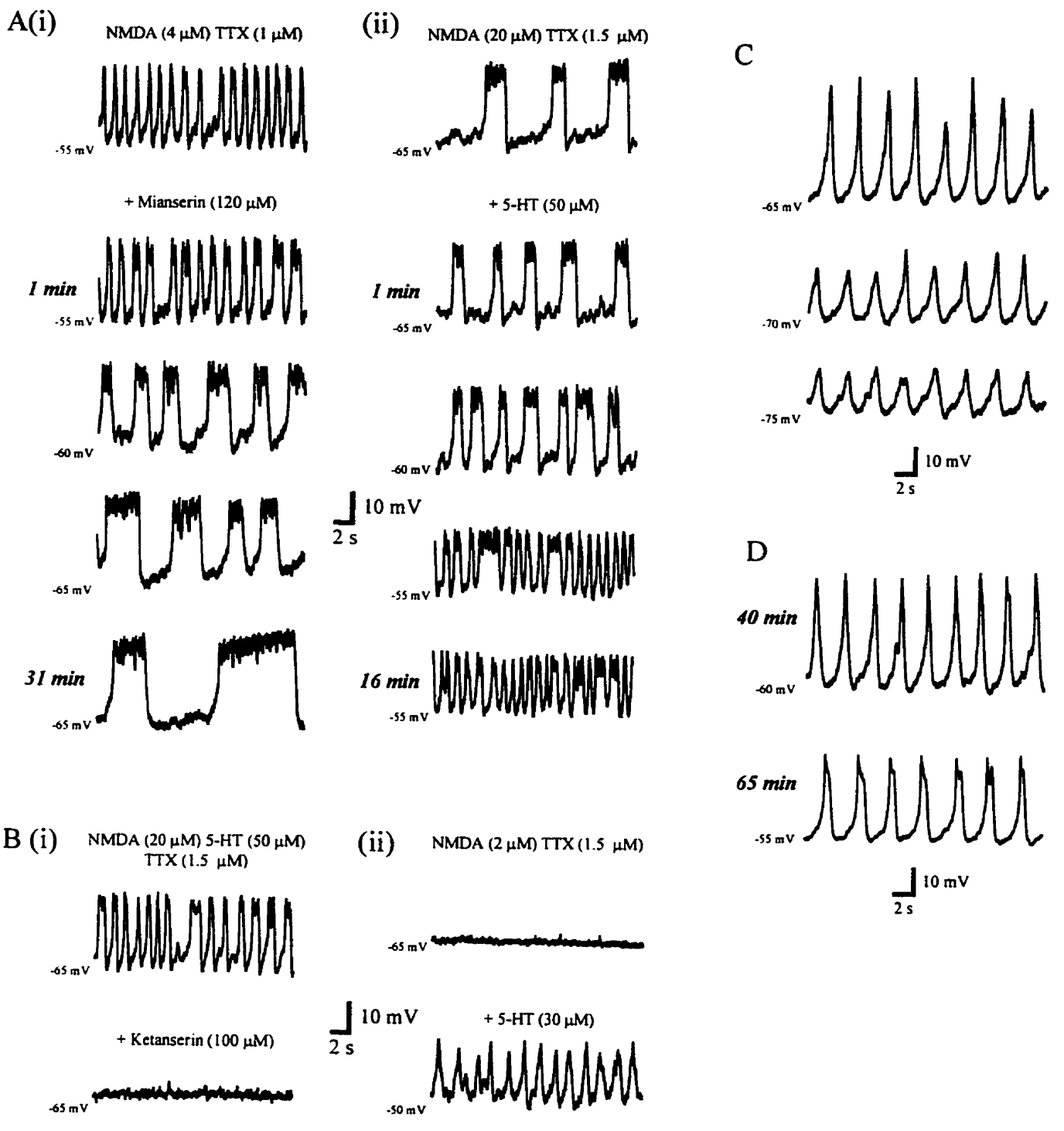

B (i)
NMDA (6 μM)
L L2
L L5
R L5
R L2
(ii)
+
Mianserin
L L2
L L5
R L5
R L2
15 sec

Figure 2. 5-HT modulation of TTX-resistant NMDA-induced voltage oscillations. ***A(i)*:** Mianserin (120 μM) transformed NMDA-induced rhythmic oscillations to long-lasting shifts of membrane voltage. ***A(ii)*:** 5-HT (50 μM) transformed long-lasting voltage shifts produced by NMDA alone into rhythmic oscillations. The time elapsed after the addition of mianserin (***A(i)***) and 5-HT (***A(ii)***) is indicated. ***B(i)*:** Oscillations induced by NMDA and 5-HT were abolished by ketanserin (100 μM). ***B(ii)*:** NMDA-induced stable membrane depolarization which was subsequently transformed to rhythmic voltage oscillations by 5-HT (30 μM). ***C:*** In the absence of 5-HT receptor blockade, oscillations persisted despite manipulation of the holding potential, although amplitude varied in relation to the holding potential. ***D:*** In the absence of 5-HT receptor blockade, TTX-resistant NMDA-mediated oscillations were well maintained for over 60 minutes. The records shown in **A(ii) and B(i)** are from the same motoneuron.


A(i)
NMDA (4 μM) TTX (1 μM)
-55 mV
+ Mianserin (120 μM)
1 min
-55 mV
-60 mV
-65 mV
31 min
-65 mV
(ii)
NMDA (20 μM) TTX (1.5 μM)
-65 mV
+ 5-HT (50 μM)
1 min
-65 mV
-60 mV
10 mV
2 s
-55 mV
16 min
-55 mV
C
-65 mV
-70 mV
-75 mV
10 mV
2 s
D
40 min
-60 mV
65 min
-55 mV
10 mV
2 s
B (i)
NMDA (20 μM) 5-HT (50 μM)
TTX (1.5 μM)
-65 mV
+ Ketanserin (100 μM)
-65 mV
(ii)
NMDA (2 μM) TTX (1.5 μM)
-65 mV
10 mV
2 s
+ 5-HT (30 μM)
-50 mV

SECTION III

NMDA RECEPTOR CHANNEL BLOCK BY MG^{2+} IS MODULATED BY SEROTONIN AND PROMOTES RHYTHMIC MOTOR ACTIVITY IN THE RAT SPINAL CORD

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ABSTRACT

N-methyl-D-aspartate (NMDA) and serotonin (5-HT) receptors contribute to the generation of rhythmic motor patterns, such as locomotion, in the vertebrate spinal cord. However, the pertinent mechanisms underlying NMDA and 5-HT receptor actions in these rhythmogenic circuits remain to be shown, as do the interactions, if any, that occur during combined activation of these receptors. In the present study, an in vitro neonatal rat spinal cord preparation was used to examine whether NMDA receptor-induced nonlinear membrane behaviour in particular is a) required for the generation of stable motor rhythms, and b) modulated by 5-HT. Because NMDA receptor channel-mediated nonlinear membrane properties are due to voltage-sensitive blockade by Mg^{2+} ions, the effect of Mg^{2+} removal on neurochemically-induced motor rhythms was examined. Locomotor-like patterns of lumbar ventral root activity observed in the presence of physiological Mg^{2+} concentrations were replaced by poorly organized patterns after removal of Mg^{2+}. In other experiments, involving whole-cell recordings of spinal motoneurons, 5-HT enhanced NMDA channel conductance and shifted the region of negative slope conductance (RNSC) in the current-voltage relationship to more hyperpolarized potentials. This effect of 5-HT on the RNSC was mimicked by decreasing the bath concentration of extracellular Mg^{2}. In summary, the results suggest that the nonlinear membrane property associated with Mg^{2+} blockade of NMDA receptor channel is critical for the production of organized locomotor activity in the mammalian preparation, and that 5-HT receptor activation modulates NMDA receptor-dependent membrane behavior by enhancing the RNSC.

Key Words: NMDA, serotonin, locomotion, neonatal rat, magnesium, modulation, pattern generation

The contribution of intrinsic neuronal properties to motor pattern generation has been studied extensively using invertebrate preparations (e.g. Meech 1979; Miller and Selverston 1982; Hartline and Graubard 1992). In contrast, relatively little is known about the possible role in rhythm generation of active membrane conductances in mammalian spinal cord neurons.

One active membrane property, expressed by vertebrate spinal cord neurons, is the voltage-sensitive conductance associated with N-methyl-D-aspartate (NMDA) receptor activation (Mayer and Westbrook 1987). This property produces a region of negative slope conductance (RNSC) in the current-voltage (I-V) relationship of the neuron (MacDonald et al 1982, Flatman et al 1983), and is due to a voltage-dependent blockade of the NMDA receptor channel by Mg^{2+} (Nowak et al 1984, Mayer et al 1984). Synaptically-isolated mammalian spinal cord neurons generate rhythmic voltage oscillations in the presence of NMDA (Hochman et al 1994a,b; Kiehn et al. 1996; MacLean et al 1997), as was previously shown in the lamprey spinal cord (Wallen and Grillner 1987). It is also established that activation of NMDA receptors in the synaptically-intact cord produces locomotion in the neonatal rat (e.g. Kudo and Yamada 1987; Smith and Feldman 1987; Cazalets et al. 1992; Beato et al. 1997), as well as other vertebrate preparations (e.g. Grillner et al 1981; Dale and Roberts 1984; Douglas et al 1993; Fenaux et al 1991; Wheatley et al 1992). Locomotor drive potentials (LDPs) in rat motoneurons are sensitive to NMDA receptor antagonists (Cazalets et al. 1996; Schmidt et al. 1989). In combination, these observations favor a role for NMDA receptor-

mediated events in the operation of rhythm generating circuits in the mammalian spinal cord.

However, it is not known whether the central role for NMDA receptor activation in spinal rhythmogenic networks should be attributed specifically to the induction of the RNSC. Alternatively, the major contribution of NMDA receptors in these circuits may be simply the support of excitatory synaptic transmission (in conjunction with non-NMDA excitatory amino acid receptors) independent of any requirement to generate a RNSC. Thus, one goal of the present study was to examine the effect on network rhythm generation of selectively abolishing NMDA receptor-mediated nonlinear voltage responsiveness while otherwise preserving the capacity for NMDA receptor activation. This was achieved through manipulation of the extracellular Mg^{2+} concentration in the bath perfusant.

We recently observed that NMDA application induces neither voltage oscillations (in synaptically-isolated motoneurons) nor locomotor network activity (in the intact spinal cord) in the presence of serotonin (5-HT) receptor blockade (MacLean et al. 1998). This interplay between 5-HT and NMDA receptor actions is consistent with observations of amphibian spinal neurons (Sillar and Simmers 1994). The exact mechanism of the 5-HT-NMDA interaction is unknown. However, it has been proposed that 5-HT enhances the voltage-dependent Mg^{2+} block of the NMDA ionophore in the amphibian preparation (Scrymgeour-Wedderburn et al. 1997). The second aim of the present study was to examine whether 5-HT receptor activation modulates the RNSC associated with NMDA

receptor activation. Our findings suggest that 5-HT *decreases* the voltage-dependent Mg^{2+} blockade of NMDA receptor-activated channels in the neonatal rat preparation.

Some of the following data has been presented previously in abstract form (MacLean and Schmidt 1997).

MATERIALS AND METHODS

Experiments were performed on 53 Sprague-Dawley rats (aged 2-8 days). Techniques for isolation of the spinal cord, extracellular recording, and neurochemical induction of rhythmic activity have been described previously (e.g. Cowley and Schmidt 1995). In brief, animals were anesthetized with ether, decapitated, eviscerated, and placed in artificial cerebral spinal fluid (ASCF), at 4 °C, containing (in mM): NaCl 128; KCl 3.0; $Na_2H_2PO_4$ 0.5; $CaCl_2$ 1.5; $MgSO_4$ 1.0; $NaHCO_3$ 21; and glucose 30 equilibrated to pH 7.4 with 95% O_2/5% CO_2. $MgSO_4$ was removed from the bath during some experiments and in two experiments (one extracellular and one intracellular) the Ca^{2+} concentration was increased by 1mM in order to maintain the same total divalent ion concentration. In three experiments, the brainstem was transected at the level of the superior colliculi and left in continuity with the bilaterally intact spinal cord. In 39 experiments the bilaterally intact spinal cord was transected at C1. These preparations were used to examine network behaviour as well as for intracellular recordings. In 11 experiments, the cord was left bilaterally intact from C1 to T13 and the right half of the lumbosacral spinal cord was

removed; these preparations were used only for experiments involving intracellular recordings. Similar observations of intracellular behaviour were obtained regardless of the type of preparation. The spinal cord was stabilized with insect pins on the bottom of a recording chamber that had been coated with Sylgard (Dow Corning Corporation). Recordings were obtained at room temperature. Rhythmic network activity was monitored via bilateral L_2 and L_5 ventral root recordings.

In 17 experiments, whole-cell patch recordings of motoneurons were obtained as previously described (Hochman et al. 1994b). Recording pipettes contained (in mM): K-gluconate 140, ethylene glycol-bis(-amino ethyl ether) N,N,N',N',-tetra-acetic acid (EGTA) 11, KOH 35, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10, and $CaCl_2$ 1. In the absence of tetrodotoxin (TTX) application during some experiments, lidocaine N-ethyl bromide (QX-314, 5 mM) was included in the patch electrode to block fast sodium channels and thereby prevent cell firing. Electrodes were made from borosilicate glass (WPI) pulled on a vertical puller (Narishige PP-83). Internal tip diameters ranged from 2 to 4 μm, and resistances measured in ACSF ranged from 3 to 5 MΩ. Cells were approached either through a pial patch made over the ventrolateral surface of the spinal cord, or through the medial surface of the lumbar spinal cord in those preparations where the right half of the lumbosacral cord was removed (see above). Cells were patched using a 'blind' approach (Blanton et al 1989), and were identified as motoneurons by their antidromic response to ventral root stimulation. The recordings were obtained with an Axopatch 1D amplifier (Axon Instruments), filtered at

2 kHz. Series resistance (22 ± 22 MΩ) was monitored continuously and compensated. In voltage-clamp mode series resistance was compensated up to 80%. In current-clamp mode series resistance was compensated by adjusting the series resistance potentiometer such that the make-and-break points of voltage transients in response to current steps were balanced (i.e. bridge balance). The electrode-bath solution liquid junction potential (10 mV) was corrected for in all recordings. Data were collected and analyzed on a 486-based computer with the pCLAMP acquisition software (v 6.0; Axon Instruments), typically at 4 kHz. Records were also digitized at 2 kHz and stored using a Vetter pulse code modulator videocassette adapter. A continuous paper copy of the data was also produced by an Astromed (MT9500) oscillographic recorder. Records were also analyzed using special purpose software on a Masscomp 5400 computer.

Current-voltage plots were generated by applying a series of voltage steps (500 ms duration) in 2.5 mV increments from -130 mV to +20 mV. The holding potential was either -80 or -90 mV. Current was measured as the average value during the final 100 ms of the voltage step.

Neurochemicals were applied as stock solutions (10 mM) to a static bath (volume = 30 ml) that was continuously oxygenated. All concentrations refer to final bath concentrations and ranged as follows: NMDA 5-20 μM, 5-HT 5-50 μM, and dihydrokainate (DHK) 20-100 μM. The final concentration of TTX was 1.5 μM in all

applications (stock solution 100 μM). Recordings were obtained after a stable response to the applied neurochemical was obtained (usually this required 5-15 min).

RESULTS

Seventeen antidromically identified motoneurons were examined. The mean biophysical data obtained in the absence of applied neurochemicals were as follows: input resistance 179.5 ± 76 MΩ, time constant 10.6 ± 5.2 ms, resting membrane potential -72.5 ± 6.1 mV.

Nonlinear membrane properties in the presence of NMDA.

NMDA induced a RNSC in the I-V relationship of 11 of 17 motoneurons voltage-clamped in the presence of TTX (Fig. 1A). The mean potential at which the negative slope region initiated was -63 ± 13 mV. The whole-cell current had a mean maximal inward current of 200 ± 102 pA. The RNSC depended on the presence of Mg^{2+} (1mM), as expected (Mayer et al 1984). Thus, the RNSC was abolished after removal of Mg^{2+} (n=10), which enabled a persistently enhanced inward current via NMDA receptor channels, even at relatively hyperpolarized membrane potentials (Fig. 1B). The RNSC was also abolished by bath application of the DL-2-amino-5-phosophovaleric acid (AP5, 10 μM, n=3; Fig. 1C), which specifically blocks NMDA receptors.

Current-clamp recordings of the response to depolarizing ramp current injection in the presence of NMDA demonstrated a nonlinear jump in membrane voltage (Fig. 2A(i)). This membrane behavior was observed in motoneurons capable of developing TTX-resistant oscillations or plateau potentials (n=8, Fig. 2A(ii)). Removal of Mg^{2+} from the ACSF abolished the nonlinear voltage jump during ramp current injection (n=10, Fig. 2B(i)) as well as oscillations and plateau potentials (n=4, Fig. 2B(ii)).

Voltage oscillations and counter-clockwise hysteresis in the RNSC of the I-V relationship.

Standard I-V plots of the type shown in Fig. 1 were generated by measuring the current flow that was evoked in response to a graded series of voltage steps, each step initiated from the same hyperpolarized holding potential (i.e. -80 to -90 mV, see Methods). However, the recruitment and inactivation of many voltage-sensitive conductances is dynamically influenced, in a time-dependent manner, by the preceding membrane voltage. Therefore, in the absence of voltage-clamp conditions, for instance during normal network operation, one would expect the expression of nonlinear membrane properties, such as the RNSC, to be influenced by membrane voltage history.

In order to gain a better appreciation of the effect of membrane voltage history, we examined the current response to long-duration ascending and descending (triangular) voltage ramps in the presence of NMDA (Fig. 3A). The ascending-descending I-V plot pairs (obtained in voltage-clamp mode) were compared to the membrane voltage behavior of the same cell during exposure to NMDA (in TTX) in current-clamp mode. Figure 3 B

shows a motoneuron with counterclockwise hysteresis in the RNSC of their current response during ramp voltage injection. This counterclockwise hysteresis in the RNSC was observed only in cells (n=3) which also developed NMDA receptor-dependent voltage oscillations in current-clamp mode (Fig 3B, right). Two other motoneurons developed a clockwise hysteresis in the RNSC of the I-V relationship in response to triangular voltage ramp injections. Instead of regular oscillatory activity in current-clamp mode, one of the two motoneurons displayed irregularly recruited plateau potentials and the other cell developed long-lasting voltage shifts (Fig. 3C) (see MacLean et al. 1998). These observations illustrate the dynamic nature of the nonlinear I-V relationship obtained in the presence of NMDA, and suggest that I-V plot pairs produced by triangular voltage ramps may be better predictors of membrane behavior than a single standard I-V plot.

NMDA receptor-mediated non-linearity contributes to locomotor network activity.

In order to examine the role of NMDA receptor-mediated nonlinearity at the (rhythmogenic) network level we selectively abolished the RNSC, while otherwise preserving the capacity for NMDA receptor activation. This was accomplished by removal of Mg^{2+} from the bath during neurochemically-induced locomotor-like behavior, as monitored by bilateral ventral root recordings. After a minimum washout of the Mg^{2+} of 15 minutes, the organized pattern of alternating L2-L5 and left-right alternation (Fig. 4A and B) was transformed into poorly coordinated patterns of phasic ventral root discharge (n = 31). Locomotor-like activity was restored after replacement of Mg^{2+} (n = 16/16). In these experiments, locomotor-like activity was induced using the excitatory

amino acid uptake inhibitor DHK (50-100 μM) and 5-HT (30-50 μM, n=23). The same results were observed using NMDA (3-5 μM) in combination with 5-HT (n=6), and 5-HT alone (n=2). The mean variance of the control cycle period was 0.57 ± 0.95 s (n=15 cycles). After washout of Mg^{2+}, the mean variance of the cycle period increased to 33.68 ± 26.24 s (n=15) and then decreased to 0.12 ± 0.18 s (n=15) after Mg^{2+} (1 mM) was restored in the bath. Similar results were obtained in the one experiment in which Ca^{2+} concentration was increased by 1 mM in order to maintain the same total concentration of divalent cations during Mg^{2+} removal.

An earlier study, using a brainstem-spinal cord version of this preparations, and brainstem electrical stimulation to induce locomotion, reported no change in the qualitative pattern of hindlimb stepping after removal of Mg^{2+} (Atsuta et al 1990). Although not an entirely analogous preparation (we did not use brainstem electrical stimulation nor leave the hindlimbs attached), in 3 preparations we did transect the neural axis at the level of the superior colliculi, instead of at C1. The rhythmic pattern produced by bath application of neurochemicals also became disorganized in these preparations after removal of Mg^{2+} (Fig 4B).

The disruption of rhythmic activity observed in Mg^{2+}-free ACSF may be directly related to the loss of the NMDA receptor-dependent RNSC, but could also have been due to a nonspecific increase in the level of excitation (i.e. NMDA receptor-mediated) in the spinal cord as a result of Mg^{2+} removal. During locomotor-like activity we increased

spinal cord excitation through K^+-induced depolarization (i.e. $[K^+]$ was increased from 3.5 to 6.5 mM (n=2)). Consistent with a previous report (Squalli-Houssaini et al 1993), manipulation of K^+ in this concentration range modulated cycle frequency but had no effect on pattern organization. To determine if the disrupted rhythms observed in Mg^{2+}-free solution might due to excess excitatory synaptic excitation we slowly decreased NMDA receptor activation in Mg^{2+}-free ACSF by addition of AP5 in small increments (2 μM up to 20 μM; n=10). AP5 decreased burst amplitude and rhythm frequency in a dose-dependent manner but never re-established a locomotor pattern before completely abolishing ventral root activity. In 13 experiments, the rhythm-inducing neurochemicals (5-HT and DHK) were removed from the bath at the same time Mg^{2+} was removed in order to determine whether the disruption of the locomotor-like pattern could be prevented by decreasing the amount of neurochemical excitation. 5-HT was then re-introduced in the bath in 5 or 10 μM increments to a final concentration of 50 μM. However, slow reapplication of 5-HT failed to elicit a locomotor-like pattern.

5-HT modulates the NMDA receptor-mediated RNSC.

5-HT (50-60 μM) alone failed to elicit a RNSC in motoneurons (n=5); addition of NMDA (10 μM) was of critical importance (Fig. 5A(i)). However six motoneurons failed to developed either a RNSC in their I-V plot (Fig. 5A(ii)) or a nonlinear voltage response to ramp current injection (Fig. 5B(i)) after bath application of NMDA (10 μM) alone. On the other hand, subsequent application of 5-HT (40-50 μM) produced a RNSC in these cells (Fig. 5A(ii)), as well as a nonlinear voltage response to ramp current

injection (Fig. 5B(ii)). Induction of the RNSC after application of 5-HT was observed in conjunction with the development of membrane voltage oscillations, recorded in current-clamp mode (Fig. 5B(ii)).

5-HT application (30-50 μM) to motoneurons that initially displayed a RNSC in the presence of NMDA alone (n=8), shifted the onset of the RNSC leftward by 18.3 ± 15.0 mV (Fig. 6A). 5-HT also increased the maximal inward current associated with the RNSC, by an average of 107± 85 pA % increase (Fig. 6A). The mean threshold for activation of the RNSC during co-application of 5-HT and NMDA was 79.5 ± 16.2 mV. Thus, the average threshold level of the RNSC was more negative than the mean resting membrane potential of motoneurons in this study (72.5 ± 6.1 mV, n=17) in TTX and in the absence of applied neurochemicals. The negative shift of the RNSC and the increase of the maximal negative slope current by 5-HT were dependent on the concentration of 5-HT in the bath (Fig. 6B(i)) and the time elapsed after 5-HT application (Fig. 6B(ii)). The effect of 5-HT was partly reversed by application of the 5-HT receptor antagonist mianserin (80 μM, n = 4) as shown in Fig. 6B(iii).

5-HT decreases the voltage-dependent blockade of the NMDA channel.

The effect of 5-HT on the RNSC was mimicked by the removal of Mg^{2+} from the bath (n=3, Fig. 7A). Although the RNSC was ultimately completely abolished in Mg^{2+}-free ACSF (Figs. 1B, and 7B bottom trace), this effect required approximately 15 minutes to

develop. During the Mg^{2+} washout period, serial I-V plots were obtained prior to total abolishment of the RNSC (Fig. 7B). The RNSC shifted increasingly leftward toward more hyperpolarized potentials. This shift also occurred despite adding an extra 1mM Ca^{2-} to the bath to maintain divalent cation charge balance in one experiment. The reversal of the 5-HT-induced leftward shift of the RNSC, observed after addition of mianserin, was itself reversed after subsequent washout of Mg^{2+} (Fig. 7B and C). These data suggest that 5-HT receptors mediated the modulation of the RNSC through regulation of the voltage-sensitive Mg^{2-}-dependent blockade of NMDA receptors.

DISCUSSION

The main finding of this study is that NMDA receptor-mediated nonlinear membrane behaviour in the rat spinal cord stabilizes the production of rhythmic motor patterns and is modulated by serotonin. The results may explain, at least in part, why NMDA receptor activation has such a prominent role in the production of vertebrate locomotor rhythms (Grillner et al 1981; Dale and Roberts 1984; Kudo and Yamada 1987; Smith and Feldman 1987; Smith et al 1988; Hernandez et al 1991; Fenaux et al 1991; Wheatley et al 1992; Cazalets et al 1992; Douglas et al 1993). In addition, the results suggest a mechanism that may account for the observation that co-application of 5-HT and NMDA more effectively induces locomotor activity than application of either neurochemical alone (Sqalli-Houssaini et al. 1993; Cowley and Schmidt 1994; Kjaerulff et al. 1994).

Although it is clear that the output of the rhythm generating network becomes disorganized in Mg^{2+}-free ACSF, these experiments do not indicate exactly which neuronal elements of the network depend on NMDA receptor-mediated nonlinear membrane properties. To determine this is a formidable challenge, since the identity of the essential neurons of the central pattern generator in the mammalian spinal cord is unknown. Thus, in the present study, we limited whole-cell recordings to motoneurons. Although motoneurons are last-order output elements only, they are functionally identifiable and in this sense represent a relatively homogenous population of spinal cord cells that can be studied. It is reasonable to speculate that some of the voltage-sensitive properties of motoneurons, as characterized in this study, may be found in other neurons involved in locomotor network operation.

Contribution of NMDA receptor-mediated nonlinearity to locomotor activity.

Previous studies have provided evidence that at least part of the rhythmic excitatory drive to spinal neurons during locomotion is mediated by NMDA receptors (Moore et al. 1987; Brownstone et al. 1994; Cazalets et al. 1996; Hochman and Schmidt 1998). In addition, NMDA receptor activation induces TTX-resistant voltage oscillations in spinal neurons (Wallen and Grillner 1987; Hochman et al 1994a,b, Sillar and Simmers 1994; MacLean 1997). Considering these observations, it seems reasonable to postulate that some spinal neurons may be endowed with a tendency to develop voltage oscillations (i.e. given the appropriate neurochemical input), or at least may possess certain nonlinear membrane properties, that may be well suited for recruitment by a rhythmogenic network. Thus, one issue addressed in the present study was whether the nonlinear I-V relationship

(associated with NMDA receptor activation) in particular, was essential for locomotor network operation. In the absence of Mg^{2+}, the voltage-sensitive blockade of NMDA ionophores and the corresponding I-V nonlinearity, was abolished. However, synaptic currents due to activation of NMDA receptors by endogenously released glutamate are preserved, and in fact enhanced, in the absence of Mg^{2+} (Fig 1B and Mayer et al. 1984). We observed that stable locomotor-like patterns were replaced by poorly organized patterns of phasic activity after removal of Mg^{2+}. Therefore, NMDA receptor-mediated nonlinearity may not be essential for phasic neuronal firing in the spinal cord, but does appear to have a critical role in the maintenance of a stable organized rhythmic pattern.

Similar effects of Mg^{2+} removal on fictive swim patterns were reported in the lamprey (Brodin and Grillner 1986) and *Xenopus* (Soffe and Roberts 1989). In addition, modeling studies of the *Xenopus* spinal pattern generator suggest that voltage-dependent Mg^{2+} blockade of NMDA ionophores stabilizes swimming activity through enhancement of post-inhibitory rebound at the single cell level (Roberts et al 1995). More recently, in *Xenopus* neurons it has been shown that removal of Mg^{2+} abolishes slow NMDA receptor-dependent oscillations of the membrane potential, the role of which appears to be the modulation of swimming activity over several consecutive cycles (Reith and Sillar 1998). A mathematical model of lamprey spinal neurons (Brodin et al 1991), which incorporates NMDA receptor-dependent nonlinear membrane properties also suggests that the voltage-dependent block of NMDA ionophores by Mg^{2+} stabilizes swim pattern (Traven et al. 1993).

A previous study using a neonatal rat brainstem spinal cord preparation revealed no qualitative difference in locomotor activity produced by brainstem electrical stimulation before and after removal of Mg^{2+} (Astuta et al 1990). However, these investigators tested the effect of Mg^{2+} removal 2 minutes after the Mg^{2+} washout. In the present study, washout of Mg^{2+} had no effect on pattern until at least 15 minutes had elapsed. Thus, incomplete washout may account for the earlier observations by Atsuta et al. (1990); even residual micromolar concentrations of Mg^{2+} may preserve the nonlinear current voltage relationship associated with NMDA receptor activation (Nowak et al 1984).

Nonlinear membrane properties and oscillations.

We recorded the current response to triangular ramp voltage injection in order to explore dynamic features of the nonlinear NMDA receptor-dependent I-V relationship. As previously demonstrated in molluscan neurons (Wilson and Watchel 1974; see also Barker and Gainer 1975), all motoneurons that displayed oscillatory behavior or plateau potentials displayed a RNSC. An 'N'-shaped I-V trajectory alone is not sufficient for the production of voltage oscillations. The relationship of the 'N'-shaped curve to the zero current axis is also important. The RNSC may cause the I-V curve to cross the zero current axis, with positive slope, at two distinct voltage levels resulting in two stable states of membrane voltage (i.e. bistability). Bistability permits the induction and termination of plateau potentials (wherein the membrane potential shifts from one stable state to the other) in response to external input (i.e. current injection or synaptic events).

However, bistability alone is also insufficient for the generation of rhythmic voltage oscillations.

Because of the slow activation or de-activation time course of many voltage-sensitive conductances, the I-V relationship of neurons under normal conditions of network operation (i.e. not voltage-clamped), is not static. Instead, the I-V relationship is influenced by membrane voltage history. Thus, neurons display different I-V relationships in response to the ascending and descending arms of a triangular voltage injection. Neurons that generate voltage oscillations cyclically alternate between one I-V curve, with one point of voltage stability (the trigger condition; Meech 1972;Hartline and Graubard 1992; Fig. 3B(arrow 'a')), and a different curve, with a different point of stability (the terminating condition; Meech 1972; Hartline and Graubard 1992; Fig. 3B(arrow 'b')). We refer to this membrane behaviour, which has been more extensively characterized in oscillating neurons in invertebrate preparations (Gola 1974; 1978; Hartline and Graubard 1992), as dynamic bistability. In the present study, motoneurons that failed to generate rhythmic voltage oscillations in the presence of NMDA and TTX displayed a clockwise hysteresis in the RNSC of their I-V plots (e.g. Fig 3C). A similar clockwise hysteresis in response to voltage ramp injections was obtained in turtle spinal neurons (non-oscillating) which developed plateau potentials in response to current injection (Svirskis and Hounsgaard 1997). In contrast, motoneurons that developed oscillatory activity demonstrated a counterclockwise hysteresis in the RNSC of their I-V trajectories (Fig. 3B). Thus, only neurons with a counterclockwise hysteresis in the RNSC have an appropriate combination of membrane conductances available for

production of voltage oscillations. The triggering condition, during which the RNSC is relatively more prominent, is associated with an I-V trajectory that lies in a region of net inward current flow and therefore, spontaneous depolarization occurs (Fig 3B). Depolarization then promotes slowly activating/deactivating conductances. The terminating condition evolves as current flow becomes net outward, driving the membrane potential in the hyperpolarizing direction until it intersects the zero current axis.

Modulation of NMDA conductances by 5-HT.

It is well known that 5-HT modulates the excitability of motoneurons (e.g. Barasi and Roberts 1974; White and Neuman 1980; Takahashi and Berger 1990; Elliot and Wallis 1992; Ziskind-Conhaim et al 1993). Multiple actions of 5-HT on neuronal responses to glutamate throughout the CNS have been described (e.g. Nedergaard et al. 1986; 1987; Reynolds et al. 1988; Murase et al 1990; Rahman and Neuman 1992; Chesnoy-Marchais and Barthe 1996). Co-localization of glutamate and 5-HT in synaptic boutons surrounding motoneuron somata (Nicholas et al. 1992) also suggests that EAA and 5-HT receptor systems may closely interact.

We previously demonstrated that 5-HT receptor activation is necessary for the production of NMDA dependent motoneuronal voltage oscillations as well as rhythmic motor network activity (MacLean et al. 1998). A preliminary report indicated that similar 5-HT modulation of NMDA receptor-dependent membrane nonlinearity may be present in cat motoneurons (Flatman and Engberg 1990). In the present series, we show that 5-

HT shifted the NMDA receptor-dependent RNSC leftward, in the hyperpolarizing direction, and that this effect was simulated by decreasing the concentration of Mg^{2+}. These results suggest that 5-HT may modulate the RNSC by decreasing the efficacy of Mg^{2+} blockade of the NMDA ionpohore. Because of the deleterious effect on pattern generation produced by suppression of the RNSC (via Mg^{2+} removal), enhancement of the RNSC by 5-HT application is expected to help promote stable locomotor network activity, as has been reported by several laboratories (Sqalli-Houssaini et al. 1993; Cowley and Schmidt 1994; Kjaerulff et al. 1994).

After application of 5-HT the mean voltage threshold for activating the RNSC was shifted to more hyperpolarized values relative to mean resting membrane potential. Thus, 5-HT likely enhances inward conductance through the NMDA ionophore in neurons that would otherwise develop relatively weak currents in response to NMDA receptor activation near resting potential. Enhancement of the RNSC is also likely to be the mechanism underlying 5-HT-induced facilitation of membrane voltage oscillations in the presence of TTX. Conversely, 5-HT receptor blockade, which has been shown to block locomotor network activity and NMDA-induced oscillations in TTX (MacLean et al. 1998), shifted the RNSC to more depolarized levels. This may substantially decrease conductance through the NMDA ionophore explaining why 5-HT receptor antagonists mimic the effect of AP5 in abolishing rhythmic network activity and TTX-resistant oscillatory activity (MacLean et al. 1998).

A negative shift of the NMDA receptor-dependent RNSC has been reported after protein kinase C activation (Chen and Huang 1992; Blank et al 1996). Single channel recordings show that PKC reduces the Mg^{2+} block of the NMDA channel (Chen and Huang 1992). It is of interest that 5-HT_2 receptor actions are mediated through the PKC pathway (e.g. see Martin and Humphrey 1994). In the present study mianserin, a 5-HT_2 receptor antagonist, partly reversed the effect of 5-HT on the RNSC.

Although NMDA receptor-induced voltage oscillations in embryonic and larval *Xenopus* spinal cord neurons also show 5-HT receptor-dependency (Sillar and Simmers 1994), several differences are noted with respect to the neonatal rat. In the amphibian preparation, 5-HT_{1A}-like rather than $5HT_2$ receptors are implicated in the modulatory action of NMDA receptors, and 5-HT appears to enhance rather than diminish the voltage-dependent blockade of NMDA channels by Mg^{2+} (Scrymgeour-Wedderburn et al. 1997). Interestingly, in *Xenopus* spinal neurons, NMDA receptor activation produces a tonic depolarization. Subsequent application of 5-HT produces oscillations characterized as rhythmic hyperpolarizations from the level of tonic depolarization produced by NMDA, consistent with an enhancement of the Mg^{2+} blockade of the NMDA channel (Sillar and Simmers 1994). In contrast, application of 5-HT to the neonatal rat spinal cord produces oscillations which depolarize from the tonic depolarization, produced by NMDA receptor activation, suggesting a reduction of the Mg^{2+} blockade of the NMDA channel (MacLean et al 1998). It appears that different mechanisms are utilized by the same neurochemicals to produce oscillations in the *Xenopus* spinal cord and the rat spinal

cord. In both cases 5-HT exerts its actions through modulation of the voltage-dependent Mg^{2+}-blockade of the NMDA channel.

In summary, the modulatory actions of 5-HT on NMDA receptor-mediated nonlinear membrane properties shown here suggest just one of many possible ways in which 5-HT and NMDA receptor actions may interact and contribute to the operation of mammalian spinal cord networks. Clearly, it would be of interest in future experiments to examine these receptor actions in locomotor network-related interneurons.

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FIGURE LEGENDS

Figure 1. NMDA receptor-mediated region of negative slope conductance (RNSC) in TTX (1.5 μM)-treated spinal motoneurons. **A.** NMDA (10 μM) produced a RNSC in the whole-cell I-V relationship of the motoneuron (trace b). **B.** Removal of Mg^{2+} from the bathing medium abolished the RNSC while allowing maximal conductance at all voltage values during exposure to 10 μM NMDA (trace b). **C.** The RNSC, in the presence of NMDA (10 μM) and 5-HT (50 μM), was abolished by AP5 (10 μM) (trace b).

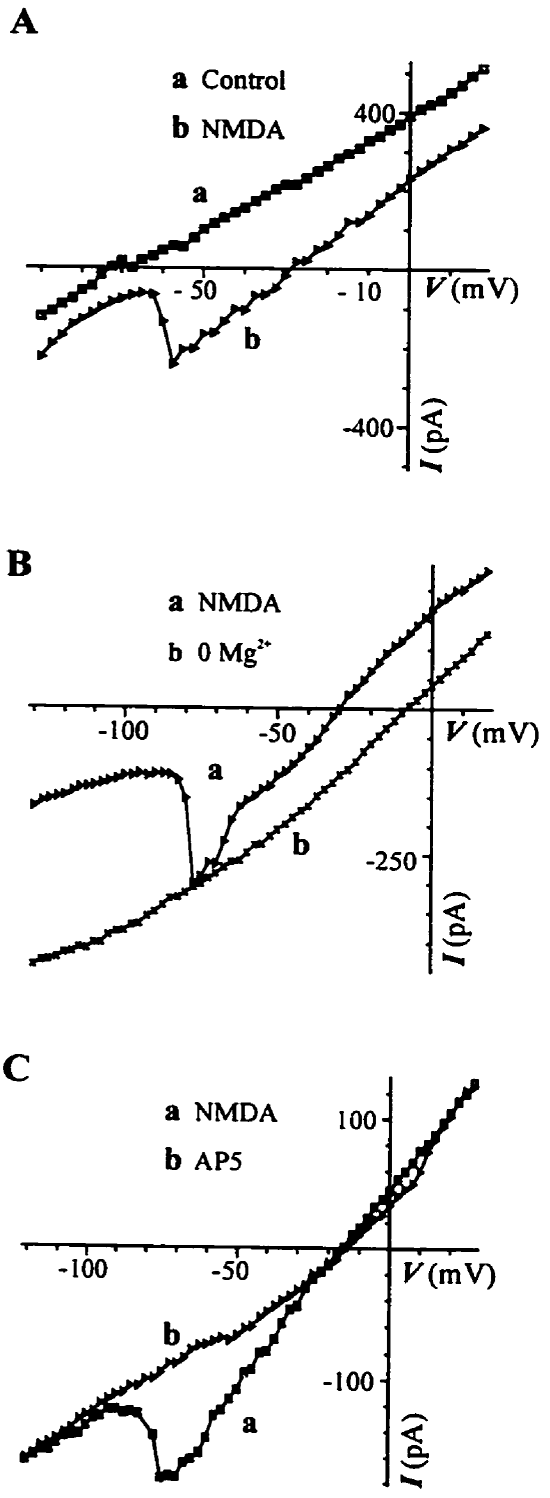

A
a Control
b NMDA
400
a
- 50
- 10
V (mV)
b
-400
I (pA)
B
a NMDA
b 0 Mg2+
-100
-50
V (mV)
a
b
-250
I (pA)
C
a NMDA
b AP5
100
-100
-50
V (mV)
b
-100
a
I (pA)

Figure 2. NMDA receptor-mediated membrane voltage nonlinearity was observed in motoneurons capable of generating rhythmic voltage oscillations and plateau potentials in TTX (1.5 μM). **A(i)**. The membrane voltage response to ascending and descending ramp current injection displayed an abrupt nonlinear increase and decrease in amplitude, respectively, in the presence of NMDA (20 μM). **(ii)**. Rhythmic voltage fluctuations were observed in the same motoneuron. **B(i)**. Removal of Mg^{2+} caused a loss of the nonlinear membrane voltage response to ramp current injection and **(ii)** abolished membrane voltage shifts. A 300 pA hyperpolarizing intracellular bias current was applied throughout these recordings.

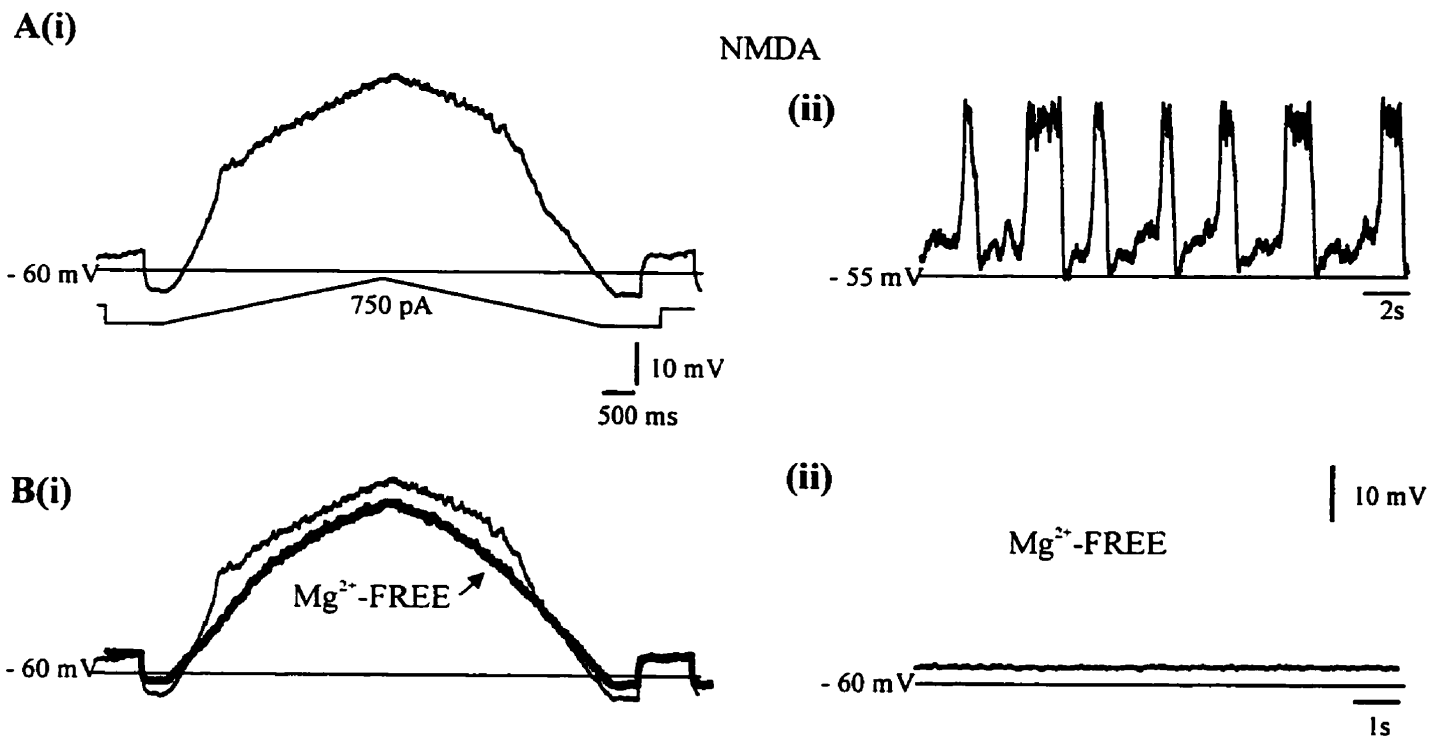

A(i)
- 60 mV
750 pA
10 mV
500 ms
NMDA
(ii)
- 55 mV
2s
B(i)
Mg2+-FREE
- 60 mV
(ii)
10 mV
Mg2+-FREE
- 60 mV
1s

Figure 3. Current response to voltage ramp injections in motoneurons in the presence of NMDA (10 μM) and TTX (1.5 μM). The quasi-steady state I-V plots in **A-C** show the response to ramp voltage injection from -130 to +40 mV, and then from +40 to -130 mV. In order to simultaneously compare currents evoked during the ascending and descending ramps at the same voltage levels (i.e. in **B**, and **C**), the repolarizing response (b) was reversed and superimposed on the depolarizing response (a) , as indicated by the arrow in **A**. In **B** the current responses a motoneuron, capable of generating rhythmic voltage oscillations in current-clamp mode (shown on right), showed a counterclockwise hysteresis in the RNSC. **C.** A clockwise hysteresis in the RNSC was observed in this motoneuron (shown on left), which displayed arrhythmic long-lasting voltage shifts rather than rhythmic oscillations during current-clamp recordings (shown on right). In these figures, the current response to the depolarizing arm of the ramp is indicated by arrow '**a**' and the repolarizing arm of the ramp is indicated by arrow '**b**'.

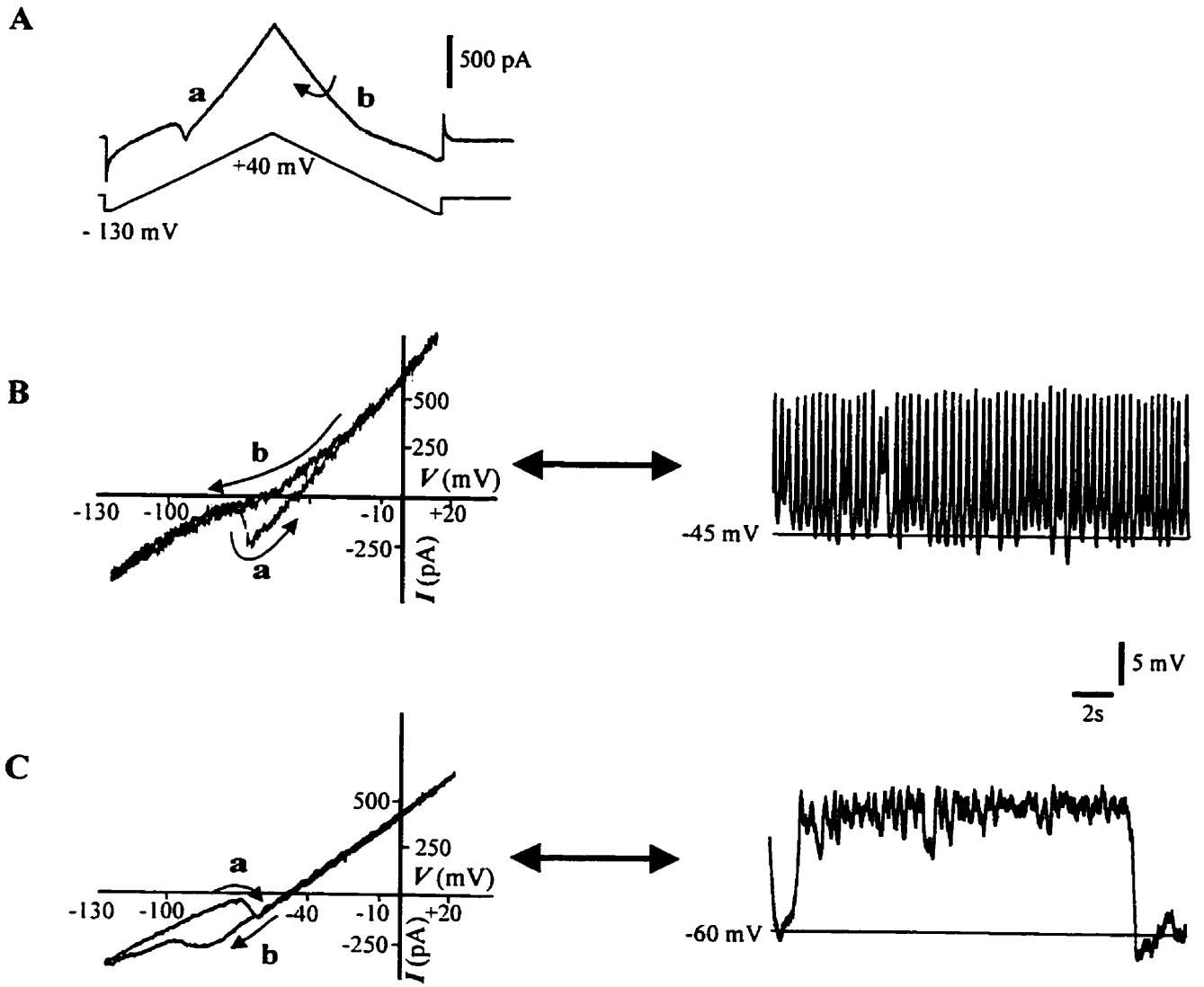

A
500 pA
a
b
+40 mV
- 130 mV
B
500
250
V (mV)
b
-130
-100
-10
+20
-250
a
I (pA)
-45 mV
5 mV
2s
C
500
250
a
V (mV)
-130
-100
-40
-10
+20
-250
b
I (pA)
-60 mV

Figure 4. Voltage-dependent block of the NMDA receptor channel by Mg^{2+} ions was necessary for rhythmic motor behavior. **A**. Rhythmic activity in an isolated spinal cord, transected at C1, was induced by 5-HT (40 μM) and DHK (100 μM), and recorded from the right and left L2 and L5 ventral roots. The pattern was disrupted by removal of Mg^{2+} and recovered after re-application of Mg^{2+} (1 mM). **B**. Rhythmic activity in an isolated brainstem-spinal cord (transected at the level of the superior colliculi) was induced by 5-HT (60 μM) and NMDA (3 μM), and recorded from the left and right L2 ventral roots. The pattern was disrupted by removal of Mg^{2+} and recovered after addition of Mg^{2+} (1 mM).

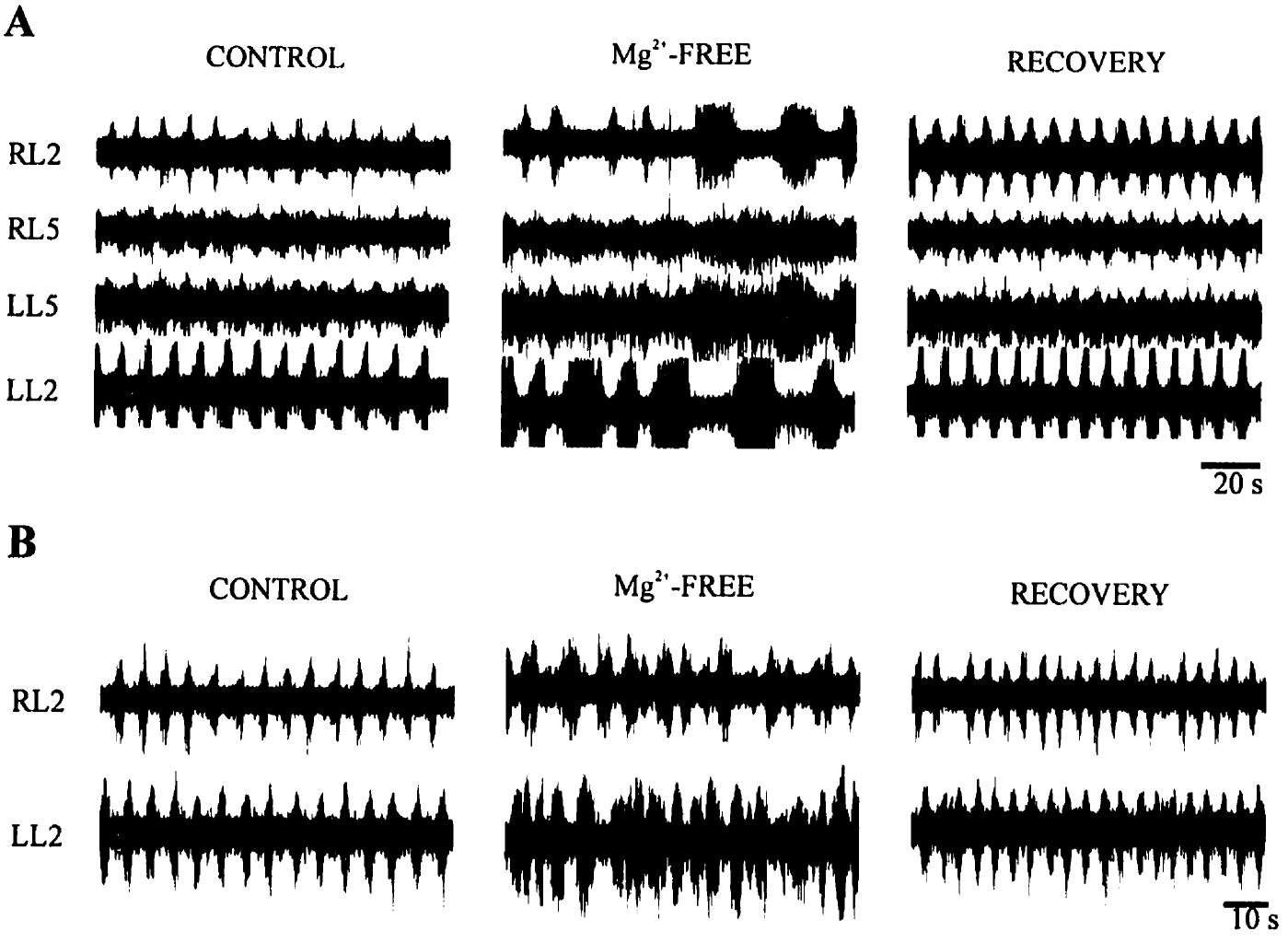

A
CONTROL
Mg2+-FREE
RECOVERY
RL2
RL5
LL5
LL2
20 s
B
CONTROL
Mg2+-FREE
RECOVERY
RL2
LL2
10 s

Figure 5. 5-HT facilitated the expression of a RNSC in motoneurons that failed to develop a RNSC when exposed to NMDA alone (TTX, 1.5 μM always present). **A(i).** Application of 5-HT (60 μM) alone did not produce a RNSC. Subsequent application of NMDA (5 μM) resulted in a RNSC. **(ii).** NMDA (10 μM) alone did not produce a RNSC in this motoneuron. Subsequent application of 5-HT (50 μM) promoted a RNSC. **B(i).** In this motoneurons, the membrane voltage response to ramp current injection in the presence of NMDA (10 μM) alone was linear, and NMDA failed to induce voltage oscillations during recordings in current clamp mode (shown on right). **(ii).** A RNSC emerged after application of 5-HT (40 μM), and in current-clamp mode rhythmic voltage oscillations were observed (shown on right).

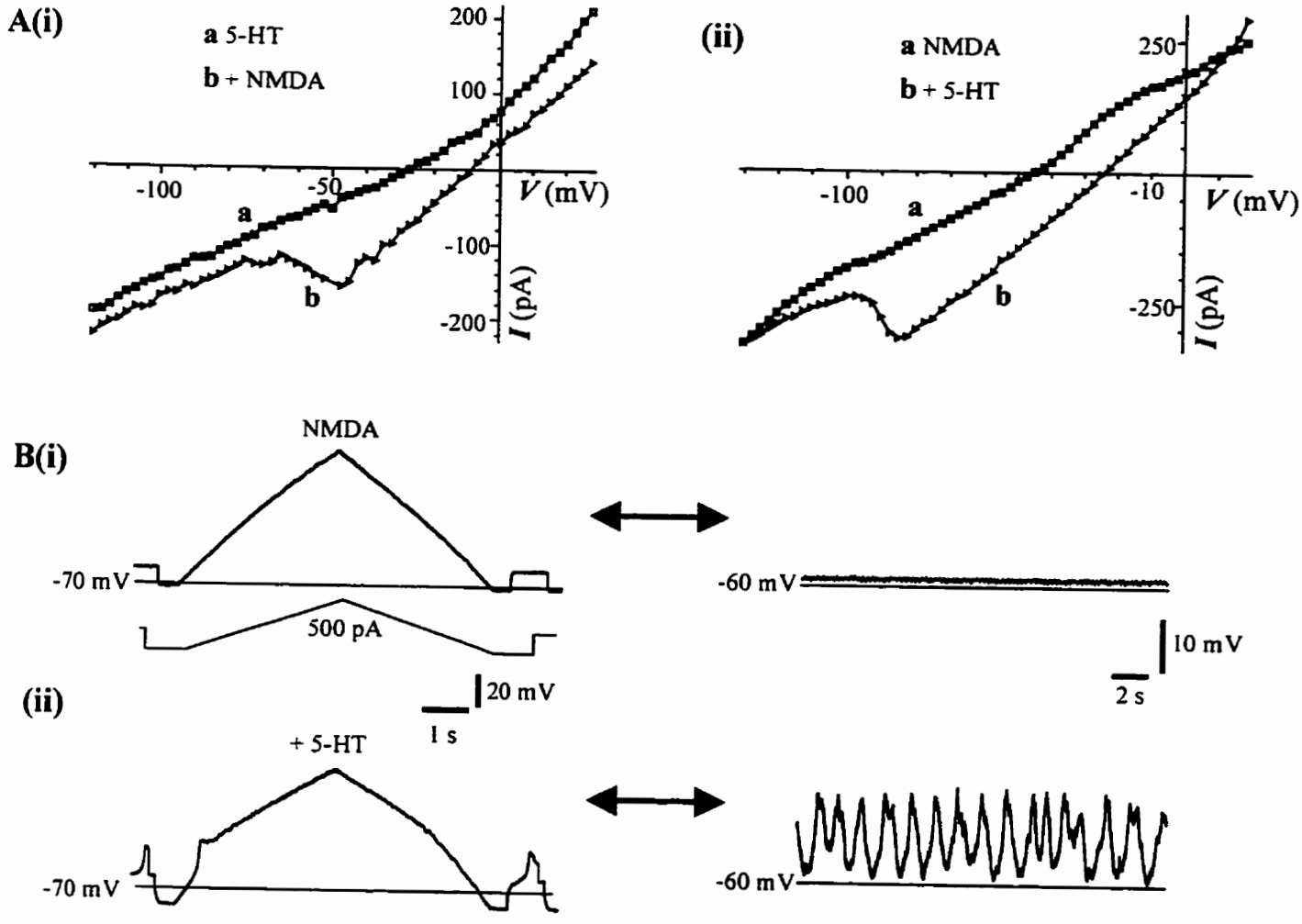
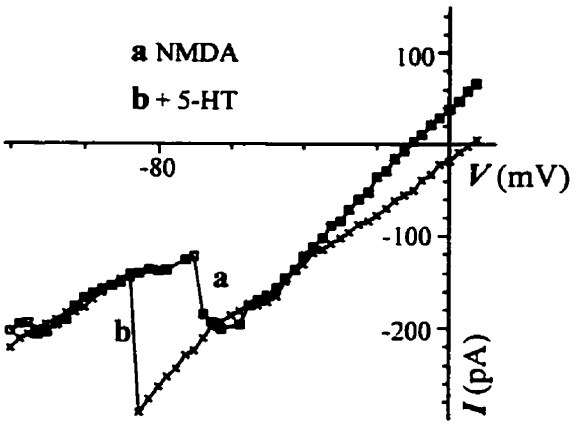
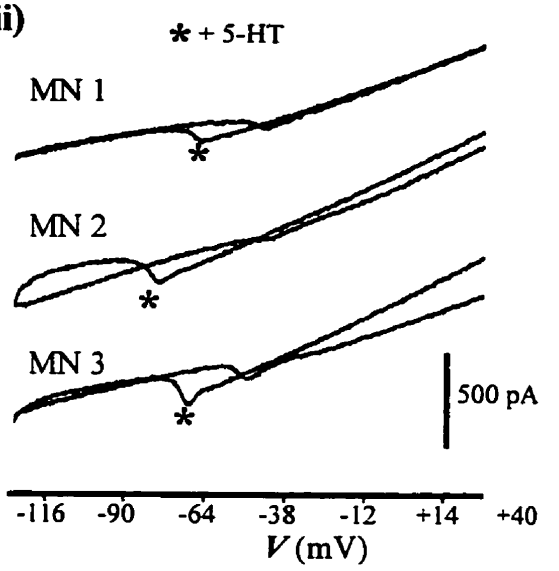

A(i)
a 5-HT
b + NMDA
200
100
-100
-200
-100
-50
V (mV)
I (pA)
a
b
(ii)
a NMDA
b + 5-HT
250
-250
-100
-10
V (mV)
I (pA)
a
b
B(i)
NMDA
-70 mV
500 pA
20 mV
1 s
-60 mV
10 mV
2 s
(ii)
+ 5-HT
-70 mV
-60 mV

Figure 6. 5-HT shifted the NMDA receptor-dependent RNSC to more hyperpolarized potentials. **A(i)**. NMDA (10 μM), in the presence of TTX (1.5 μM), produced a RNSC that was initiated at approximately -70 mV(a). Application of 5-HT (50 μM) shifted the onset of the RNSC to approximately -85 mV, and increased the peak current by 90 pA (b). **(ii)**. In each of three motoneurons (labeled MN 1-3), the current response to a depolarizing ramp voltage injection (from -130 to +40 mV), in the presence of NMDA and TTX (1.5 μM), showed a RNSC. After addition of 5-HT (50 μM), indicated by asterisk, the RNSC was shifted to more hyperpolarized potentials. **B(i)**. The 5-HT-induced leftward shift of the RNSC was concentration dependent. After application of 5-HT (30 μM) the onset of the RNSC induced by NMDA (10 μM) (a) was shifted from -62 to -65 mV (b). Subsequent application of an additional 20 μM 5HT (final concentration 50 μM) shifted the RNSC onset region to -87 mV (c). **(ii)**. The degree of RNSC shift was also dependent on the duration of exposure to 5-HT. Fifteen minutes after of application of 5-HT (50 μM) the onset of the RNSC shifted from -52 mV (a) to -87 mV (b). After an additional 5 minutes, the RNSC onset region shifted to -92 mV (c). **(iii)**. The effect of 5-HT on the RNSC was partly reversed by mianserin. The onset of the RNSC during exposure to NMDA (10 μM), in the presence of TTX (1.5 μM), occurred at -62 mV (a). After application of 5-HT (50 μM) the onset of the RNSC shifted to -87 mV (b). Subsequent application of mianserin (80 μM) shifted the voltage of activation to -77 mV (c). The same motoneuron is illustrated in **B(i and iii)**.

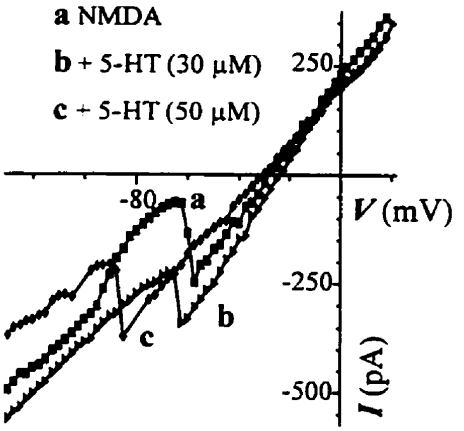
A(i)



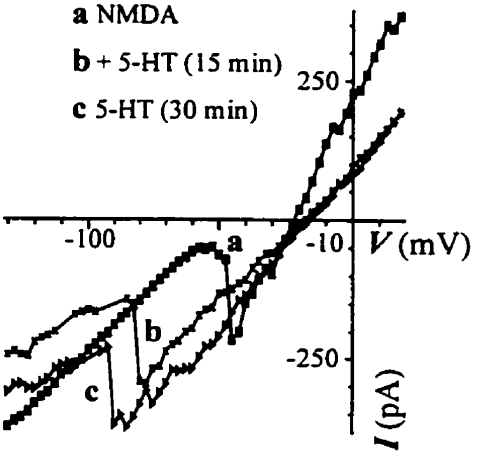
(ii)



B(i)



(ii)



(iii)

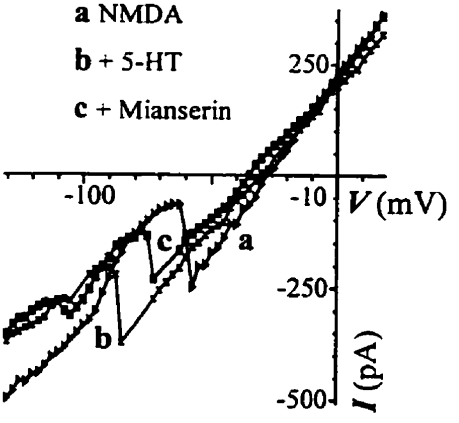
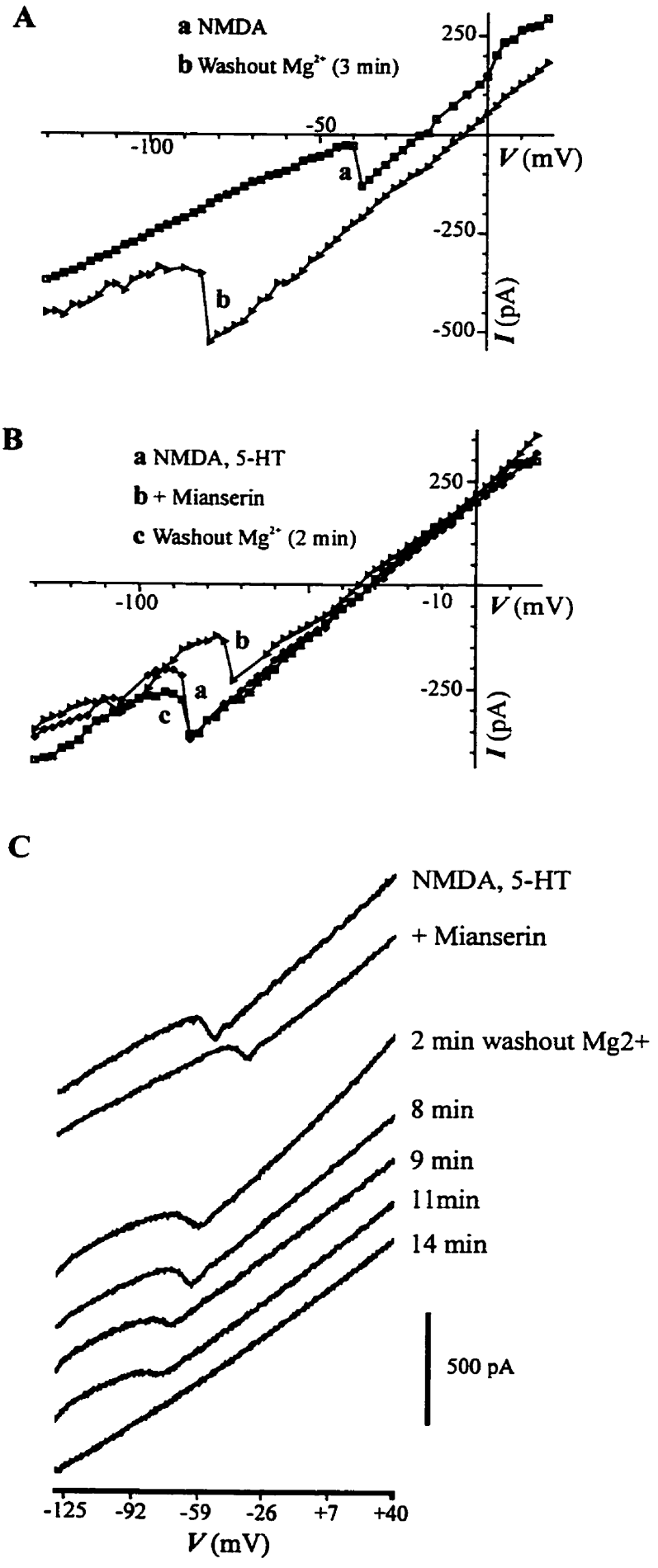


Figure 7. The effect of 5-HT was mimicked by washout of Mg^{2+}. **A.** The onset of the RNSC occured at -40 mV (trace 'a') in the presence of NMDA (10 μM) and TTX (1.5 μM). Three minutes after replacing normal ACSF with Mg^{2+}-free ACSF the onset of the RNSC shifted to -90 mV (trace 'b'). **B and C** (same motoneuron). The partial reversal of the 5-HT effect produced by mianserin was itself reversed two minutes after replacing normal ACSF with Mg^{2+}-free ACSF. The onset of the RNSC shown in **B** shifted from -87 mV in the presence of NMDA and 5HT to -77 mV after application of Mianserin. Two minutes after washout of Mg^{2+} the onset of the RNSC returned to -87 mV. As shown in **C**, the leftward shift of the RNSC increased as the concentration of Mg^{2+} progressively decreased during the washout. Ultimately the RNSC was no longer apparent as NMDA receptor channels became totally unblocked and permitted maximal current flow even at hyperpolarized membrane potentials.


A
a NMDA
b Washout Mg2+ (3 min)
250
-50
-100
V (mV)
-250
-500
I (pA)
B
a NMDA, 5-HT
b + Mianserin
c Washout Mg2+ (2 min)
250
-100
-10
V (mV)
-250
I (pA)
C
NMDA, 5-HT
+ Mianserin
2 min washout Mg2+
8 min
9 min
11min
14 min
500 pA
-125 -92 -59 -26 +7 +40
V (mV)

General Discussion

Principle Observations - Synopsis

The first section of the thesis tested the hypothesis that NMDA-induced voltage oscillations produced by intrinsic membrane properties can be recruited by synaptic events. The data demonstrates that the ability of spinal cord lumbar motoneurons to generate NMDA receptor-dependent nonlinear behaviors (plateau potentials and voltage oscillations) is not limited to motoneurons that are synaptically isolated, as the same properties can be elicited in motoneurons under the influence of functionally intact circuitry. Motoneuronal oscillations in the presence of an intact network were capable of recruiting action potentials and plateau potentials suggesting the potential for a functional role in the generation of motor output. Clearly, action potential generation will influence motor output. Trajectories of the NMDA-induced voltage oscillations were similar in the presence and absence of functionally intact circuitry suggesting that this intrinsic property persists in the presence of an intact network. Membrane voltage oscillations and plateau potentials, even in the presence of functionally intact circuitry, could be initiated, terminated, and entrained by intracellular current pulse injection indicating an intrinsic origin. However, these oscillations could also be influenced by synaptic activity being

initiated (although the mechanism underlying these oscillations was not determined), and terminated by afferent synaptic input indicating that synaptic inputs were capable of influencing this activity. Membrane voltage oscillations were of a higher mean frequency (4.5 Hz) when the network was intact as compared to the frequency after application of TTX (2.0 Hz) further demonstrating that the oscillations could be influenced by synaptic activity. In combination, the data supports the concept that NMDA-induced voltage oscillations, produced by intrinsic membrane properties, can be recruited and influenced by synaptic events.

The second part of this thesis tested the hypothesis that NMDA receptor-dependent nonlinear membrane properties are modulated by 5-hydroxytryptamine (5-HT), which has a permissive effect on the generation of voltage oscillations. This section demonstrates that the production of rhythmic voltage oscillations in synaptically isolated spinal motoneurons requires both NMDA and 5-HT receptor activation. 5-HT induced oscillatory activity in neurons that initially express only tonic depolarization of membrane potential or plateau potentials in the presence of NMDA. Conversely, 5-HT_2 receptor antagonists abolished NMDA receptor-mediated membrane voltage oscillations. 5-HT_2 receptor antagonists also abolished the rhythmic motor output of intact spinal motor networks. These results suggest that an interplay between 5-HT and NMDA receptor actions is critical for the production of rhythmic behavior both at the single cell and network level.

The third section of this thesis tested the hypothesis that 5-HT promotes NMDA receptor-dependent voltage oscillations by decreasing the efficacy of the voltage-dependent Mg^{2+} blockade of the NMDA channel, and that the nonlinear membrane property imparted by the voltage-dependent Mg^{2+} blockade of the NMDA channel is necessary for locomotion. Voltage clamp recordings demonstrated that 5-HT potentiates the current through the NMDA channel and shifts the voltage for activating the region of negative slope conductance (RNSC) to more hyperpolarized potentials. In a small number of cases in which NMDA receptor activation alone failed to produce a RNSC, subsequent application of 5-HT promoted a RNSC. 5-HT alone did not produce a RNSC. The effect of 5-HT on the NMDA receptor-dependent RNSC was mimicked by decreasing concentrations of extracellular Mg^{2+}, suggesting that 5-HT may reduce the Mg^{2+} blockade of the NMDA channel. The nonlinear property of the NMDA channel, imparted by the Mg^{2+} ion, was critical for NMDA-induced oscillations. When the Mg^{2+} ion was removed from the bath during neurochemically-induced locomotor behavior (in the absence of exogenous NMDA), this activity was replaced by poorly organized patterns of phasic activity. This suggests that stable long-lasting rhythmic patterns require endogenous activation of nonlinear NMDA receptor-mediated properties. These findings suggest 5-HT mediates its modulatory influence on NMDA-mediated nonlinearity by enhancing the RNSC and secondly that the RNSC imparted by Mg^{2+} ions on the NMDA receptor-channel is critical for stable locomotor activity.

This project has examined the fundamental issue of the role of nonlinear motoneuronal membrane properties in the generation of mammalian locomotor behavior. The results

indicate that a) the network may call upon NMDA-dependent nonlinear membrane properties to facilitate rhythm generation, b) an endogenous transmitter (5-HT) present in the network is capable of modulating NMDA-dependent membrane nonlinearity and promoting oscillatory behavior, and c) one important mechanism through which 5-HT may modulate NMDA receptor-mediated effects is through regulation of the voltage-dependent Mg^{2+} blockade, which is necessary for locomotor pattern generation.

NMDA effects on spinal motoneurons.

Voltage clamp recordings in the presence of NMDA and TTX revealed a RNSC in the steady state current-voltage relationship in the majority of motoneurons examined. The RNSC was abolished by application of a specific NMDA antagonist DL-2-amino-5-phosophovaleric acid (AP5). The potential at which this regenerative inward current was initiated was ~ -63 mV. Whole cell current had a mean maximal inward current of ~ 200 pA. This RNSC was dependent upon the presence of Mg^{2+} in the bath medium. The RNSC was abolished following removal of Mg^{2+} from the perfusate.

When examined in current clamp mode, motoneurons exhibited four different membrane behaviors in response to NMDA application, as outlined below.

1) Tonic Depolarization. In some motoneurons a sustained depolarization was the only response observed during NMDA application in the presence of TTX (Section 2 and

Section 3). These motoneurons usually failed to demonstrate a RNSC (Section 3). It is not clear why these motoneurons failed to show a RNSC in the presence of NMDA alone. It is likely that the RNSC produced by NMDA receptor-activation was masked by competing outward currents as the only current blocked in these experiments was the fast transient Na+ conductance (by TTX). Thus these recordings measured all but one of the motoneuronal currents and not an isolated NMDA conductance. Although such an observation has not been reported previously in the literature this apparent discrepancy may be the result of our examination of the motoneuron as an electric whole rather than focusing on one of it's component currents. The masking of the NMDA receptor-dependent RNSC may have been exacerbated due to washout of the transient component of the NMDA current which accounts for ~50 % of all NMDA mediated current (see Mody et al 1988).

Motoneurons which did display a RNSC following NMDA receptor activation developed one of the other three types of membrane voltage behavior described below.

2) Oscillations. In some motoneurons bath application of NMDA, in the presence of TTX, produced rhythmic fluctuations of membrane voltage (Hochman et al 1994b; Sections 1 and 2). These fluctuations are referred to as 'oscillations', and are characterized by the presence of a peak depolarized phase that is of brief duration compared to the rising and falling phases of the voltage fluctuation. Oscillation amplitude was dependent on motoneuronal membrane holding potential, an observation which is consistent with what has been reported in many other preparations (e.g. Grillner

and Wallen 1985; Kim and Chandler 1995; Scrymgeour-Wedderburn et al. 1997). The frequency of motoneuronal TTX-resistant oscillations however was not consistently related to membrane holding potential. This observation is in contrast to observations of NMDA-induced oscillations in other systems (e.g. Durand 1991; Wallen and Grillner 1987), including pacemaker potentials of invertebrate neurons (e.g. Mathieu and Roberge 1971). This discrepancy may be the result of inadequate space clamp preventing sufficient control of distal dendritic NMDA channels. Electrotonic coupling was proposed by Sillar and Simmers (1994) to account for a similar independence of membrane potential and motoneuron oscillation frequency in *Rana Temporaria* spinal cord. Because electrotonic coupling is present in neonatal rat spinal motoneurons (Walton and Naverrete 1991), electrotonic coupling may also account for our observations.

3) Plateau Potentials. Motoneurons could also display rhythmic NMDA-induced membrane voltage fluctuations characterized by a depolarized phase that was relatively persistent compared to the abrupt rising and falling phases (Section 1). These voltage fluctuations were initiated, terminated and entrained by intracellular current pulse injection. Plateau potentials occurred as all-or-none events and were sometimes superimposed on underlying rhythmic voltage oscillations. Although these plateau potentials were dependent upon NMDA receptor activation it remains to be shown whether a separate inward conductance contributes to this membrane behavior.

4) Long-lasting Membrane Voltage Shifts. Motoneuronal NMDA receptor activation may result in the recurrence of long-lasting voltage shifts (LLVSs) (Section 2). LLVSs are characterized by a long duration (1-5 s), slow frequency (0.1-0.3 Hz) which may be rhythmic or arrhythmic, and of a large amplitude (~21 mV) which is relatively insensitive to the applied holding voltage. Preliminary evidence has indicated that LLVSs do not satisfy the two primary requirements in order to be classified plateau potentials (Hartline and Graubard 1992). That is, LLVSs could not be initiated or terminated by intracellular current pulse injection (data not shown) suggesting that this membrane behavior, although bistable, is inherently different from plateau potentials described earlier in this thesis and in other preparations (e.g. Hartline and Graubard 1992). In addition, the plateau potentials reported in cat and turtle spinal neurons were facilitated by 5-HT (Hounsgaard and Kiehn 1989) whereas the expression of LLVSs observed in response to NMDA is promoted by 5-HT receptor antagonists (Section 2).

Modulation of NMDA receptor-dependent nonlinearity by 5-HT.

NMDA alone is capable of producing a RNSC in most motoneurons, however, nonlinear membrane properties are not synonymous with membrane voltage oscillations. 5-HT modulates the NMDA receptor-dependent RNSC, promoting membrane voltage oscillations. Similar effects of 5-HT have been demonstrated by intracellular recordings of cat neocortical neurons. That is, NMDA, in the presence of TTX, produced tonic depolarization of neocortical cell membrane potential and subsequent application of 5-HT

produced membrane potential oscillations (Nedergaard, Engberg and Flatman 1986, 1987). A preliminary report indicated a similar modulatory action by 5-HT on NMDA actions which resulted in the generation of membrane potential oscillations in cat spinal motoneurons (Flatman and Engberg 1990). In amphibian spinal neurons, 5-HT is required for the maturation of locomotor rhythms (Sillar et al. 1995) and the expression of intrinsic voltage oscillations (Sillar and Simmers 1994; Scrymgeour-Wedderburn et al. 1997).

In addition, data from embryonic and larval *Xenopus* spinal cord neurons suggests that 5-HT_{1A} receptor activation enhances the voltage-dependent blockade of NMDA channels by Mg^{2+} as evidenced by a greater apparent input resistance in the presence of 5-HT and NMDA, compared to NMDA alone (Scrymgeour-Wedderburn et al. 1997). In contrast, our observations suggest that 5-HT, via 5-HT_2 receptor activation, shifts the voltage of activation of the NMDA-dependent RNSC to more hyperpolarized potentials by *reducing* the voltage dependent Mg^{2+}-blockade of the NMDA channel. Interestingly, in *Xenopus* spinal neurons, NMDA receptor activation produces a tonic depolarization. Subsequent application of 5-HT produces oscillations characterized as rhythmic hyperpolarizations from the level of tonic depolarization produced by NMDA, consistent with an enhancement of the Mg^{2+} blockade of the NMDA channel (Sillar and Simmers 1994). In contrast to the amphibian preparation, application of 5-HT to the neonatal rat spinal cord produces oscillations which depolarize from the tonic depolarization produced by NMDA receptor activation, suggesting a reduction of the Mg^{2+} blockade of the NMDA channel (Section 2). It appears that different mechanisms are utilized by the same neurochemicals

to produce oscillations in the *Xenopus* spinal cord and the rat spinal cord. In both cases 5-HT exerts its actions through modulation of the voltage-dependent Mg^{2+}-blockade of the NMDA channel. Nedergaard and colleagues (1987), however, suggest that the 5-HT modulation of NMDA voltage responses in the cat neocortex is through a mechanism other than the modulation of the voltage dependent Mg^{2+}-blockade of the NMDA channel. They arrive at this conclusion because they found an increased depolarization in response to NMDA following perfusion of 5-HT, both in the presence and absence of Mg^{2+}. While the increase in depolarization they observed may be independent of Mg^{2+}, it is not clear whether the 5-HT promotion of voltage oscillations was also independent of Mg^{2+}. No voltage clamp recordings were performed in the presence of NMDA before and after 5-HT, and as a result, it is difficult to comment on whether modulation of the voltage-dependent Mg^{2+}-blockade of the NMDA channel was the mechanism by which 5-HT promoted membrane voltage oscillations in these neocortical neurons.

We have demonstrated that the voltage of activation of the RNSC after 5-HT (mean = -79 mV) is below the resting membrane potential (mean = -72 mV). Thus, if there is no change in the currents which produced the stable resting membrane potential, this point of balance (i.e. stable) is lost, and providing that the neuron has sufficient voltage-dependent outward conductances to repolarize the neuron to this *previous* point of stability voltage oscillations will result. This action of 5-HT on the NMDA RNSC is likely the main mechanism by which 5-HT promotes NMDA-dependent membrane voltage oscillations. A similar negative shift of the NMDA-dependent RNSC has been reported after activation of protein kinase C (PKC; Chen and Huang 1992; Blank et al 1996). Single

channel recordings indicate that PKC reduces the Mg^{2+} block of the NMDA channel (Chen and Huang 1992). This may be the mechanism by which 5-HT modulates the NMDA receptor. Although preliminary, mianserin a 5-HT_2 receptor antagonist, partly reverses the action of 5-HT on the activation voltage of NMDA evoked RNSC. 5-HT_2 receptors are known to act though the PKC pathway (see Martin and Humphrey 1994) making it a prime candidate for the second messenger system mediating the results reported here.

A similar modulation of NMDA receptor-dependent RNSC by 5-HT may occur in cat spinal motoneurons, as suggested by a preliminary report which indicated that co-application 5-HT and NMDA resulted in the generation of oscillations in the membrane potential of cat spinal motoneurons (Flatman and Engberg 1990). Experiments to demonstrate a negative shift in the threshold for activation of the NMDA RNSC by 5-HT in cat motoneurons would be compelling evidence in favor of the concept put forth in the general introduction; that is, NMDA receptor-mediated nonlinear membrane properties are the major substrate of cat motoneuron nonlinearity. However, such experiments in the cat spinal cord have not been done at this point in time.

Although the action of 5-HT on the Mg^{2+}-blockade of the NMDA channel is likely the main mechanism by which 5-HT promotes motoneuron voltage oscillations, it has been well documented that 5-HT influences the excitability of motoneurons by many other mechanisms (e.g. Barasi and Roberts 1974; White and Neuman 1980; Elliot and Wallis 1992; Ziskind-Conhaim et al 1993). As a result, it is also quite possible that other actions

of 5-HT on motoneurons contribute to the promotion of membrane voltage oscillations. (1) 5-HT potentiates low voltage-activated (LVA) Ca^{2+} channel conductances in spinal motoneurons (Berger and Takahashi 1990). An enhancement of LVA conductances would increase the speed at which membrane potential of the spinal motoneurons would depolarize to threshold for NMDA receptor-dependent RNSC, facilitating the expression of voltage oscillations. (2) 5-HT has also been found to enhance the inward rectifier current in spinal motoneurons (Takahashi and Berger 1990) and facial motoneurons (Larkman and Kelly 1992). An enhancement of an inward rectifier (I_h) would also facilitate oscillatory activity of the motoneurons through a similar mechanism suggested for the LVA current. (3) 5-HT decreases the outward Ca^{2+}-dependent K^+ channels in lamprey ventral neurons (Wallen at al 1989). This caused a broadening of the NMDA-dependent voltage oscillations, similar to that with the use of the a Ca^{2+}-dependent K^+ channel antagonist apamine (Wallen and Grillner 1987; Kim and Chandler 1995). Of course decreasing this conductance would promote plateau potentials (as defined above) rather than faster frequency voltage fluctuations (i.e. oscillations). Thus multiple effects of 5-HT in addition to 5-HT effect on NMDA-dependent RNSC may account for the promotion of membrane potential oscillations in mammalian motoneurons.

Functional implications of nonlinear membrane properties in neurons.

Nonlinear membrane behaviors, produced by NMDA receptor activation, have several potential roles in determining neuron behavior and output.

Nonlinear membrane properties promote membrane voltage oscillations. Intracellular recordings of *Aplysia* abdominal ganglia L2-L6 neurons, which demonstrate pacemaker potentials, revealed that the RNSC is the most critical determinant of whether a cell is capable of demonstrating membrane voltage oscillations. (Wilson and Watchel 1974; see also Barker and Gainer 1975a; Eckert and Lux 1975). Our examination of spinal motoneurons also indicated that a RNSC must be present in order to observe voltage oscillations. Of course a RNSC alone does not produce membrane voltage oscillations but such a region is critical in order to elicit oscillations. As demonstrated in Section 1, motoneuronal voltage oscillations are capable of recruiting action potentials and plateau potentials, and in this way oscillations may limit output to discrete time intervals during each cycle (see below).

Because NMDA receptor-dependent nonlinearity is a conditional property it is very difficult to determine, for example, the role of intrinsic properties in the production of output triggered by synaptic inputs such as locomotor drive potentials (LDP) in motoneurons. Although we report that NMDA is capable of producing motoneuronal oscillations in the presence of an intact network, it is unclear how large a role these membrane behaviors play in LDP formation. Oscillations are a manifestation of the nonlinear membrane properties produced by NMDA receptor activation, thus making them a useful tool to examine the modulation of NMDA-dependent nonlinearity. This is not to suggest however, that motoneurons oscillate during locomotion, rather that

common membrane properties activated during voltage oscillations are also called upon to contribute to LDP formation.

Nonlinear membrane properties may limit temporal dispersion of synaptic input. Injection of artificial sinusoidal oscillations into neurons of the inferior olive indicate that impinging synaptic inputs arriving at different times within the cycle can produce the same output (Lampl and Yarom 1993). Synaptic inputs which arrived any time during the rising phase of the oscillation caused the neuron to spike at the same time relative to the cycle frequency, at the peak of the depolarization. Phasic synaptic inputs could arrive within a particular window of time but the output remained invariant thus preventing temporal dispersion. Lampl and Yarom (1993) also found that synaptic inputs were weighted differently depending on when within the oscillation cycle they occurred. Synaptic inputs which arrived at the peak of the oscillation were much more likely to affect neuron output than inputs that arrived in the trough of the oscillation.

A similar effect can result in the absence of oscillations. Nonlinear membrane properties effectively produce two states of excitability within the neuron (see below). Synaptic inputs that arrive when the neuron is in a state of low excitability are less likely to affect the output of the motoneuron than a synaptic input that arrives when the neuron is in a high state of excitability. In this way particular synaptic inputs can be filtered in and out depending upon their temporal relationship to the excitability state of the neuron. This phenomena has been termed 'temporal contrast' by Hartline and Graubard (1992).

Nonlinear membrane properties result in an increased sensitivity to synaptic input by the postsynaptic cell. Nonlinear membrane properties can place the cell in a high gain state and, as a result, motoneuronal output can far outlast synaptic input. This is evidenced by the prolonged depolarizations observed in cat spinal motoneurons in response to relatively brief afferent input (e.g. Hounsgaard et al 1985). The RNSC provides an intrinsic source of regenerative depolarization, and if the depolarized point of null current is more depolarized than the threshold for action potential generation, bursting occurs. The RNSC also allows excitatory inputs to depolarize the postsynaptic neuron beyond the sum of their inputs (i.e. amplification). Inhibitory inputs can also be amplified by nonlinear membrane properties. A brief inhibitory input can produce rapid repolarization of a membrane potential provided that this conductance, in combination with the intrinsic repolarizing conductance, results in sufficient membrane hyperpolarization, to reinstate the Mg^{2+}-blockade of the NMDA channel (thereby moving the membrane potential to the more hyperpolarized of the potentials which cross the null current axis). Analysis of the post-synaptic currents occurring in spinal interneurons located in the vicinity of the central canal revealed that a surprisingly small number of synaptic events (8-300) generates the rhythmic activity seen in these neurons (Raastad et al 1996, 1997). This observation suggests that intrinsic properties may indeed be necessary to amplify synaptic events in these neurons. Or that the neurons recorded from have little, if anything, to do with locomotion.

Nonlinear membrane properties increase the flexibility of the motor output. As previously described, many different neuromodulators are capable of producing burst activity in the neurons of the pyloric central pattern generator (Harris-Warrick et al 1992). Each neurochemical produces burst activity in the pyloric neurons through different ionic mechanisms. As a result, although the neurons burst in a number of neurochemical environments they behave differently depending on the neurochemical used (Harris-Warrick et al 1992). These different intrinsic behaviors result in unique motor patterns produced by the pyloric CPG. Clearly, the NMDA receptor-dependent RNSC in vertebrate neurons is subject to modulation by 5-HT just as other motoneuronal membrane properties are modulated. It is reasonable to suggest that other endogenous neuromodulators, in the presence of endogenous glutamate release, could also produce a number of nonlinear membrane behaviors. As a result, motor output would reflect on the specific membrane properties that the motoneuron is exhibiting as determined by its neurochemical environment at that time.

Contribution of NMDA receptor-dependent nonlinearity to locomotor activity.

Although excitatory currents contributing to the LDP in spinal motoneurons demonstrate a voltage dependent increase in amplitude (Moore et al 1987; Brownstone et al 1994; Hochman and Schmidt 1998) and oscillations of membrane voltage persist in the intact network and can be recruited by synaptic events (Section1), this evidence does not

directly address whether the nonlinearity imparted by NMDA receptor activation contributes to locomotor activity. NMDA receptor-mediated non-linear membrane properties can be abolished by removal of Mg^{2+} from the bath (Brodin and Grillner 1986; Soffe and Roberts 1989; Reith and Sillar 1998) and this results in a maximal conductance through the NMDA channel at all potentials (Mayer et al 1984). As predicted, TTX-resistant NMDA-induced motoneuronal oscillations were abolished by the removal of Mg^{2+} from the bathing medium. In the rat spinal cord, stable locomotor activity patterns produced by bath application of 5-HT are replaced by poorly organized phasic activity in the ventral roots following removal of Mg^{2+}. As rhythmic motor activity was disrupted following removal of Mg^{2+} (in the absence of exogenous NMDA receptor activation) it is likely that endogenous activation of NMDA receptor-dependent nonlinear membrane properties is necessary for stable motor patterning in the mammalian spinal cord. However, it is possible that the disruption of the locomotor pattern reflects the loss of motoneuronal nonlinear membrane properties alone and is not necessarily reflective of a disruption of the behavior of the locomotor CPG. Similar results of disruption of fictive swim patterns following removal of Mg^{2+} have been reported in the lamprey (Brodin and Grillner 1986) and in the *Xenopus* (Soffe and Roberts 1989). Modeling studies of the *Xenopus* spinal pattern generator suggest that voltage-dependent Mg^{2+} blockade of NMDA receptors stabilizes swimming activity through enhancement of postinhibitory rebound within each individual neuron (Roberts et al 1995). Rowat and Selverston (1997) applied mathematical models of the stomatogastric ganglion and examined the activity patterns of coupled neurons that had nonlinear membrane properties. The activity patterns were generated using chaos mathematics, and they found that using

inhibitory synaptic connections could transform random activity to a stable bursting pattern in each cell (Rowat and Selverston 1997). Excitatory synaptic inputs however, were unable to establish a stable pattern of activity. The authors suggest that this stabilization is the direct result of the rapid repolarization (a product of the nonlinear membrane properties) of the cell membrane and the resulting postinhibitory rebound (Rowat and Selverston 1997). Examination of fictive locomotor patterns in the larval *Xenopus*, produced by NMDA, revealed a slow (0.5 Hz) rhythmic fluctuation of ventral root activity in which burst intensity, duration and frequency cyclically increased at a similar frequency to the 5-HT/NMDA dependent voltage oscillations reported in *Xenopus* spinal neurons (Reith and Sillar 1998). This activity was blocked by the 5-HT_{1A} receptor antagonist pindobind and by removal of Mg^{2+} from the bath indicating that it was dependent on 5-HT receptor activation as well as the voltage-dependent block of the NMDA ionophore. It is hypothesized that this slow fluctuation of ventral root activity may act as a boosting mechanism for motor output.

One previous study using a neonatal rat brainstem spinal cord preparation reported no qualitative difference in locomotor activity produced by brainstem stimulation before and after removal of Mg^{2+} (Astuta et al 1990). In our experiments however, we found that there was a disruption of the locomotor pattern following removal of Mg^{2+} in both the brainstem spinal cord preparation and in the isolated spinal cord. It is quite possible that due to the long (>15 min) washout time of Mg^{2+} required to observe an effect on the locomotor pattern, these authors did not achieve a Mg^{2+}-free state as μM concentrations

of Mg^{2+} can produce a nonlinear current voltage relationship of the NMDA channel (Nowak et al 1984).

Final Thoughts.

Nonlinearity is but one of the membrane properties which motoneurons express during locomotion (e.g. Brownstone et al 1992; Brownstone and Hultborn 1992; Schmidt 1994; Krawitz et al 1997) increasing the complexity of their operation immensely. Study of motoneurons is extremely appealing, especially in the in vitro spinal cord preparation, as they provide an identifiable neuronal population. Examination of motoneurons allows one to study principles of motoneuron operation in the hope that these principles hold true throughout the locomotor network and in this regard motoneurons are a very useful tool. However, future experiments hoping to unravel the neural basis of locomotion must begin to examine interneurons which are active during locomotor activity.

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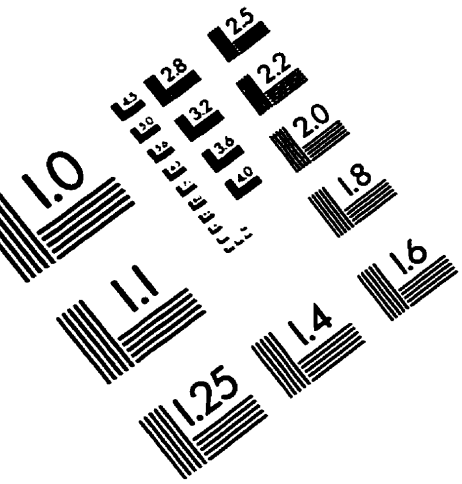
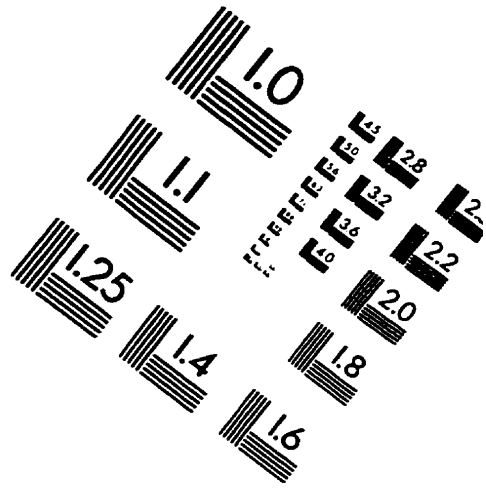
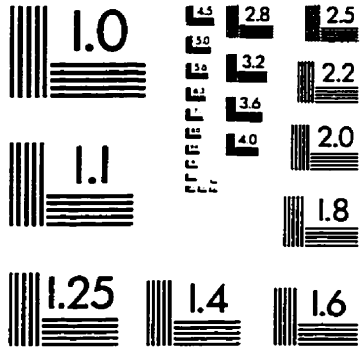
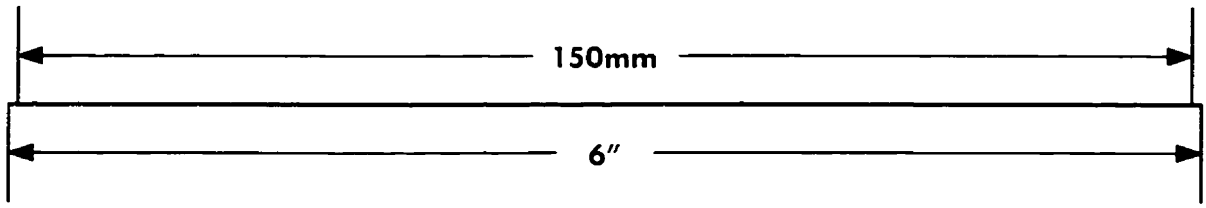
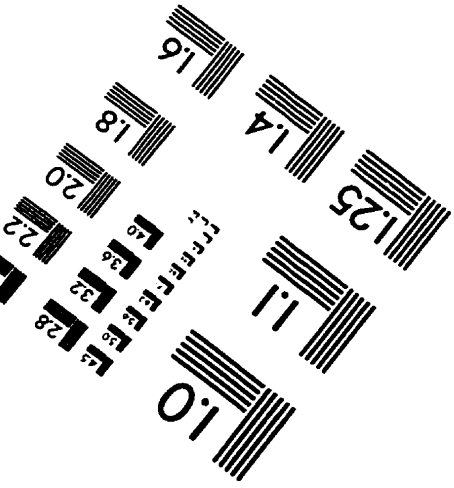
IMAGE EVALUATION TEST TARGET (QA-3)

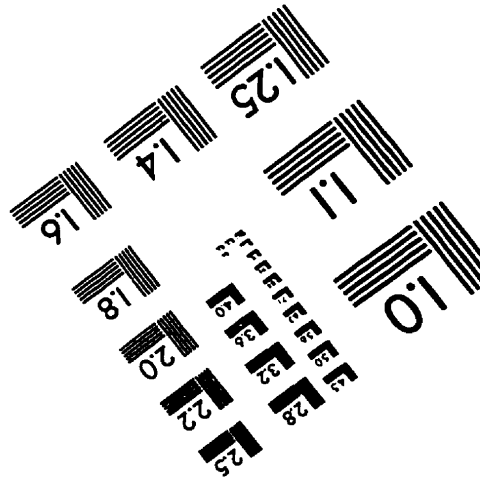












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