LAMINA VII NEURONS ARE RHYTHMICALLY ACTIVE DURING LOCOMOTOR-LIKE ACTIVITY IN THE NEONATAL RAT SPINAL CORD

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BY

JASON N. MACLEAN

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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Acknowledgments

"I've got science for any occasion, postulating theorems, formulating equations ...drop-ping science like when Galileo dropped his orange."

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Abstract

The midsagittaly-sectioned rat spinal cord with thoracic segments intact retains the capacity for locomotor-like activity. Intracellular recordings were used to characterize the activity and concurrently label lumbar neurons in lamina VII, an area implicated in the generation of locomotion. Sharp electrodes were shown to preferentially impale large neurons in the lumbar spinal segments, identified with intracellular label. These large neurons undergo rhythmic voltage oscillations, presumably synaptically driven, during locomotor-like activity induced by bath application of N-methyl-D-aspartate and 5-hydroxytryptamine. This supports the hypothesis that synaptic activity recruits neurons in lamina VII during locomotion. These neurons were further characterized by their depolarizing responses to inputs from segmental afferents and from the ventrolateral funiculus, a region of the spinal white matter thought to carry descending fibers involved in the initiation of locomotion. This work represents an important advance towards the elucidation of the cellular components comprising the locomotor CPG, providing the first intracellular recordings from interneurons in the neonatal rat lumbar spinal cord during locomotor-like activity.

List of Abbreviations

ACSF	Artificial Cerebrospinal Fluid
DOPA	dihydroxyphenylalanine
St	Semitendinosus
PBSt	Posterior Biceps Semitendinosus
NMDA	N-methyl-D-aspartate
5-HT	5-hyroxytryptamine/serotonin
MLR	Mesencephalic Locomotor Region
MRF	Medial Reticular Formation
VLF	Ventrolateral Funiculus
NA	noradrenaline
6-OHDA	6-hydroxy-dopamine
5,6-DHT	5,6-dihydroxytryptamine
AP5	2-amino-5-phosphonovaleric acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPG	central pattern generator
IaIN	Ia Inhibitory Interneuron
RC	Renshaw Cell
LDP	Locomotor Drive Potential
MEC	mecamylamine
MN	Motoneuron
EIN	Excitatory Interneuron
LIN	Lateral Interneuron
CCIN	Contralaterally and Caudally Projecting Interneuron
eIN	Descending Excitatory Interneuron
iIN	Commisural Inhibitory Interneuron

T	Threshold
P	Postnatal
L	Lumbar
ChAT	Choline Acetyl Transferase
TTX	Tetrototoxin
EMN	Extensor motoneuron
FMN	Flexor motoneuron
coFRA	contralateral flexor reflex afferents
iFRA	ipsilateral flexor reflex afferents
FRC	Flexor Renshaw cell
ENG	Electroneurogram
PBS	Phosphate buffered saline
ERC	Extensor Renshaw cell
EAA	Excitatory Amino Acid

Introduction

The circuitry necessary for locomotion is contained within the spinal cord.

As early as 1906, researchers were aware that locomotion could be elicited in the absence of descending cerebral, cerebellar, or brainstem input. Sherrington visually observed that 'reflexive stepping' could be easily elicited in chronic spinal cats demonstrating that some locomotor movements could be organized without descending input from the brain or brainstem (Sherrington, 1906). Researchers then removed afferent input to the spinal cord by sectioning the dorsal roots in order to examine hindlimb function in the absence of sensory inputs. Following dorsal rhizotomy and transection of the spinal cord, Graham Brown (1911) observed spontaneous alternating contractions of pairs of antagonistic hindlimb muscles. As a result, he suggested that this muscle activity was generated centrally within the spinal cord and not by a complex chain of reflexes. He hypothesized that a simple neuronal organization, which he termed the half-center, could produce the alternation between flexor and extensor motoneurons. He postulated that there were two half-centers, one comprised of extensor motoneurons and one comprised of flexor motoneurons connected by recurrent inhibitory motor axon collaterals (Brown, 1914) (Fig 1A). The important feature of this hypothesis is the mutual inhibitory connection between the flexion centers and extension centers. Tonic excitation of the half-centers would not produce an equal balance of activity between the two. Rather, one half-center would become dominant and silence the other half-center. Eventually this inhibition would lessen due to fatigue resulting in a switch in dominance to the other half-center. As a result, rhythmic activity between flexors and extensors could be produced with a simple tonic excitation applied to the motoneurons.

Using intracellular recordings of single motoneurons and extracellular recordings of interneurons in the lumbar spinal cord, Lundberg and colleagues revealed mutually inhibitory reflex pathways as predicted by Brown. Under certain conditions these pathways were capable of generating rhythmic alternation between flexors and extensors (Jankowska et al., 1967a) (Fig 1B). In acute spinal cats given dihydroxyphenylalanine (DOPA) (metabolized into noradrenaline and dopamine), stimulation of ipsilateral high-threshold cutaneous, muscle and joint afferents resulted in a reflex discharge of a long latency, long duration recorded intracellularly in flexor motoneurons. Stimulation of contralateral high threshold afferents evoked similar reflex excitation of ipsilateral extensor motoneurons. When both ipsi- and contralateral afferents were stimulated within 2 ms of each other, the two pathways were mutually inhibitory, indicating a reciprocal organization. After the administration of DOPA and nialamide (monamine oxidase inhibitor), single stimuli delivered to high threshold afferents triggered short periods of alternating activity in flexor and extensor nerves which Jankowska et al (1967a) likened to spinal stepping. As a result the reciprocal connections between flexors and extensors were believed to be mediated by inhibitory interneurons rather than by recurrent motoneuron axon collaterals as proposed by Brown. The long duration, long latency responses were believed to be mediated by a chain of interneurons. The long duration of the response could be the result of a positive feedback loop, and the long latency could be the product of both the number of interposed interneurons and/or their requirement of considerable temporal summation for their activation (Jankowska et al., 1967a). This work introduced the concept of a group of interneurons organized with inhibitory and excitatory connections so as to be capable of the production of the locomotor rhythm. Further insight into the neuronal components responsible for rhythm generation would require intracellular recordings from functionally identified interneurons. These interneurons would be further classified by their morpholgy, location, and through the afferent and descending inputs they receive (Jankowska et al., 1967b).

The Central Pattern Generator

The intrinsic neuronal network responsible for the generation of locomotion is termed the locomotor central pattern generator (CPG)(Grillner 1981). According to this definition the neurons comprising the CPG may have different functions. It is possible that the CPG may be subdivided into two functionally separate but hierarchically connected subcomponents, (1) interneurons whose activity determine the frequency of locomotion which act on (2) last-order interneurons (those which synapse directly onto motoneurons) (Grillner 1981). The CPG may also be composed of a mosaic of unit CPGs, a unit being the minimal subset of neurons for both rhythm and pattern generation. Unit CPG's could generate alternating activity between antagonistic muscles working around a single joint and be selected and recombined by a supraspinal command for a variety of movements (Grillner 1981). Kiehn suggests the 'start up' period of unorganized flexor/extensor activity following application of 5-HT is due to the mosaic of burst generators at each joint becoming synchronized (Kiehn et al., 1994b).

Two models for the mammalian CPG have been proposed but subsequently refuted through the examination of interneuronal and motoneuronal activity during locomotion. Miller and Scott (1977) proposed that a system of antagonist motoneurons, Ia inhibitory interneurons and Renshaw cells (the two best understood mammalian interneurons at that time) comprised the spinal locomotor generator (Fig 2A). Ia inhibitory interneurons are last-order interneurons which receive strong input from ipsilateral group I spindle afferents. These interneurons mediate Ia reciprocal inhibition, inhibiting the antagonist motor nuclei of the muscle which they innervate. Renshaw cells are activated by axon collaterals from motoneurons. Renshaw cells mediate recurrent inhibition onto their respective motoneurons and the Ia inhibitory interneurons, the latter projecting to the antagonist motor nuclei. This electronic model indicated that an oscillatory firing pattern in the presence of tonic excitation applied to both MN and IaIN produced an alternation

between flexor and extensor motoneuronal activity. The alternation between flexor and extensor activity was determined by phasic IaIN inhibition of motoneurons. The pattern of IaIN activity was determined by the inhibitory action of the RCs. The rhythmic alternations between flexors and extensors was produced by the delay associated with the RC recurrent pathway and it's influence on IaINs.

Pearson and Duysens proposed a flexor burst generator (Fig 2B) which could explain the fact that the duration of the swing phase of the step cycle remains constant during changes in the overall step cycle duration while that of the stance phase varies considerably in the cat and the cockroach (Pearson and Duysens, 1976). They interpreted this as indicating that once activated, the cellular components comprising the flexion center were active for a set duration and that extension merely followed flexor activity. Further evidence for rhythmic flexion was the observation that some mesencephalic cats walked spontaneously on a treadmill and showed a normal swing phase while no extensor activity was observed during stance; the leg was extended passively by the treadmill (Pearson and Duysens, 1976). Thus flexion could be present, but independent, from extension. As a result, a system of interneurons which periodically produced bursts of activity in motoneurons during swing (mainly flexors) and concomitantly inhibit the motoneurons which are active during the stance (extensors) was proposed (Fig 2B). They assumed that the swing generator was periodically activated by central and reflex inputs (leg position) and remained active for a relatively constant period of time independent of the cycle time. Extensor motoneurons would be activated by disinhibition, resulting in the alternation between flexion and extension.

Both hypotheses were examined by Jordan and colleagues (Jordan, 1981; Shefchyk et al., 1982; and Jordan, 1983). By measuring membrane conductance changes in motoneurons during fictive MLR-induced locomotion, they found that neither flexor nor extensor motoneurons showed a particular pattern of conductance changes during the depolarized or hyperpolarized phases of the step cycle (however, see Schmidt et al.,

Afferent input onto the CPG.

Although the isolated spinal cord is capable of generating locomotor activity, successful locomotor behavior requires sensory feedback from the moving limbs. Some sensory signals may affect the pattern of locomotion through reflex pathways also operational at rest, while others may affect the pattern of the step cycle through direct actions on interneurons comprising the rhythm generator. Understanding these direct connections may offer insights into the organization of interneurons comprising the rhythm generator. Resetting is a term used to describe the phenomena by which a specific afferent input perturbs the ongoing rhythm, by either lengthening the duration of muscle activity and shortening the duration the duration of the antagonists activity, or by terminating muscle activity and initiating activity in muscle antagonists. In either case the next phase of muscle activity in the locomotor cycle does not occur when expected. Entrainment describes the phenomena in which an endogenous rhythm is synchronized to external periodic afferent inputs (Pavlidis, 1973). Specific proprioceptive inputs seem to have a critical effect on the timing of the different phases in the locomotor cycle. Hip position, loading of the ankle extensors, enhanced activity of flexor muscle high-threshold afferents or an abrupt change in length of ankle extensors are capable of resetting or entraining the ongoing locomotor rhythm (Grillner et al., 1978, Conway et al., 1987, Perreault et al., 1995, Guertin et al., 1995). Normal transition from stance to swing in the chronic spinal cat does not occur if the hip extension is manually prevented (Grillner and Rossignol, 1978). Swing could occur if the hip is moved to the position (approximately 80°) which corresponded to the stance/swing transition point and not before. This effect is mediated by muscle afferents, since dennervation of the hip joint capsule did not prevent entrainment of the ongoing locomotor rhythm by manual movement of the hip (Kriellaars et al., 1994), while cutting muscle afferents did. The unloading of the ankle extensor muscles is necessary to allow the CPG to switch from stance to swing phase (Pearson and

Duysens, 1976). If an additional load was placed on the ankle extensors during the stance phase in thalamic cats, the swing phase was prevented. Stimulation of ankle extensor afferents at group Ia (Guertin et al., 1995) and Ib (Conway et al., 1987) strength during flexor nerve activity terminates that nerve activity and promotes generation of ipsilateral extension and contralateral flexor activity with an earlier onset than would be anticipated without the stimulus. When stimulation occurred during the extension phase, the extensor activity was prolonged and the flexor burst delayed. Stimulation of flexor afferents at five times threshold during the flexion phase terminated activity in flexor nerves and initiated activity in extensors (Perreault et al., 1995). Since this 'shortening' of the locomotor cycle duration occurs at 5T but not at 2T stimulus strengths, group II afferents most likely mediate this effect. Stimulation of flexor muscle afferents during extension did not perturb the ongoing rhythm.

Examination of afferent input onto the CPG in the neonatal rat can also offer insights into the organization of the CPG and may provide a means to categorize cells in this preparation. The effects on the centrally generated rhythm from low threshold afferents in the *in vitro* neonatal rat preparation has been examined by Kudo and Kiehn (Kiehn et al., 1992; Iizuka et al., 1994; Kiehn and Kjærulff, 1994a). During locomotor activity induced with the application of NMDA and 5-HT, a brief train of stimuli in low threshold afferents of L3 or L2 dorsal roots (1.7xT) caused a resetting of the ongoing rhythm. However, stimulation of L4 or L5 dorsal roots had little or no effect on the rhythm (Kiehn et al., 1992). Stimulation of afferents in the quadriceps nerve had similar effects to that seen after stimulation of L2 or L3 (Kiehn et al., 1992). This stimulation resulted in resetting to flexion in contrast to what is observed in the cat where stimulation resets to extension. However recent work by Cowley and Schmidt (1994a) suggests that Kiehn and colleagues may have used the incorrect ventral root electroneurograms (ENG) as monitors for extension and flexion. Kiehn et al (1992) used L5 ENG as a monitor of flexor motoneuron activity and the L3 ENG as a monitor of extensor motoneuron activity,

but Cowley and Schmidt (1994a) have demonstrated that although ventral roots contain both fexor and extensor motoneuron axons, activity in L5 ventral root was related to tibial nerve discharge, an extensor, and activity in L3 ventral root was related to peroneal nerve discharge, a flexor. Thus it is possible that the resetting reported by Kiehn et al (1992) could be interpreted as resetting to extension, as observed in the cat, and not resetting to flexion. In the adult cat, resetting appears to be mediated by extensor group I afferents and flexor group II afferents (Conway et al., 1987; Guertin et al., 1995; Perreault et al., 1995). However, there is limited quantitative data on the degree of nerve myelination and conduction velocity (e.g. Cheng et al., 1992) in the neonatal *in vitro* preparation, and little is known about the development of proprioceptive organs (Kudo and Yamada, 1985; Kiehn et al., 1994a). As a result, it is difficult to know which muscle afferents are stimulated at a given stimulus strength although this has been determined for cutaneous afferents (e.g. King et al., 1990). The observation that stimulation of muscle afferents can reset the rhythm at P0-P3 however, indicates that these afferents do have connections to the CPG and thus afferent input provides one criteria through which to examine putative interneuronal components of the mammalian locomotor CPG.

The CPG in lower vertebrates

The interneuronal components comprising the CPG for locomotion have been studied in lower vertebrates in great detail in both lamprey (for review, see Grillner et al., 1991) and *Xenopus* larvae (for review, see Arshavsky et al., 1993). In the lamprey, propulsive force during swimming is due to laterally directed undulations propagating along the body in a caudal direction. The propagation of the wave caudally is due to an intersegmental coordination causing a delay, which is proportional to 1% of the total cycle

time, between each consecutive segment (Wallen and Williams, 1984). The circuitry underlying locomotion, as in other vertebrates, is comprised of three main components: 1) the circuitry responsible for segmental motor activity, 2) sensory input onto the segmental circuitry, and 3) a descending reticulospinal system. Segmental circuitry includes: motoneurons (MN), one population of excitatory interneuron (EIN), and two populations of inhibitory interneurons, lateral interneurons (LIN) and contralaterally and caudally projecting interneurons (CCIN) (Fig 3A). EINs excite all ipsilateral interneurons (both excitatory and inhibitory) and motoneurons, and CCIN inhibit all contralateral interneurons and motoneurons. LINs project ipsilaterally to CCINs. Inhibition of CCINs occurs at the end of the cycle as the threshold of LINs for action potentials is very high. This produces a disinhibition of the contralateral side (see Grillner et al., 1991).

The *Xenopus* swims in response to cutaneous stimulation (Sillar and Roberts, 1993). As in the lamprey, this activity is characterized by alternating activity between the left and right sides of the same segment while motoneuron activity in more caudal segments is delayed. There are only four populations of neurons in the spinal cord of the Xenopus larvae which are active during locomotion. These include; the dorsal sensory interneurons located in the initiation pathway; ventral motor neurons (MN); and two types of premotor interneurons (Roberts et al., 1990), a descending interneuron (eIN) which excites ipsilateral interneurons and motoneurons through activation of excitatory amino acid receptors (Dale and Roberts, 1985) and commissural interneurons (iIN) which inhibits contralateral eIN and MN at strychnine-sensitive (glycinergic) synapses (Fig 3B) (Dale et al., 1985). Alternation between the two sides is dependent upon the coordination of excitation and inhibition by the iIN. Each neuron fires only one action potential per cycle due to a strong accommodation (Sillar and Roberts, 1993). Reactivation of eIN is mediated by rebound excitation which follows inhibition from iIN.

Even for the lower vertebrates, however, the circuitry for locomotor output is not completely understood. The proposed model for the lamprey does not account for it's

ability to swim backwards nor the constant phase lag of 1% of the total cycle. The *Xenopus* model fails to explain the conservation of the rhythmic activity following removal of one side of the spinal cord, which removes the inhibitory input from the commisural interneurons.

Localization of the CPG in the mammal.

Unlike the lower vertebrates, the complexity of the mammalian spinal cord has made identification of neurons comprising the locomotor CPG extremely difficult. The largest hindrance to the understanding of the locomotor central pattern generator in the mammal is the lack of identified neurons which can be used to test various hypotheses. A further understanding of rhythm generation in terms of connections and intrinsic cell properties requires intracellular recording from functionally identified interneurons and the use of both electrophysiological and morphological techniques (Jankowska et al., 1967b). In order to undertake this type of study, it is necessary to know where to start looking for putative components of the CPG.

In 1967 Jankowska and colleagues discovered a group of interneurons in Rexed lamina VII which were organized reciprocally as had been predicted by the half-center hypothesis. After intravenous injection of DOPA, two types of interneurons were discovered which mediated the late onset, long duration effects of high-threshold afferents. Type A received excitation from ipsilateral high-threshold afferents and inhibition from contralateral high-threshold afferent stimulation. Type B received inhibition from ipsilateral high-threshold afferents and excitation from contralateral high-threshold afferents. The reciprocal organization revealed following DOPA administration suggested strongly that these interneurons were interposed in the locomotor pathway (Jankowska et

al., 1967b). This work suggested that reciprocally organized interneurons (in lamina VII of the lumbar spinal) were involved in the generation of the locomotor rhythm. An extracellular survey of the lumbosacral portion of the spinal cord a few years later revealed that interneurons in the spinal cord were rhythmically active during MLR evoked locomotion in the cat (Orlovsky and Feldman, 1972). This study reported that the majority of rhythmically modulated neurons were located in the ventral intermediate gray of the spinal cord and the motor nucleus, but they also recorded similar activity throughout the gray matter. Field potential mapping during MLR-evoked fictive locomotion in the lumbar spinal cord of the cat indicated that lamina VII was a major location of activity (Jordan, 1991a, Noga et al., 1995). Extracellular recordings in a spontaneously locomoting decorticate rabbit also pointed to the lumbar medial grey as being very active during locomotion (Viala et al., 1991). The main problem with this and other surveys (e.g. Baev et al., 1979) was that they involved recordings from unidentified neurons. They did not use criteria such as specific inputs from afferents to characterize the neurons and focused only on the neurons activity during locomotion, although this was not the object of these studies. Their significance was however, that they indicated the regions of greatest activity during locomotion and thus focused the locations for further studies using intracellular recording methods.

In 1990, Shefchyk and colleagues recorded rhythmic activity during fictive locomotion in a specific set of interneurons in the fourth lumbar segment (L4) (Shefchyk et al., 1990). These L4 interneurons, previously described by Jankowska and colleagues (Cavallari and Jankowska, 1987; Edgley and Jankowska, 1987a,b; Jankowska and Skoog, 1986), received powerful input from group II afferents, were located in lamina VII and VIII, and projected caudally to motoneurons in lower lumbar segments where they produced monosynaptic excitation or disynaptic inhibition of motoneurons. Interneurons in lamina VII, some receiving input from group II afferents, also receive direct synaptic

input from the MLR, strengthening the case for their role in locomotion (Edgley et al., 1988; Shefchyk et al., 1990; Jordan et al., 1991b) .

Activity dependent labeling allows researchers to identify neurons involved in a behavior *en bloc*. Activity-dependent label is probably taken up through endocytosis into active synaptic terminals labeling the neurons' soma and dendrites (Lichtman et al., 1985). During locomotion in the rabbit, cat, and neonatal rat, activity dependent labels; 2-deoxy[C]-glucose (metabolic indicator) (Viala et al., 1988), c-fos immunohistochemical method (Dai et al., 1990), and sulforhodamine (Kjærulff et al., 1994), respectively, revealed that neurons in circumscribed regions of the spinal cord, particularly lamina VII, are active during locomotion. Injection of fluorescent microspheres into cat hindlimb motor nuclei in the cat retrogradely labeled interneurons in lamina VII suggesting they have inputs directly onto motoneurons (Hoover and Durkovic, 1992). Injection of wheat germ agglutinin conjugated to horseradish peroxidase also retrogradely identified interneurons in this area as having direct inputs onto motor neurons in both the cat and the rat (Harrison et al., 1984). The labeling and the electrophysiological studies indicate that neurons in lamina VII are involved in the production of locomotion.

The in vitro Neonatal rat preparation

Different preparations and biological systems offer different advantages and disadvantages for the study of locomotor mechanisms. These strengths and limitations must be recognized when choosing a particular biological model to examine a particular question.

The lamprey provides a basic neuronal model for examining motor function. The lamprey brainstem-spinal cord preparation can be maintained in vitro for several days

(Rovainen, 1979). The small number of large neurons in lamprey spinal cord make visual identification and subsequent intracellular recordings, even paired neuronal recordings, possible (e.g. Buchanan et al., 1989). The extracellular medium's composition can be tightly controlled as can concentrations of pharmacological agents. This allows a researcher to examine motor behavior in a dose-dependent manner and to determine the ionic mechanisms underlying these behaviors. Motor activity is produced by identified interneurons with a reciprocal organization similar to what is proposed by the half-center hypothesis making the lamprey a useful model to examine cellular function which may be applicable to higher vertebrate locomotion. However, the lamprey spinal cord lacks the complex cellular organization of the mammalian system and the complex muscular organization necessary for overground locomotion, thus straining an organizational connection between this model and mammals.

The *in vivo* cat provides a model which possesses a hindlimb muscular organization for overground locomotion. Hindlimb muscle and joint nerves are relatively easily dissected allowing precise examination of particular muscle activity during locomotion. This allows well defined inputs onto interneurons and motoneurons to be examined. This precise examination allows behavioral inferences based on these observations to be possible. An intact preparation does not, however, permit the extracellular medium to be easily controlled. The presence of the blood brain barrier necessitates the use of neurotransmitter precursors, thus the researcher can only estimate the concentration of the pharmacological agent the neurons are exposed to. Iontophoretic application of drugs can avoid this problem but this method of applying drugs is difficult.

An *in vitro* neonatal rat preparation was developed by Otsuka and Konishi in 1974 which provides a compromise between the strengths of the *in vivo* cat preparation and the *in vitro* lamprey preparation. Due to its small size and small amount of myelination, the neonatal rat spinal cord can survive for a number of hours with the passive diffusion of metabolites and gas. The blood brain barrier is immature, therefore the concentration of

The induction of locomotion.

Pharmacological

Glutamate, an excitatory amino acid, can activate two types of receptors named after selective agonists to the N-methyl-D-aspartate (NMDA) and non-NMDA (kainate and quisqualate) receptors. Both NMDA and kainate have been shown to induce locomotion in *in vitro* preparations (e.g. Smith and Feldman 1987; Kudo and Yamada, 1987; Brodin et al., 1985). The NMDA receptor is characterized by voltage sensitivity (MacDonald and Wojtowicz, 1982; Nowak et al., 1984), slow kinetics (Lester et al., 1990), and permeability to Ca^{2+} (Mayer et al., 1987), three features which make it unique. At normal resting membrane potentials the NMDA-sensitive conductance is prevented by a voltage-dependent block of Mg^{2+} at the NMDA receptor ion channel. The activation of NMDA receptors at hyperpolarized membrane potentials will cause the activation of only a small inward conductance. Depolarization sufficient to remove the Mg^{2+} from its ion channel binding site, however, increases the amplitude of the NMDA receptor-mediated inward cation current (Na^{2+} is the major inward conductance), which produces an accelerated increase in amplitude. This rapid depolarization initiates the entry of Ca^{2+} into the neuron through the NMDA channel. Calcium entry through the NMDA channel and voltage-sensitive Ca^{2+} channels activates a hyperpolarizing outward conductance via Ca^{2+}-dependent K^{+} channels. As the neuron hyperpolarizes, the Mg^{2+} block of the NMDA channel increases, decreasing the NMDA- dependent inward current, accelerating the neuron's hyperpolarization. With the neuron hyperpolarized, Ca^{2+} no longer enters and is sequestered into internal stores and/or extruded. This inactivates the Ca^{2+}-dependent K^{+} current and the neuron is once again under the influence of the small inward conductance of the NMDA channel, repeating the cycle. Thus, NMDA alone is capable of producing rhythmic oscillations in membrane voltage, or bistability, a feature which may give it an important role in a rhythmic behavior such as locomotion.

Recent work (Hochman, et al 1994a,b) identified a population of interneurons in lamina X which displayed non-linear membrane properties in the presence of NMDA (reflective of the non-linear property of the NMDA receptor ionophore) as well as similar non-linear membrane properties in neonatal rat spinal motoneurons. Cells which demonstrate this endogenous rhythmicity are excellent candidates for putative rhtyhm generating components of the locomotor CPG.

Bath application of NMDA initiates locomotion in a number of preparations. Dale and Roberts (1985) demonstrated that the voltage sensitivity of the receptor contribute to motor output in the *Xenopus* embryo. NMDA is also capable of inducing fictive locomotion in the lamprey (Grillner, 1981) and in the *in vitro* neonatal rat preparation (Smith and Feldman 1987; Kudo and Yamada, 1987). Intrathecal injection of NMDA over the lumbar segments of the cat spinal cord initiates locomotor activity in the presence and absence of afferent feedback (Douglas et al., 1993). Activation of kainate receptors gives rise to swimming in the lamprey (Brodin et al., 1985) which is less stable and of a higher frequency than that observed during NMDA-induced swimming.

The spinal cord of mammals contains descending monoaminergic pathways consisting of serotoninergic (5-HT), noradrenergic and dopaminergic projections (for review see Grillner 1975). Intravenous application of DOPA, a noradrenergic precursor, causes increased synthesis and release of noradrenaline which leads to an activation of noradrenergic alpha receptors (Aden, Jukes and Lundberg 1966). L-DOPA can induce locomotion in spinal cats (Jankowska et al 1967a) and in neonatal rats (Van Hartesveldt et al 1991) suggesting noradrenaline plays a role in the generation of locomotion. There is no evidence that dopamine plays a role in locomotion in the cat (Rossignol et al 1986), however, in the *in vitro* neonatal rat (Astuta et al 1991), dopamine has been shown to induce locomotion. In chronic spinal cats, 5-HTP administered alone increases the tonic level of excitation in motoneurons but does not induce locomotion. However, when 5-HTP is administered simultaneously with clonidine (a noradrenergic agonist), a robust

locomotor pattern is produced (Barbeau and Rossignol 1991). The bath application of 5-hydroxytryptamine/5-HT is also capable of producing a locomotor pattern as recorded from ventral roots (Cazalets et al 1990, Cazalets 1992) and peripheral nerves (Cowley and Schmidt 1994b) in the *in vitro* neonatal rat preparation. Cowley and Schmidt (1994b) demonstrated that serotonin produced rhythmic motor activity consistent with motor activity found in intact preparations with greater regularity than with the application of NMDLA. When both NMDA and 5-HT are applied simultaneously, they produce a locomotor pattern of greater stability than either drug alone (Squalli-Houssani et al 1993). A possible explanation of the increased stability of the locomotor pattern following application of 5-HT are plateau potentials. Intracellular recordings of turtle spinal cord interneurons demonstrated that following injection of a depolarizing current pulse, some interneurons remained in a prolonged depolarized state following termination of the depolarizing current (Hounsgaard and Kjærulff, 1992). This phenomenon was characterized by bistability, that is the prolonged depolarization which could be terminated with a hyperpolarizing current pulse (Hounsgaard and Kjærulff, 1992). Plateau potentials are mediated by nifedipine-sensitive Ca^{2+} entry through L-type channels (Hounsgaard and Kiehn, 1989). In turtle and cat motoneurons bistability is not an endogenous property, but is dependent upon the presence of monoamines (Hounsgaard et al., 1988; Conway et al., 1988). Thus the modulatory action of 5-HT, and possibly the other monoamines, on NMDA currents and bistability probably plays a considerable role in the initiation and the regulation of locomotion.

Electrical

A major breakthrough in the study of locomotion occurred with the characterization of the mesencephalic locomotor region in the cat (located at the level of the inferior colliculus near the caudal end of the nucleus cuneiformis) (Shik et al 1966). Other regions of the brainstem are also capable of generating locomotion (for reviews see

Shik et al 1976; Grillner 1975). This allowed locomotion to be induced reliably in the decerebrate cat without the use of pharmacological agents and the problems associated with their use in the *in vivo* preparation (discussed above). Increasing the strength of electrical stimulation of the MLR decreases the duration of the cycle, suggesting a simple command can induce the spinal cord to generate the complex muscle synergism for gaits of different frequencies. Studies in the paralyzed decerebrate cat have shown that continuous stimulation of the MLR at 5-30 Hz can produce locomotion for periods of minutes to hours. Skinner and colleagues (Skinner et al 1984; Astuta et al 1988; Iwahara et al 1991) have demonstrated that MLR stimulation, as well as stimulation of the medioventral medulla, at 3 Hz can induce locomotor-like activity in the neonatal rat brainstem-spinal cord preparation. The production of the locomotor pattern with the stimulation of the MLR is likely mediated in part by the descending monoaminergic systems. However the monoamines appear not to be necessary since depletion of spinal cord NA and 5-HT contents by injection of 6 hydroxy-dopamine (6-OHDA) and 5,6-dihydroxytryptamine (5,6-DHT) did not abolish MLR evoked locomotion (Steeves et al 1980), although this depletion was not complete.

The primary MLR pathway originates in the cuneiform nucleus in the rostral pons, synapses in the medial reticular formation (MRF) in the lower brainstem, and descends to the lumbar spinal cord in the ventrolateral funiculus. Experiments using focal, reversible cooling of descending pathways have shown that the VLF is the primary descending pathway for the initiation of locomotion (Steeves and Jordan 1980; Shefchyk et al 1984; Noga et al 1991).

The pharmacology of ventral root responses to VLF single shock stimulation in the neonatal rat has been thoroughly described by Wallis an Wu (1993) and by Elliot and Wallis (1993). They demonstrated that VLF stimulation produced both ipsi- and contralateral ventral root depolarization that involves both fast (100 ms, 50% decay time) and slow (12-14 s, 50% decay time) components. They suggested both components are

glutaminergic as they were blocked by the application of AP5 (selective NMDA antagonist) and CNQX (selective non-NMDA antagonist). Both components were modulated (particularly the slow responses) by endogenous 5-HT. Wallis, Wu and Wang (1993) showed that single stimuli delivered to the VLF depressed the ventral root response to dorsal root stimulation. This effect was eliminated with the 5-HT antagonist ketanserin. They concluded that stimulation of the VLF descending pathway inhibits the ventral root response to dorsal root stimulation through a release of 5-HT, suggesting 5-HT is one of the neurotransmitters released by the VLF.

Aims of the study

Since 1967 it has been established that a group of interneurons organized with inhibitory and excitatory connections most likely comprise the locomotor central pattern generator. In lower vertebrates, intracellular characterization of interneurons during swimming has lead to the identification of populations of interneurons with known connections and cellular properties. Very few interneurons in the mammal have been characterized to this extent. In the cat, RC, IaIN, and group II interneuron activity during locomotion has been characterized with extracellular recordings. These three populations of interneurons have been further characterized by the descending and afferent inputs they receive, their pharmacology, projections/target cells, morphology and location (see above; for review see Jankowska 1992). While IaIN and RC cells do contribute to the final motor output, they are not thought to contribute to the generation of the motor rhythm. A further understanding of rhythm generation in terms of connections and intrinsic cellular properties requires intracellular recordings from functionally identified interneurons and the use of morphological and electrophysiological techniques. Labeling and

electrophysiological studies indicate that neurons in lamina VII are involved in the production of locomotion. Since a population of lamina X neurons in the neonatal rat also possess intrinsic oscillatory properties (Hochman et al 1994a), it is possible that membrane oscillatory properties are recruited during locomotion. Thus, intracellular recording techniques are required to determine the contribution of intrinsic and synaptic cellular events to rhythmic activity. As a result, intracellular recordings of lumbar spinal neurons were performed during drug-induced locomotor-like activity.

The present study determined if neurons in lamina VII of the neonatal rat spinal cord are rhythmically active during locomotor-like activity. Using intracellular recording methods the neurons were characterized by their morphology and location as well as their segmental and descending synaptic inputs. A hybrid approach was used to investigate hindlimb locomotor activity in the *in vitro* neonatal rat spinal cord in a preparation in which the lumbosacral portion of the cord is hemisected but the entire thoracic spinal cord remains intact. The exposed medial lumbar gray matter is then accessible to microelectrodes while drug-induced locomotor activity is retained (Cowley and Schmidt 1993; Kudo and Yamada 1987).

The present results show that large neurons in lamina VII undergo rhythmic membrane voltage oscillations during drug-induced locomotor-like activity. The response to stimulation of segmental afferents and of the rostral ipsilateral VLF was depolarization. This work represents a significant step towards the characterization of the CPG as so few interneurons in the mammalian spinal cord have been characterized or identified sufficiently for proper examination of the theories of mammalian locomotor pattern generation.

Methods

Sprague-Dawley rats (aged 1-5 days) were decapitated and eviscerated, then quickly chilled to 4° C in oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) which contained in mM: NaCl, 125; KCl, 5; $NaHCO_3$, 26; NaH_2PO_4, 1; D-Glucose, 10; $MgSO_4$, 2; and $CaCl_2$, 2. The spinal cord was exposed by ventral laminectomy. Following warming to room temperature, superfusion with N-methyl-D-aspartate (NMDA; 2.5 -7.5 μM) (Sigma) and 5-HT (20 - 75 μM) (Sigma) induced air-stepping in approximately 50% of the preparations. The spinal cords of these locomoting preparations were transected at a mid-cervical level and isolated with dorsal and ventral roots attached. The lumbo-sacral portion of the spinal cord was midsagittally-sectioned, the right hemicord discarded, and the preparation placed medial side up in a recording chamber which was continuously gravity perfused with oxygenated ACSF. The recording chamber was clear silicone elastomer (Sylgard) bottomed, 3.5cm x 1.5cm with a separate reservoir for vacuum removal of the perfusing solution. The preparation was heavily pinned with 0.1 mm insect pins along the white matter of the hemisected lumbar spinal cord as well as at the most rostral and caudal points of the spinal cord.

Suction electrodes, glass capillaries with a filament, outside diameter 1.2 mm and inside diameter 0.9 mm, were pulled on a Flaming/Brown micropipette puller (Sutter Instrument Co.). The electrode tip was broken on a sharpening stone, rinsed of any debris, polished over a Bunsen burner, then an alternation between fire polishing and filing of the tip ensued until an inside tip diameter of 150-260 μm was achieved. The suction electrodes were placed on ventral roots L2 or L3, and L5 for recording. Population synaptic responses were filtered at 0.1 Hz and continuous ventral root recordings were filtered at 10 Hz in order to observe DC shifts in the ventral roots. Filtering and preamplification were performed by Cyberamp 380 and AI 401 headstages (Axon

Instruments). Rhythmic activity in the ventral roots was elicited by superfusion with NMDA and 5-HT at the same concentrations which induced air-stepping. Rhythmic activity was considered locomotor-like if the activity recorded on two ipsilateral ventral roots were approximately 180° out-of-phase.

Intracellular recordings were made using sharp electrodes, borosilicate glass capillaries, outside diameter 1.2 mm and inside diameter 0.68 mm, pulled on a Flaming/Brown micropipette puller (Sutter Instrument Co.) (input resistance 120 - 160 M Ω) filled with 2 - 3M potassium acetate (pH 10) and 1% biocytin (Sigma) in spinal segments L2 to L5. Recordings were performed using the Axoclamp 2A amplifier (Axon Instruments). Neurons were filled with biocytin by injecting hyperpolarizing current pulses (maximum 0.5 nA, 50% duty cycle) for approximately 10 minutes.

Prior to the application of NMDA/5-HT, trains of stimuli were delivered to the VLF in the cervical region of the isolated spinal cord (30-100 μA, 0.3 ms, 25-50 Hz, for 0.5-2.0 s). The cervical enlargement of the spinal cord was rolled to one side exposing the lateral surface. A suction electrode (150-200 μm) was placed 0.5mm dorsolateral to the ventral root exit zone. If a 1 s train did not elicit tonic activity lasting a minimum of 5 s as recorded in the ventral roots, the electrode was moved less than one tip diameter dorsal or ventral and the stimulus train was delivered again. A suction electrode was placed on dorsal root L3, L4 or L5 in order to examine afferent inputs onto the intracellularly recorded neurons. Stimulus intensities in both cases were determined as multiples of the ventral root threshold.

After the recordings were completed the tissue was fixed in 4% paraformaldehyde, frozen, then sectioned at 40 μm and placed in phosphate buffered saline (PBS) for 24 hours. The tissue was processed (60 revolutions per min for 3 hours) with streptavidin-FITC (Amersham) and PBS-Triton X-100 at a ratio of 1:100 to identify labeled neurons. The tissue was further processed (60 revolutions per min for 48 hours) with a primary monoclonal antibody to rabbit Choline acetyltransferase (1:1000) (ChAT) (Chemicon) and

PBS-Triton X-100. The primary antibody was then labeled with secondary antibody, sheep anti-rabbit Cy3 (1:100) (Sigma)(60 revolutions per min for 1.5 hours). Once mounted on glass slides the tissue was cover slipped with anti-fade medium (glycerol, 1M Tris buffer solution and paraphenylenediamine).

ChAT staining was used to insure that biocytin-filled neurons were not motoneurons, partition cells, preganglionic sympathetic or parasympathetic neurons (Phelps et al., 1984). Biocytin-FITC labeled neurons were photographed using a microscope equipped with epifluoresence, the images were digitized and reconstructed using a photoCD (Kodak) and a Macintosh computer to determine soma location. In 5 preparations, the slices containing the labeled neurons were also stained with cresyl violet allowing soma diameters (mean of long and short axes) of surrounding neurons to be compared to those of the recorded (labeled) neurons. Electrophysiological analyses were performed on a Macintosh computer using Axograph 2 while waveforms were analyzed with special purpose software on a Masscomp 5400 computer.

Results

Development of methods

In order to examine whether lamina VII interneurons were active during locomotor activity in the neonatal rat, a preparation was required in which intracellular recordings could be easily carried out and which was also capable of undergoing rhythmic locomotor activity. The initial preparation used Sprague-Dawley rats P10-14, which were anesthetized with Halothane (Ayerst), then decapitated and chilled in 4° C in oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) (see above). The spinal cord was exposed using a dorsal laminectomy. Following isolation of the spinal cord with dorsal and ventral roots intact, the length of the cord was completely hemisected using a guillotine. The duration of this dissection was approximately 20 minutes. Older animals were used in order to examine interneuron activity in the most developmentally mature preparation possible. However, the isolated spinal cords of older animals failed to exhibit synaptic or rhythmic activity. This was most likely the result of the animal not being chilled quickly enough due to its large mass, insufficient oxygenation of the spinal cord due to the diffusional barrier of myelin present at this age, and/or the long duration of the dissection in these older animals.

In order to increase the survival rate of the isolated spinal cord, younger animals were tried. Eventually neonatal rats P1-5 were used, as was the case in most other neonatal rat preparations that were used to examine locomotion (e.g. Cowley and Schmidt, 1994a). Concurrently, a ventral approach for the laminectomy, following evisceration was used. These two changes dramatically increased the health of the preparations, and they now routinely exhibited ventral root activity following stimulation of the dorsal roots. However, the completely hemisected spinal cord did not become rhythmic following application of NMDA and 5-HT. A lesioning study by Cowley and

Schmidt revealed that midsagittal sectioning of the lumbar spinal cord while the thoracic cord remained intact was still capable of producing locomotion (Cowley and Schmidt, 1993). Based on these observations, we altered our protocol and only the lumbar spinal cord was hemisected while the rostral cord remained intact. This ensured the production of locomotor-like activity, although this was never confirmed with the hindlimbs attached, and provided access to the medial grey matter for intracellular microelectrodes. The midsagittal sectioning was done with a 0.1 mm insect pin. However, the partially hemisected spinal cord did not always become rhythmically active following application of NMDA and 5-HT. It was determined that not all preparations prior to isolation of the spinal cord responded to NMDA and 5-HT. Prior to the isolation of the spinal cord, while the hindlimbs were still attached, NMDA and 5-HT were applied. The presence of evoked air-stepping was used as an indication that the preparation would likely be viable after further dissection, approximately 90% of the isolated spinal cords exhibited rhythmic activity using this selection criteria. Thus the presence of air-stepping functioned as a gauge of the quality of the dissection.

Figure 4 shows the rhythmic activity induced with NMDA (2.5 μM) and 5-HT (40 μM) as recorded in lumbar ventral root electroneurograms in the intact spinal cord (A), the lumbar midsagittally-sectioned spinal cord (B) and following removal of the right hemicord (C) note that the frequency decreases from 0.3 Hz to 0.15 Hz following the midsagittal sectioning. This frequency following midsagittal sectioning corresponds closely to the frequency (0.16 ± 0.04 Hz) reported by Kudo and Yamada (1987) which they observed following hemisection of the neonatal rat spinal cord. Recovery of the rhythm was time dependent (approx. 20 min) as it eventually increased to 0.2 Hz or 67% of the original frequency.

Rhythmically Activated Interneurons

Rhythmic activity

Intracellularly recorded cells (n = 13) displayed rhythmic oscillations in membrane voltage termed locomotor drive potentials (Jordan 1983) which were phase related to rhythmic ventral root activity (Fig 5A). Eighty-one percent of cells with a stable holding potential accompanied by robust rhythmic activity in the ventral roots exhibited rhythmic activity. The averaged filtered drive potential for a given cell ranged in amplitude from 0.6 to 4.6 mV (mean = 1.9 mV). The frequency of the LDPs ranged from 0.13 to 0.43 Hz (mean = 0.23 Hz). When averaged and the cycle/amplitude normalized, the drive potentials had a similar shape in all neurons sampled (Fig 5B). A raw record of a neuron firing action potentials at two different membrane potentials is illustrated in Fig 6A(i). Four cells fired action potentials during the depolarized phase of the drive potential. Figure 6A(ii) shows the relationship between the instantaneous firing frequency and step cycle in one cell. In all 6 cells the instantaneous spike frequency was modulated in phase with the cycle. Synaptic activity appeared to be superimposed on the drive potential in each cell. This postsynaptic activity appeared to recruit action potentials (Fig 6B(i)). A raster plot (Fig 6B(ii)) of sequential spike-triggered sweeps of activity in Fig 6B(i) more clearly illustrates the apparent recruitment of action potentials by synaptic events. Bath application of TTX (0.5 - 1μM) resulted in a cessation of activity including drive potentials in both the ventral roots and two interneurons tested. Furthermore, with one of these interneurons the concentration of NMDA was increased from 20 to 100 μM and did not produce any rhythmic behavior in the presence of TTX.

Passive Membrane Properties

Cells in which passive membrane electrical properties were examined before the bath application of NMDA and 5-HT (n = 8) displayed an average input resistance of 101

± 27 (S.D.) MΩ and a membrane time constant of 9.0 ± 4.4 ms, as measured with 120 ms hyperpolarizing current steps (1.5 nA, stepping up 0.5 nA). The average resting membrane potential of the cells was -69 ± 7 mV and the threshold for spiking was -53 ± 4 mV. Application of NMDA and 5-HT resulted in a mean membrane depolarization of 6 ± 4 mV.

Morpholgy

Labeled rhythmically active cells (n = 7) were located ventral and lateral to the central canal in lamina VII (Fig. 7A) from lumbar segments L2-L5 (note: unlabeled neurons whose location was determined by electrode placement are also included in this figure as are the location of 3 non-rhythmic labeled cells). The general morphology of 3 representative rhythmically active neurons is presented in Figure 7B. Neuronal processes of the labeled neurons were predominantly dorsoventrally oriented and five neurons had processes extending into motor nuclei. The bar graph presented in Figure 7C illustrates a comparison of the cell diameters of 5 sampled neurons in 5 preparations to those of nearby neurons, not recorded from. The graph demonstrates that the sample was biased toward larger neurons (Fig. 7D). The mean diameter of the cell soma ranged from 14 to 19 μm, (mean 16 μm) corresponding to the upper 15% of the cells measured (Fig 7C). Two neurons located immediately dorsal to, and one ventral to, the central canal did not display drive potentials during rhythmic ventral root activity. These cells were smaller, on average (mean soma diameter = 12 μm) than the rhythmically active lamina VII neurons.

Segmental input

Prior to the application of NMDA and 5-HT, stimulation of the dorsal root afferents at 2T and 5T evoked membrane depolarization in all eight interneurons examined. At 2T, the depolarization ranged from 3.2 mV to 20.5 mV (mean = 8.1 mV)

and at 5T the depolarization ranged from 5.3 mV to 22.8 mV (mean = 13.4 mV). Depolarization was sufficient in five neurons to elicit an action potential. Activity in the ventral roots was elicited at the same, or slightly larger, stimulation strengths as the interneurons (10-30 μA, 300 μs), and was used as an indicator of the monosynaptic reflex evoked by stimulation of dorsal root afferents. The mean latency of the initial activity in the ventral roots following stimulation of the dorsal root, as measured from the stimulus artifact (cord dorsum unavailable) was 4.33 ms. All of the neurons in lamina VII received synaptic input following the initial activity in the ventral roots. The mean latency from the initial activity in the ventral roots at 2T was 7.6 ms (2.0 ms to 11.6 ms) and at 5T was 6.3 ms (4.2 ms to 11.7 ms)(Fig 8). Note that depolarization in the interneuron following stimulation of the dorsal root outlasts the activity in the ventral root (Fig 8).

Descending Input of the Ventrolateral Funiculus

Ventral root response to single stimuli

Ventral root responses to single VLF stimuli consisted of a fast component and a slow component similar to that described by Wallis and Wu (1993) (e.g. Fig 9A). The fast component following single VLF stimuli were larger in the L3 ventral root than those in more caudal roots (Fig 9A). Mean amplitudes of the integrated ventral root responses for VLF stimulation at 5T was 0.66 ± 0.49 mV (n=12) for L3 and 0.28 ± 0.16 mV (n=15) L4/5 ventral roots. Latencies however were not significantly different for L3 and L4/5 with 5T stimulation (21.9 ± 9.26 ms and 21.4 ± 7.8 ms respectively). Increasing the stimulus strength from 2T to 5T increased the peak response amplitude of L3 ventral roots from 0.32 ± 0.21 mV (n=14) to 0.66 ± 0.49 mV (n=12) without affecting the response latency (28.2 ± 11.5 ms at 2T and 21.9 ± 9.3 ms at 5T). The slow component of the response was not always present although when present, it could reach 20-30% of the fast component amplitude. This variability was probably due to slight differences in recording

electrode placement as long duration activity could be elicited even when the responses to single shocks had no slow component. The proximity of the recording electrode to the ventral root exit zone was critical to prevent degradation of the signal since the slow component was of such smaller amplitude than the fast and could be easily lost in this manner. Ventral root responses to single VLF stimuli were potentiated and long-lasting following a 10s, 50 Hz conditioning train of VLF stimuli (Fig 9B) at 1 min (Fig 9C) and at 3 min (Fig 9D). Amplitude of ventral root responses were acquired prior to (0.24 mV) and 1 min (1.06 mV) and 3 min (0.45 mV) following the cessation of the conditioning stimuli.

Ventral root and motoneuron response to trains of VLF stimuli

Stimulation of the VLF using 0.3ms stimuli delivered at 5T and 1 Hz did not produce an increase in ventral root activity (Fig 10A), however the same stimuli delivered at 5 Hz were sufficient to cause a slight increase in ventral root activity (Fig 10B). Increasing the frequency of stimulation elicited proportionally larger (with a shorter rise time)(Fig 10 C and D) increases in ventral root activity which outlasted the stimulus by up to 30 s. A long lasting depolarization was observed in which intracellularly recorded motoneurons (n=7) and the proximal ventral roots continued to depolarize following termination of the stimulus train (Fig 10D). Intracellular records of motoneuronal responses to high frequency VLF stimulation is provided to better illustrate the continued depolarization following cessation of the stimulus. Motoneurons were identified by low input resistance (< 60 MΩ), their location (lamina IX/deep ventral horn), morphology (mean soma diameter > 22 μm) , ChAT staining (n=5) and the presence of an after-depolarization. Antidromic stimulation was not used to identify motoneurons because the recording electrodes used on the ventral roots could not be used for stimulation.

VLF input onto interneurons

Stimulation of the VLF at 2T and 5T prior to the application of NMDA and 5-HT evoked a membrane depolarization in the seven interneurons examined, two of which were rhythmically activated after drug administration. At 2T the depolarization ranged from 3.5 mV to 4.1 mV (mean = 3.8 mV) (n = 5) and at 5T the depolarization ranged from 1.7 mV to 3.4 mV (mean = 2.6 mV) (n = 2). All of the neurons received synaptic input following the initial activity in the ventral roots. The mean latency from the initial activity in the ventral roots at 2T was 26.5 ms (19.5 ms to 31.2 ms) and at 5T was 37.0 ms (36.0 ms to 37.8 ms) (Fig 11A). Application of stimulus trains produced a depolarization which outlasted the stimulus in the interneurons examined (n = 4) (Fig 11B).

Pharmacology of the ventrolateral funiculus evoked responses

The pharmacology of the ventrolateral funiculus evoked actions was examined in 9 separate preparations. Figure 12A demonstrates that kynurenate decreased fast component of the ventral root responses to single VLF stimuli (n=2). Figure 12B(ii) illustrates the reduction of the slow component of the single stimulus ventral root response after the administration of the NMDA receptor antagonist AP5 (20 μM) (n=4). In contrast, the non-NMDA receptor antagonist 10 μM CNQX (Fig 12B(iii)) reduced the fast component of the single stimulus ventral root response by more than 40% (n=3). The catecholamine antagonist propranolol (10 μM) (Fig 12B(iv)) had no effect on the fast component of the single stimulus ventral root response but did reduce the slow component (n=3). The monoaminergic antagonist yohimbine at 40 μM (Fig 12B(v)) had no effect on the single stimulus ventral root response (n=2).Washout controls were used between each one of the trials illustrated in Fig 12, but are not shown.

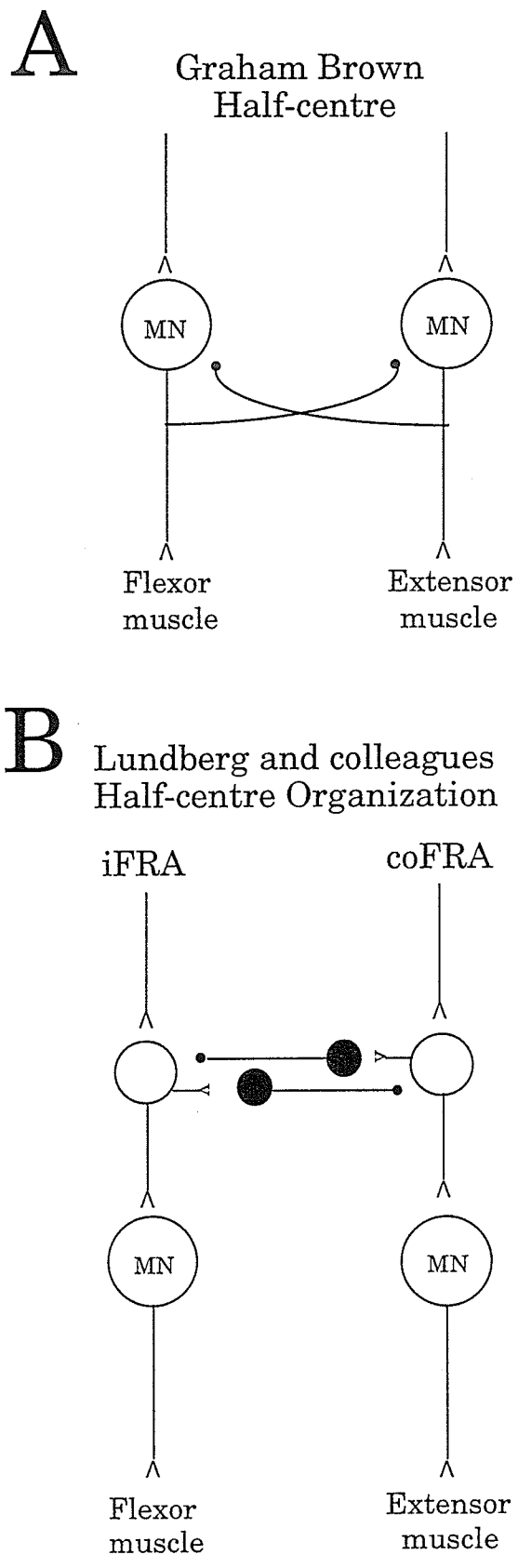
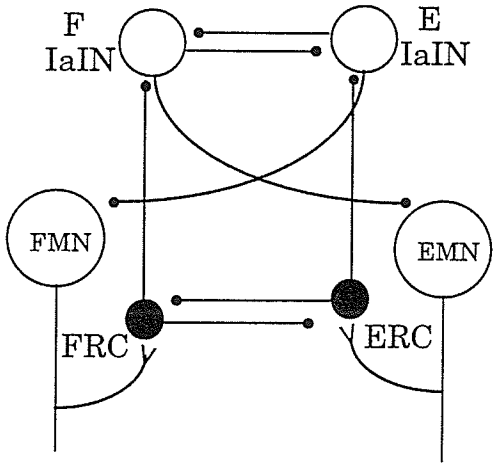

A
Graham Brown
Half-centre
MN
MN
Flexor
muscle
Extensor
muscle
B
Lundberg and colleagues
Half-centre Organization
iFRA
coFRA
MN
MN
Flexor
muscle
Extensor
muscle

Figure 1.

Figure 1

A. The half-center hypothesis as proposed by Graham Brown. (Adapted from Lundberg, 1981). Note the recurrent inhibitory axon collaterals. Solid circles represent inhibitory synapses and open triangles excitatory synapses. ***B.*** The reciprocal organization between flexors and extensors revealed with the administration of DOPA. (Adapted from Gossard and Hultborn, 1991). Solid circles represent inhibitory synapses open triangles excitatory synapses. A single interneuron diagrammed represents a chain of interneurons.

A


F
IaIN
E
IaIN
FMN
EMN
FRC
ERC

B

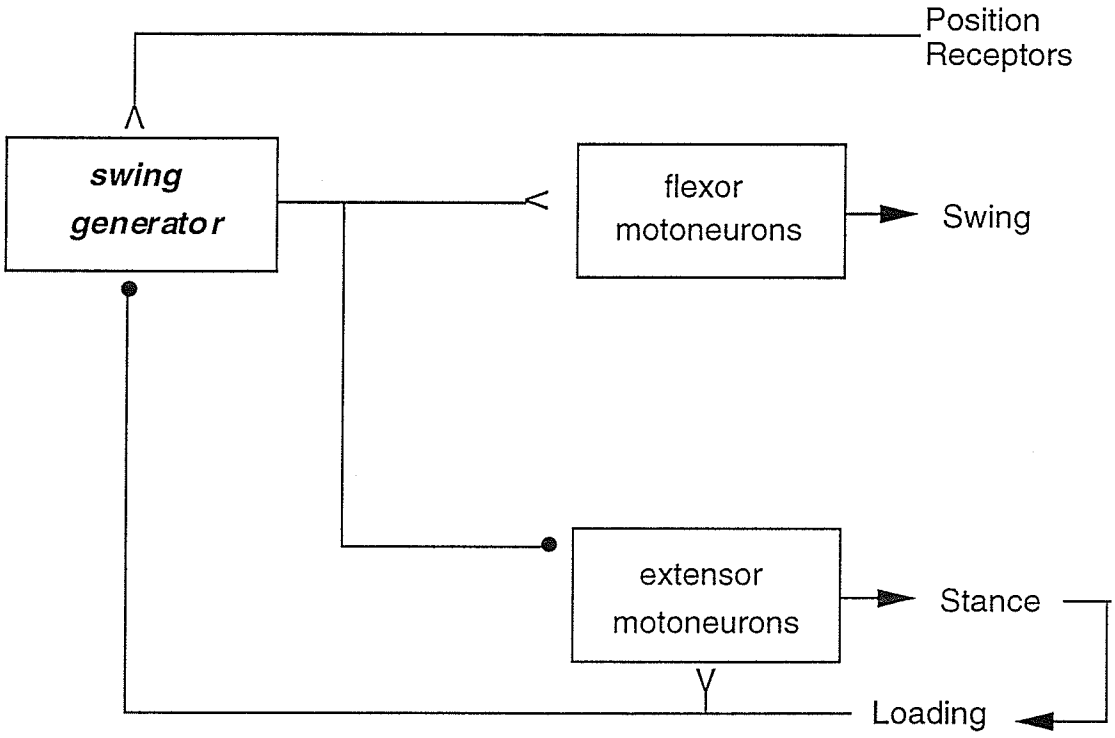
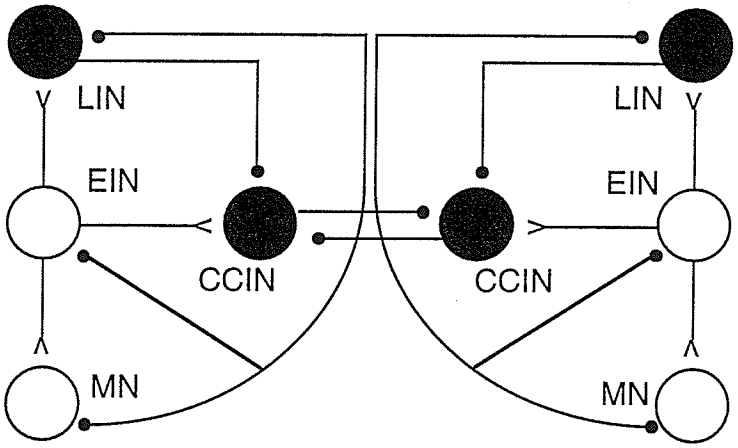

Position
Receptors
swing
generator
flexor
motoneurons
Swing
extensor
motoneurons
Stance
Loading

Figure 2.

Figure 2

A. The Miller and Scott model illustrating the electrical connections between the 'cells'. (Adapted from Miller and Scott, 1977). RC indicates a 'Renshaw cell' and IaIN indicates 'Ia inhibitory interneuron' and MN indicates 'motoneuron'. Solid circles represent inhibitory synapses and open triangles excitatory synapses ***B.*** The Pearson and Duysens model for the swing generator. (Adapted from Pearson and Duysens, 1976.) Solid circles represent inhibitory synapses and open triangles excitatory synapses

A



B

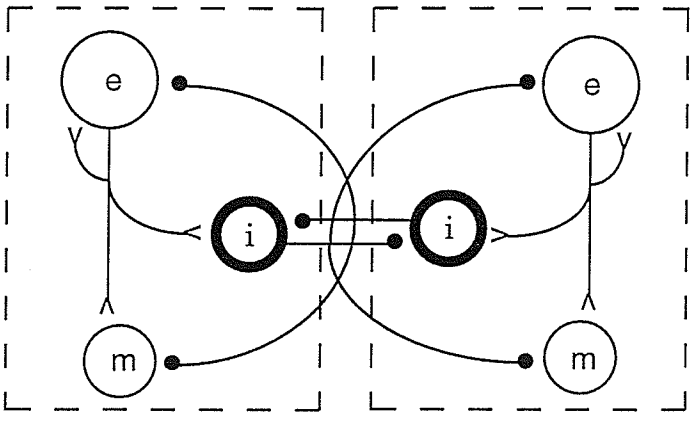


Figure 3.

Figure 3

A. A model depicting the proposed segmental circuitry in the lamprey. (Adapted from Grillner et al., 1991). LIN indicate lateral interneurons; EIN, excitatory interneurons; CCIN, contralaterally and caudally projecting interneurons and MN, motoneurons. Solid circles represent inhibitory synapses and open triangles excitatory synapses. ***B.*** The proposed segmental cellular organization for the Xenopus embryo. (Adapted from Roberts et al., 1986). "e" indicates descending excitatory interneurons, "i", commisural inhibitory interneurons and "m" motoneurons. Solid circles represent inhibitory synapses and open triangles excitatory synapses.

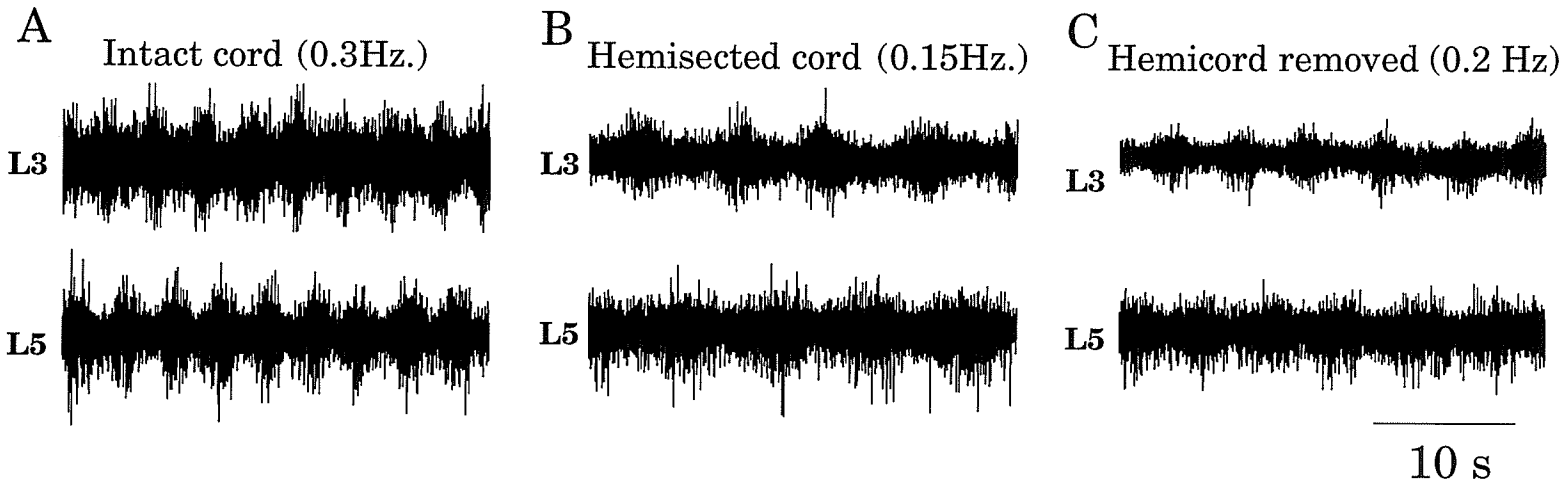

A
Intact cord (0.3Hz.)
L3
L5
B
Hemisected cord (0.15Hz.)
L3
L5
C
Hemicord removed (0.2 Hz)
L3
L5
10 s

Figure 4.

Figure 4

Rhythmic activity induced with 2.5 μM NMDA and 40 μM 5-HT recorded from lumbar ventral root electroneurograms (L3 and L5) is conserved from the intact spinal cord (*A*), the lumbar hemisected spinal cord (*B*) and following removal of the right hemicord (*C*). The frequency decreased from 0.3 Hz to 0.15 Hz following the midsaggital sectioning of the lumbar spinal cord. Following recovery for 30 min the frequency of activity increased to 0.2 Hz.

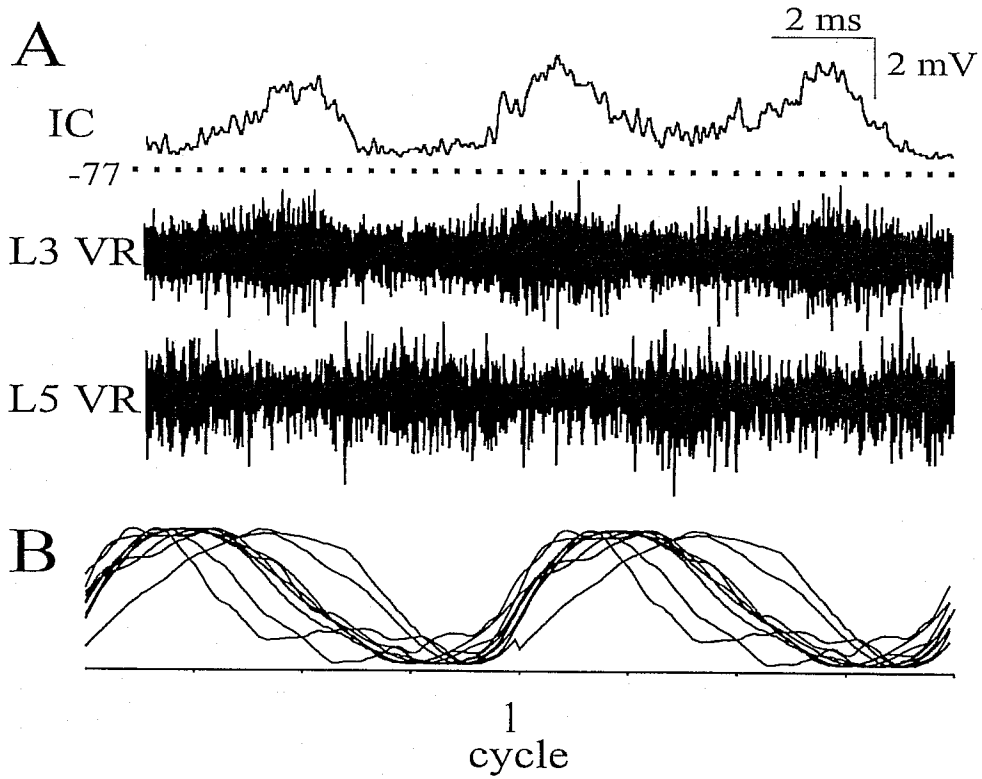
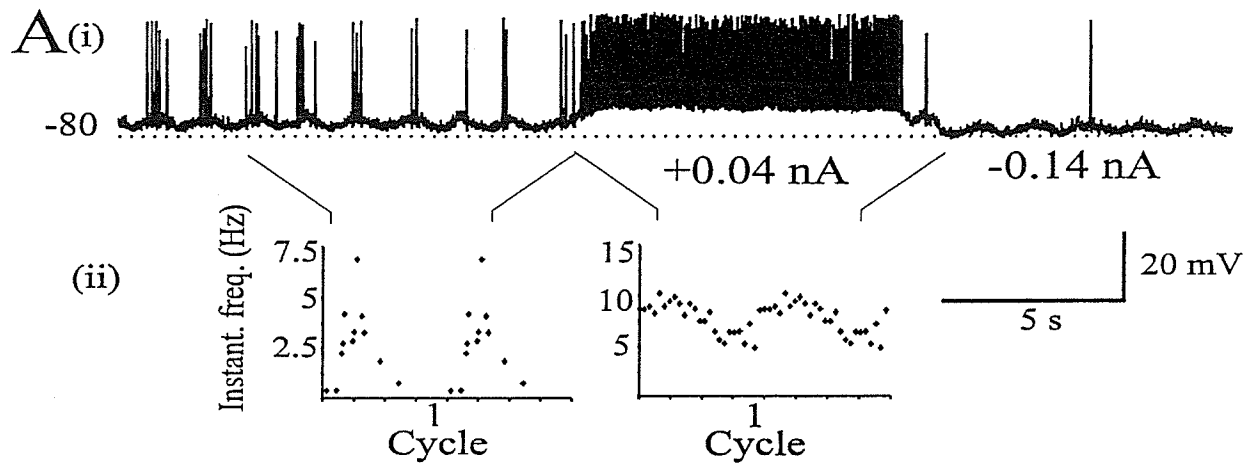


Figure 5.

Figure 5

A. An intracellular (IC) record, from an interneuron low-pass filtered at 5 Hz, coupled with two extracellular ventral root recordings (L3 and L5). ***B.*** Intracellular waveforms are averaged and the cycle duration's and amplitudes normalized to illustrate that drive potentials are similar in shape in all neurons sampled. Waveforms are plotted twice to aid in visualizing the cyclic nature.


A(i)
-80
+0.04 nA
-0.14 nA
(ii)
Instant. freq. (Hz)
7.5
5
2.5
15
10
5
1
Cycle
1
Cycle
20 mV
5 s

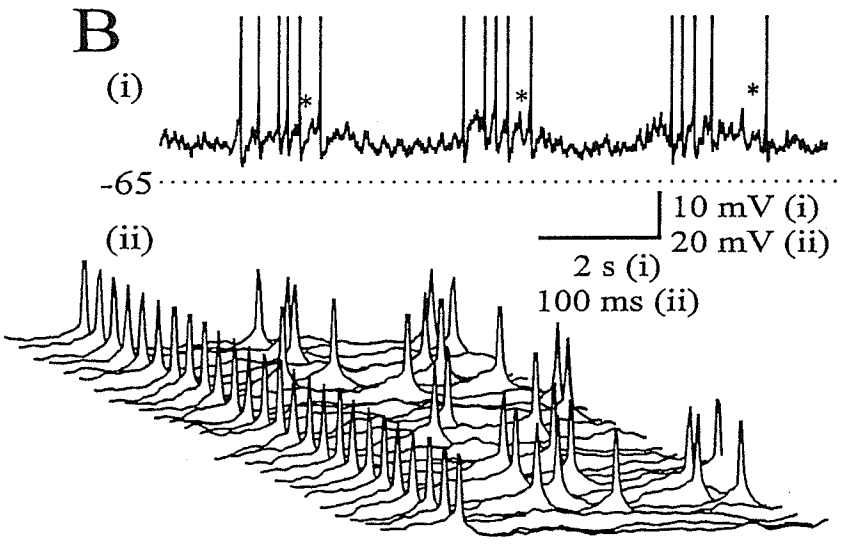

B
(i)
-65
(ii)
10 mV (i)
20 mV (ii)
2 s (i)
100 ms (ii)

Figure 6.

Figure 6

A(i). The raw record of rhythmic activity in an interneuron during rhythmic activity in the ventral roots. ***A(ii).*** Action potential instantaneous frequency (ordinate) is plotted against normalized cycle length (abscissa) to demonstrate cyclic modulation of firing frequency at 2 different potentials. ***B(i).*** A second neuron which fired action potentials on the depolarized phase on the drive potential. Asterisks (*) identify synaptic potentials. ***B(ii).*** Raster plot of sequential spike-triggered sweeps of activity in B(i) demonstrating the apparent recruitment of action potentials by depolarizing synaptic events. Spikes are truncated in (i) and (ii) for illustrative purposes.

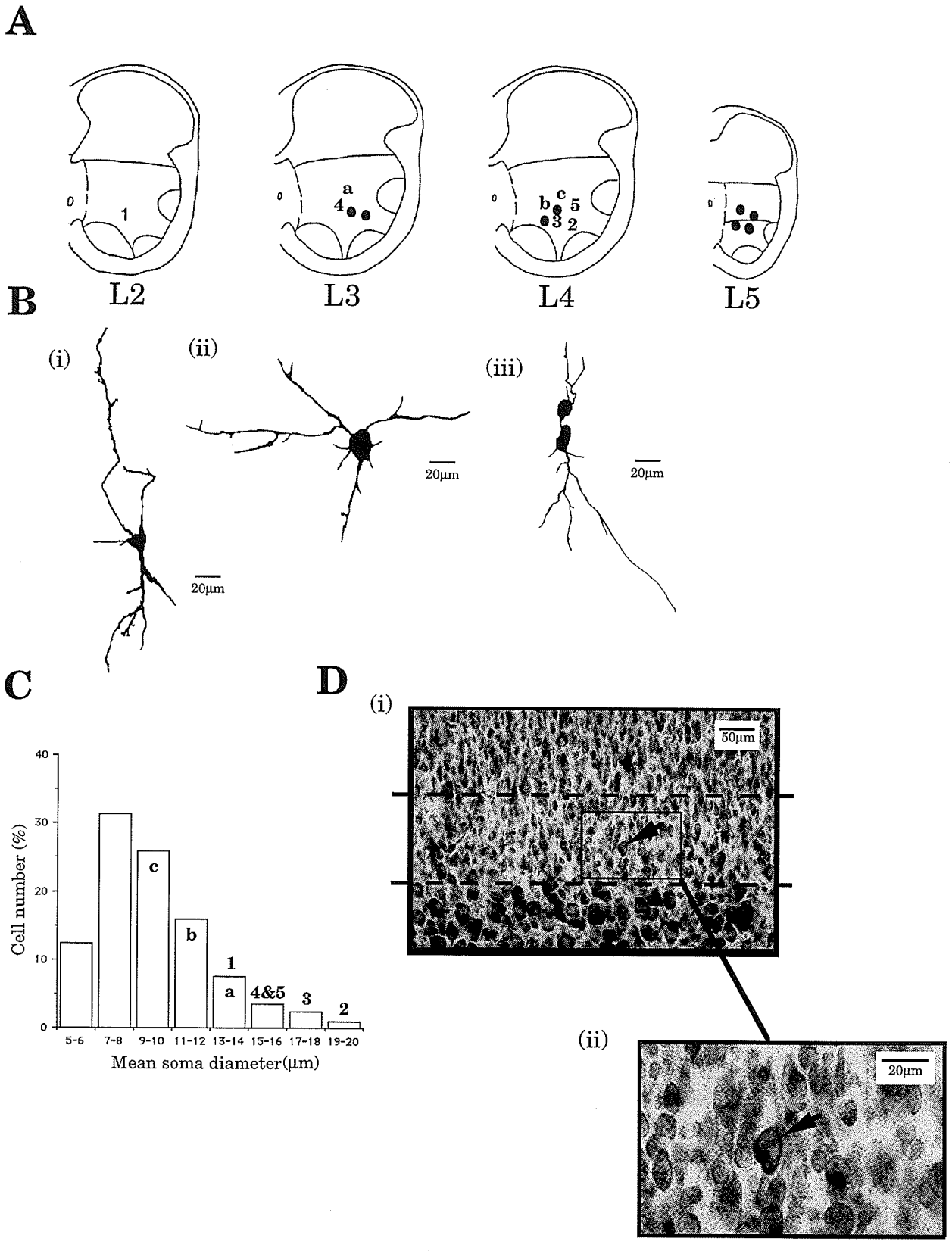

A
1
a
4
b
c
5
3
2
L2
L3
L4
L5
B
(i)
(ii)
(iii)
20μm
20μm
20μm
C
Cell number (%)
40
30
20
10
0
c
b
1
a
4&5
3
2
5-6
7-8
9-10
11-12
13-14
15-16
17-18
19-20
Mean soma diameter(μm)
D
(i)
50μm
(ii)
20μm

Figure 7.

Figure 7

Morphology and location of 16 recorded neurons. ***A.*** Approximate location of intracellularly recorded neurons. (spinal cord outlines adapted from Kjærulff and Kiehn, 1994). Numbers (1-5) denote biocytin-filled cells which were later stained with cresyl violet. Dots (●) denote labeled cells or those estimated by electrode placement and depth. Lowercase letters (a-c) denote labeled cells which were not modulated during locomotion. ***B.*** Three examples of reconstructed neurons (cells 1, 2 and 4 respectively in A) which displayed rhythmic activity. ***C.*** Distribution of cell soma diameters surrounding and including labeled cells in 5 preparations. Numbers and letters correspond to those in (A) and indicate the diameters of labeled interneurons. ***D.*** Example of Nissl-stained section illustrating the area of cells used for size measurements in (C). The dashed lines indicate the dorsoventral borders of the region from which soma diameters were measured. The arrow indicates the intracellularly recorded (labeled) interneuron which corresponds to neuron 2 in (A), (C) and reconstructed cell B(ii).

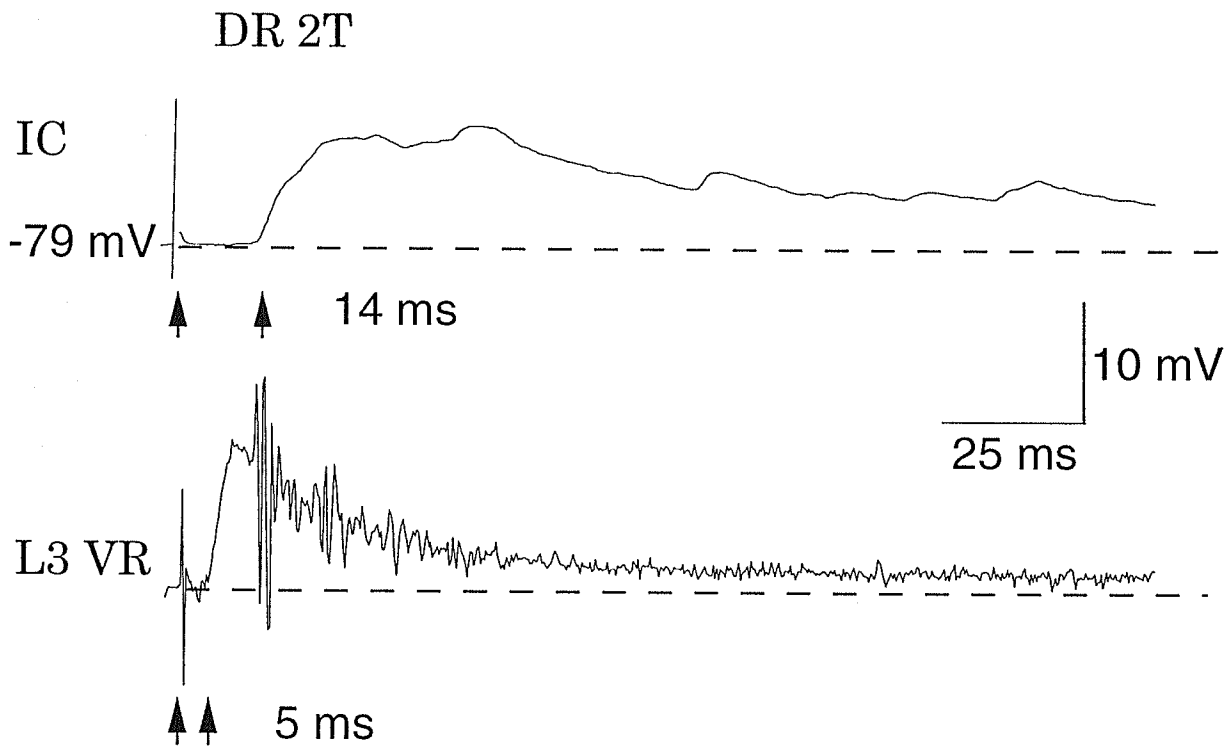


Figure 8.

Figure 8

Response in the L3 ventral root and interneuron evoked by stimulation of the L3 dorsal root. Dorsal root stimulation produces a depolarization in the ventral root at a latency of 5 ms. The depolarization recorded in the interneuron occurred at 14 ms.

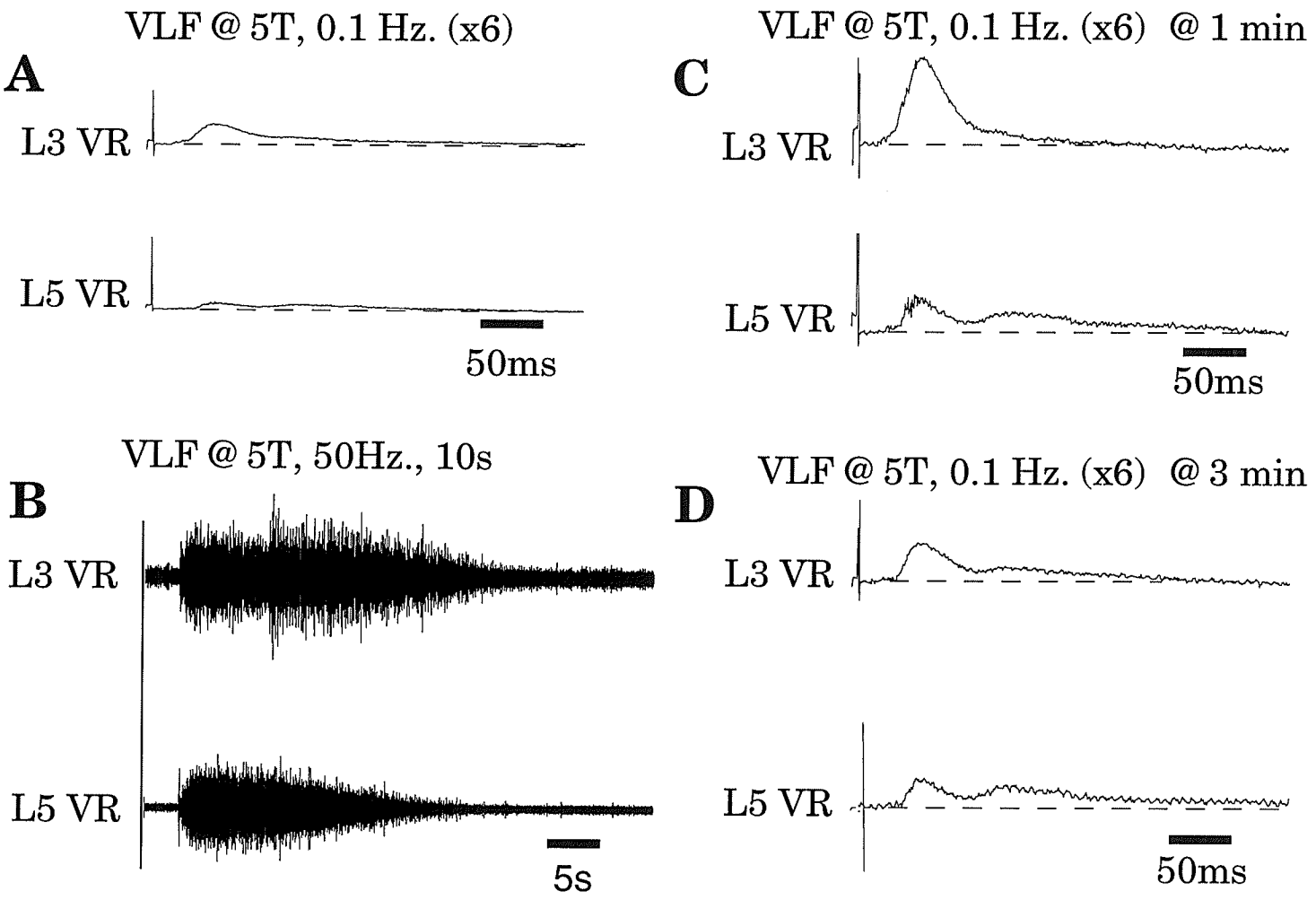

A
VLF @ 5T, 0.1 Hz. (x6)
L3 VR
L5 VR
50ms
B
VLF @ 5T, 50Hz., 10s
L3 VR
L5 VR
5s
C
VLF @ 5T, 0.1 Hz. (x6) @ 1 min
L3 VR
L5 VR
50ms
D
VLF @ 5T, 0.1 Hz. (x6) @ 3 min
L3 VR
L5 VR
50ms

Figure 9.

Figure 9

L3 and L5 ventral root responses to single VLF stimulus are potentiated. Ventral root responses to single VLF stimulus ***A***, prior to a 10 s conditioning stimulus train delivered at 50 Hz ***B***. Ventral root responses to single VLF stimulus at 1 min ***C*** and 3 min ***D*** following high frequency stimulation of the VLF

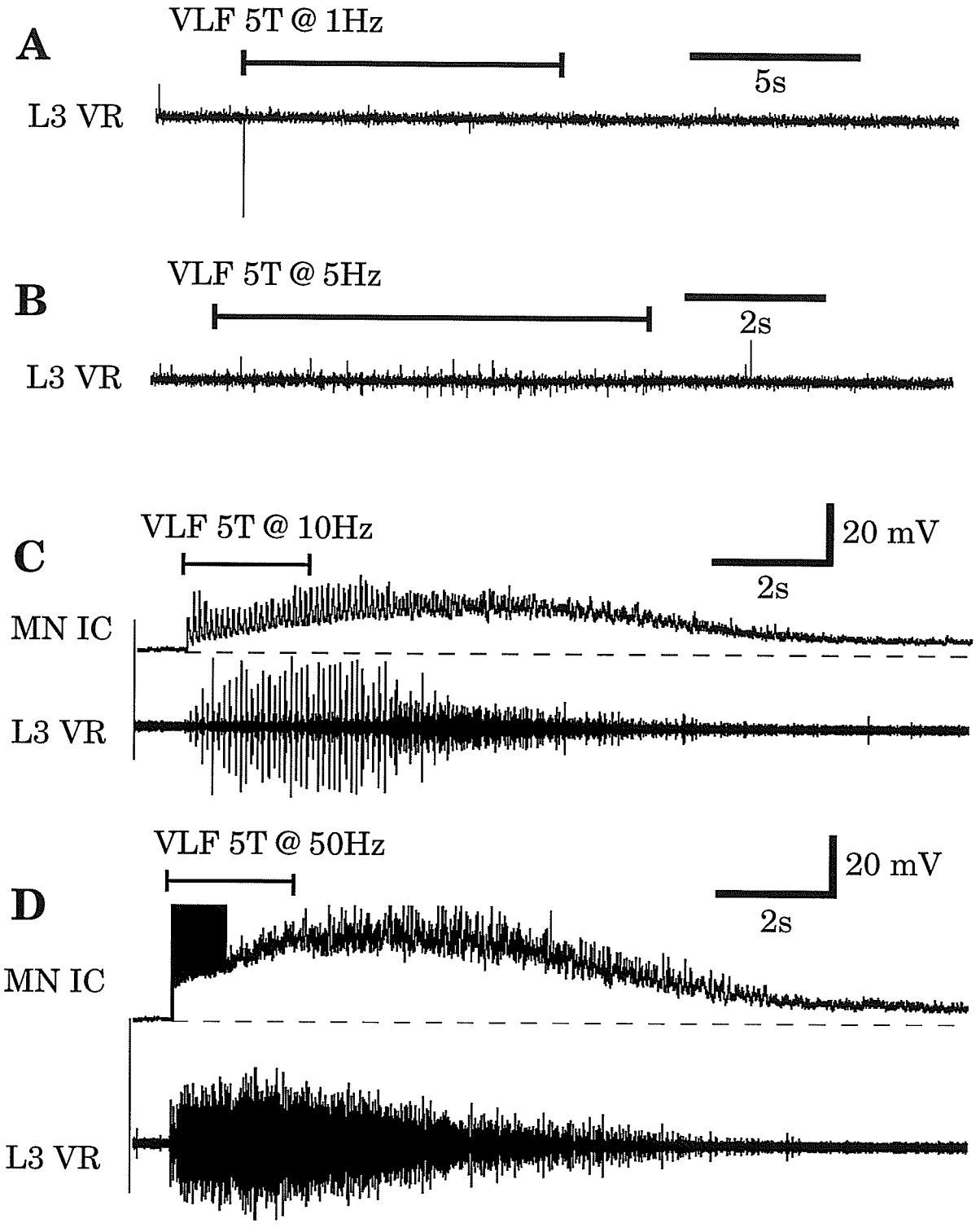

A
VLF 5T @ 1Hz
5s
L3 VR
B
VLF 5T @ 5Hz
2s
L3 VR
C
VLF 5T @ 10Hz
20 mV
2s
MN IC
L3 VR
D
VLF 5T @ 50Hz
20 mV
2s
MN IC
L3 VR

Figure 10.

Figure 10

Thoracic VLF stimulation delivered at 1 ***A***, 5 ***B***, 10 ***C*** and 50 ***D***, Hz evokes depolarization of L3 ventral root and motor neuron ***C*** and ***D*** that persists beyond the stimulus at 10 Hz and above.

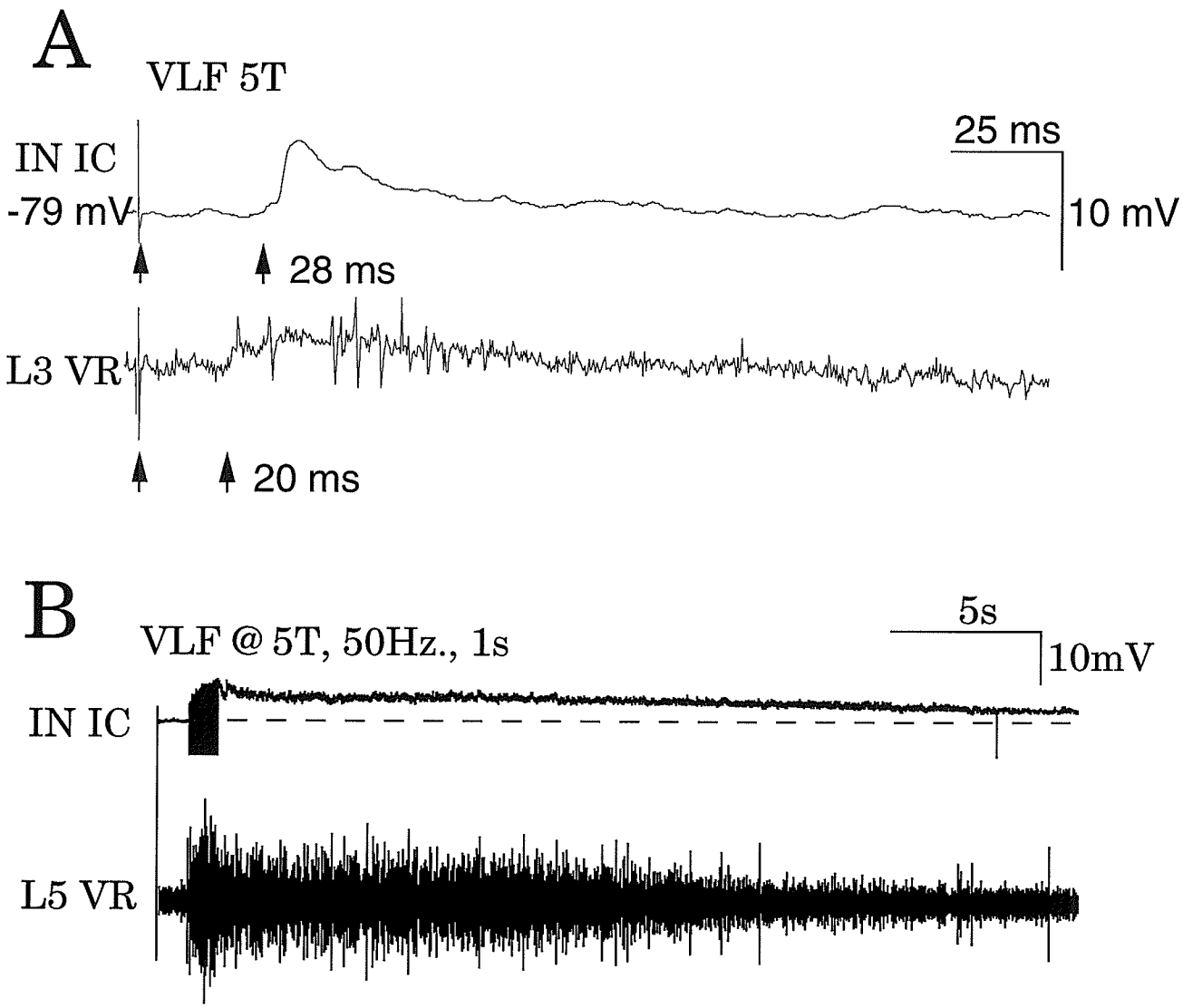

A
VLF 5T
IN IC
-79 mV
25 ms
10 mV
28 ms
L3 VR
20 ms
B
VLF @ 5T, 50Hz., 1s
5s
10mV
IN IC
L5 VR

Figure 11.

Figure 11

A. Response in the L3 ventral root and interneuron following stimulation of the thoracic VLF. VLF stimulation produces a depolarization in the ventral root at 20 ms and in the interneuron at 28 ms.

B. Stimulation of the VLF at 50 Hz produces a prolonged depolarization in both the L5 ventral root and the interneuron.

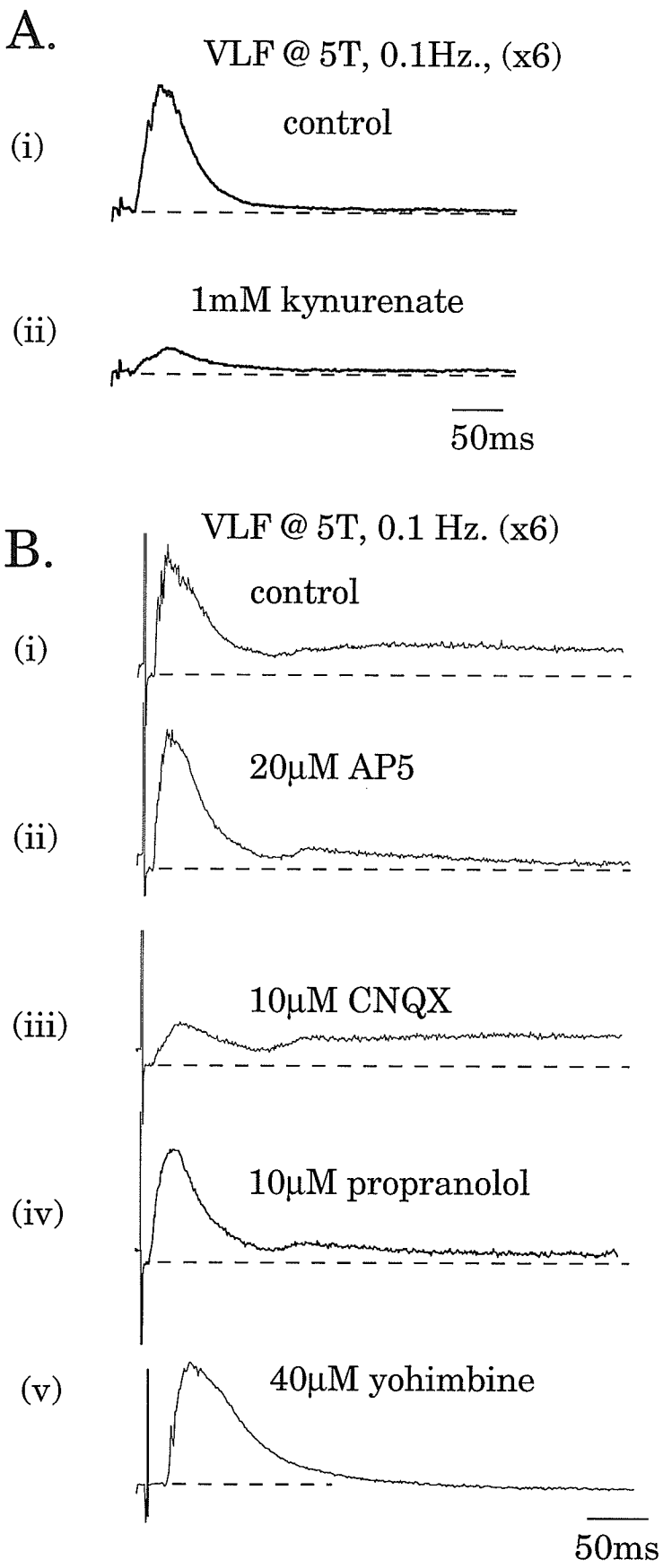

A.
VLF @ 5T, 0.1Hz., (x6)
(i)
control
(ii)
1mM kynurenate
50ms
B.
VLF @ 5T, 0.1 Hz. (x6)
(i)
control
(ii)
20μM AP5
(iii)
10μM CNQX
(iv)
10μM propranolol
(v)
40μM yohimbine
50ms

Figure 12.

Figure 12

L3 ventral root responses to single stimuli delivered to the thoracic VLF (5T, 0.1 Hz) are illustrated in A and B.

A. Application of kynurenate (1 mM) results in a considerable decrease of the response of L3 ventral root to single VLF stimuli.

B. AP5 (20 μM) ***(ii)*** and propranolol (10 μM) ***(iv)*** reduce the slow component's duration to single VLF stimuli. CNQX (10 μM) ***(iii)*** reduces the fast component of the response to single VLF stimuli. Bath application of yohimbine in a separate cell from the one illustrated above, at 40 μM ***(v)*** has little effect on the response to single VLF stimuli. Note between each application of an antagonist a control washout was used to insure a return to baseline activity, these are not illustrated.

Discussion

We have observed that interneurons in ventrolateral lamina VII undergo rhythmic membrane voltage oscillations during drug-induced locomotor-like activity. The cells described in the present work may overlap with the population of relatively large neurons activity-dependent labeled with sulforhodamine during locomotion in the intermediate gray of the neonatal rat lumbar spinal cord described by Kjærulff and Kiehn (1994). The rhythmic activity or drive potentials described in the present study appear to be generated from similar synaptic input (similarity of shape of LDP between all cells) which recruited action potentials in only 30% of the neurons. In the neurons examined (n=2), it appears that the NMDA receptor plays a negligible role in the generation of the locomotor drive potentials. The bath application of TTX eliminated the LDP in the intracellularly recorded neurons and all activity in the ventral roots. An increase in the concentration of NMDA from 20 μM to 100 μM in the one neuron examined produced a slight depolarization and some disorganized activity in the cell but failed to produce any rhythmic oscillations such as those reported by Hochman and colleagues (see Hochman et al., 1994a,b). In this case, it is not clear whether or not the LDP, prior to the application of TTX, was driven by inhibitory synaptic inputs, but it does appear that the LDP was not NMDA receptor mediated. Data from both cells suggest that this population of interneurons do not express an endogenous bursting property in the presence of NMDA. This observation indicates that possibly an endogenous bursting property in the presence of NMDA is not a criteria which must be met by a putative component of the locomotor rhythm generator, perhaps Ca^{2+}-oscillations similar to those which produce plateau potentials (e.g. Hounsgaard and Kjærulff, 1992) is the only criteria which must be met. Extreme caution is required when drawing any conclusion about a population of interneurons based on an analysis of two cells.

The low number of neurons which exhibited action potentials may in part be due to partial hemisection which decreases locomotor frequency (Kudo and Yamada, 1987). Sillar and Roberts (1993) observed that as the frequency of swimming decreased in *Xenopus* embryos, the likelihood of an interneuron spiking decreased although the cell continued to receive synaptic input. Midsagittal sectioning of the cord decreased the frequency of activity. Hemisection of the spinal cord may have also affected the synaptic inputs the cells received, Kudo and Yamada (1987) observed that the asymmetry between flexor and extensor activity duration at high frequencies was no longer present, suggesting that while the rhythm can be generated in a hemicord, the intact spinal cord is necessary for the complete rhythm.

Two cells just dorsal to the central canal were quiet during rhythmic activity in the ventral roots. This suggests that cells which undergo LDPs may be located within circumscribed areas as indicated by activity-dependent labeling of neurons during locomotion in the neonatal rat (Kjærulff et al., 1994). There also appears to be a size specific criteria for cells exhibiting LDPs. Quiet cells located both ventral and dorsal to the central canal were smaller than those which exhibited rhythmic activity. This observation could have been artificially produced because the sharp electrodes used had a greater likelihood of impaling large neurons and as a result, only a low number of small interneurons were sampled.

Rhythmically active interneurons received depolarizing synaptic input from both segmental afferents and the ventrolateral funiculus while quiescent (absence of 5-HT or NMDA). Quantitative data concerning the degree of axon myelination and conduction velocities are limited (e.g. Chen et al., 1992) for neonatal rats, therefore it is difficult to attribute specific afferents to the stimulation and difficult to discuss latencies (Kudo and Yamada, 1985; Kiehn et al., 1994a). However, a short train of stimuli delivered to dorsal roots L2 or L3 at 1.7 x T (threshold determined by cord dorsum) is capable of resetting the locomotor rhythm in the neonatal rat, indicating that low threshold afferents have

access to the CPG for locomotion (Kiehn et al., 1992). The latency of dorsal root evoked responses in all the interneurons was longer than the latency of the initial activity in the ventral root. The interneurons examined have the same threshold for segmental input or slightly less than the threshold for ventral roots response. Since the cells receive long latency but similar threshold input relative to the ventral roots the input onto the interneurons from segmental afferents may involve a polysynaptic pathway. This assumes that they receive inputs from the same afferents as the motoneurons. It is also possible that the motoneurons receive input from a population of faster conducting afferents than the interneurons. This is not likely because ventral roots never exhibited activity at lower thresholds than the interneurons. However, neither possibility can be ruled out at this time.

The interneurons were depolarized following both high frequency and single shock stimulation of the ventrolateral funiculus. The latency for the single stimuli VLF input onto the cells was greater than that for the ventral roots suggesting they are not interposed between the motor neurons and the VLF pathway, or are part of a longer pathway between the motor neurons and the VLF that may contribute to the prolonged ventral root discharge observed following high frequency stimulation of the VLF (note: this is purely speculative there is no evidence for this).

It is impossible to definitively state whether the cells recorded from in this study overlap with one of the interneuronal populations (IaIN, RC, and ventral group II) extensively characterized in the cat. Historically these interneurons have been characterized by the proprioceptive inputs they receive and by their actions on other neurons. In this report it is difficult to determine what proprioceptive inputs these interneurons received due to the lack of quantitative data concerning the threshold of afferents in the neonatal rat, and also due to the limitation of stimulating many unidentified afferents in the dorsal roots. While the interneurons in the present study appeared to be last order as suggested by their morphology and location, the actions of these interneurons on motoneurons were never elucidated. The IaIN interneurons (Feldman and Orlovsky,

1975), RCs (McCrea et al., 1980) and ventral midlumbar group II interneurons (Shefchyk et al., 1990) in the cat all exhibit LDPs during locomotion. Both IaIN and ventral midlumbar group II interneurons are located in the intermediate grey of the spinal cord (Jankowska and Lindstrom, 1972; Bras et al., 1989) as are the interneurons examined in this study. Both IaINs and midlumbar group II interneurons have different projections than our interneurons. IaIN project long distances rostrocaudally to motoneurons (Jankowska and Lindstrom, 1972) and the midlumbar group II interneurons project caudally to motoneurons (Bras et al., 1989). The interneurons described in the present work appear to be 'local' interneurons, since they do not appear to project outside of the segment in which they are located. The extent of the projections, however, could be age dependent and change with the maturation of the animal. Anatomically the interneurons reported in this study cannot be distinguished from IaIN, however, the interneurons described here do not appear to be monosynaptically activated from the afferents which mediate early ventral root activity suggesting that they are not IaIN which are activated monosynaptically by group I afferents. Overlap with ventral midlumbar group II interneuronal populations cannot be ruled out anatomically or electrophysiologically, but this is not to suggest that the results presented in this study strongly imply that the interneurons presented in this report are group II interneurons. Assuming the location of RC are the same in the neonatal rat as in the cat (lamina IX; Jankowska and Lindstrom, 1971) we can be sure that the cells reported here are not RC based solely on their location.

It is unlikely that the lamina VII cells recorded from are motoneurons in a dorsomedial nucleus. Previous examination of the passive membrane properties of motoneurons in the neonatal rat identified by antidromic stimulation indicate that the input resistance of the motoneurons is 18.1 MΩ ± 7.0 (Fulton and Walton, 1986). Motoneurons we recorded from had a mean input resistance of 52 MΩ. This discrepancy is likely due to the differences of micropipette resistance's used (120-160 MΩ vs. 55-60 MΩ) and thus differences in the micropipette diameter, the micropipettes we used being smaller and thus

less harmful to the motoneurons. Healthy motoneurons consistently exhibited an after-depolarization as previously reported by Fulton and Walton (1986). The recorded interneurons had a mean input resistance of 101 MΩ, putting them well outside the range of motoneurons and they did not express an after-depolarization. Neonatal rat motoneurons stain positively for ChAT as do preganglionic sympathetic neurons in the dorsal commissural nucleus in L2 and parasympathetic neurons in L5 (Phelps et al., 1984). No cell which exhibited ChAT staining, nor any cell located in an area of ChAT staining, was included in this study. Note all tissue was stained for ChAT.

Stimulation of a specific descending pathway in the ventrolateral funiculus elicits frequency dependent, long duration responses in the ventral roots. Recent work has demonstrated that in the intact spinal cord, high frequency stimulation of the VLF is capable of eliciting alternating activity between L3 and L5 ipsilateral ventral roots as well as alternation between ipsilateral L3 and contralateral L3 ventral roots suggesting this pathway plays a role in the initiation in locomotion in the neonatal rat (D.S.K. Magnuson; personal communication). It is possible that the long lasting depolarization following high frequency stimulation of the VLF reported in the present study is linked to locomotion, although rhythmic activity due to VLF stimulation was not observed in the partially hemisected spinal cord

As described by Wallis and Wu (1993) and Elliot and Wallis (1993), ventral root responses to single stimulus VLF input consist of a fast non-NMDA receptor (CNQX sensitive) mediated component and a slow component that appears to involve an NMDA receptors (AP5 sensitive). The present work corroborates these findings, demonstrating that both the fast and slow components of the ventral root response to single shock VLF input are sensitive to the non-specific excitatory amino acid antagonist kynurenate. The non-NMDA antagonist CNQX depresses the fast component to single shock stimulation of the VLF. The NMDA antagonist AP5 and the monaminergic/catecholaminergic antagonist propranolol decreases the long duration responses to single shock VLF

stimulation. The similarity of action of these two antagonists implies that the fast component of the response is mediated by an interaction between NMDA and the monamines. Recent work in the amphibian *Rana temporaria* has demonstrated that the presence of both 5-HT and NMDA was necessary for neurons which had been rhythmically active during locomotor activity to demonstrate oscillatory membrane behavior in the presence of TTX (Sillar and Simmers 1994). Application of NMDA or 5-HT alone in a TTX treated preparation was unable to produce any rhythmic activity suggesting an interaction between the two was necessary for this behavior. We also demonstrated that the monoaminergic antagonist yohimbine (both a noradrenergic alpha 2 antagonist and a 5-HT antagonist) (see Weiner, 1980) has little effect on the ventral root response to single VLF stimuli. Why both monaminergic antagonists yohimbine and propranolol have different effects on the ventral root response to single shock VLF stimulation is not clear.

Recent papers by Pinco and Lev-Tov (1994) and Wallis and Wu (1993) demonstrated some facilitation of ventral root or motoneuron responses to either high frequency or paired pulse VLF stimulation. Our findings differ from this work in the magnitude and nature of the facilitation. In the present study, ventral root responses to stimulus trains did not reach their peak until 2 to 10s after cessation of the stimulus whereas previous reports indicate that the peak response coincided with the termination of the stimulus (e.g. Pinco and Lev-Tov, 1994). This may be due to the fact that in our study the pathway being stimulated is less defined than those used in the other studies. In addition, the work of Pinco and Lev-Tov (1994) focused on an ascending VLF pathway as opposed to the descending pathway. The arrival of the response peak several seconds following stimulation in the present study suggests that the pathway has access to spinal cord circuitry that exhibits endogenous mechanisms such as plateau potentials, endogenous burst properties, or wind-up which may act to enhance excitatory inputs activated by high frequency stimulation of the VLF.

Recent work by Cowley and Schmidt (1994a) has shown that ventral roots are poor monitors of phasic hindlimb flexor and extensor nerve activity during locomotion because ventral roots have both flexor and extensor efferents coursing through them (Cowley and Schmidt, 1994a). This necessitates the use of individual peripheral flexor and extensor motor nerves as monitors of motor output in future studies if one wishes to refer to induced activity as locomotion. It is probably safe to refer to the activity reported here as locomotor-like, since the concentration of the drugs used in this study are known to induce locomotion as monitored in peripheral nerves in the neonatal rat (e.g. Cowley and Schmidt, 1994b). It is also important to note that in order to refer to the ventral root recorded motor activity as locomotor-like, it was necessary for the L2 or L3 ventral roots and L5 ventral roots to be out of phase.

While the neurons described in the present study may be involved in the generation of the motor pattern for locomotion, there are several reasons why we do not think they are responsible for rhythm generation. There was no evidence that any of these interneurons possessed endogenous bursting capabilities (see below), although this does not necessarily exclude them from being components in the locomotor CPG. However, the fact that these large interneurons were in the location of a population of interneurons brightly labeled during locomotor activity-dependent labeling (Kjærulff et al., 1994) suggests that the present sample is not reflective of neurons labeled with an activity-dependent marker during locomotion. The cells labeled by Kjærulff et al (1994) presumably were firing action potentials in order to be labeled, as sulforhodamine is taken up by endocytosis at active synapses (Lichtman et al., 1985), and only 4 of 13 interneurons fired action potentials in the present study, although such a small sample size calls into question such a conclusion. Both the ventrolateral lamina VII location and the dorsoventral orientation of the recorded interneurons, and that 5 of 7 cells had processes extending into the motor nuclei, suggests that they are premotor neurons. We did attempt to get quantitative evidence of a direct synaptic connection between the recorded interneurons and

motoneurons through the use of spike triggered averaging but we were unable to devise a means to excite the cell for long enough periods while maintaining adequate impalements.

There is evidence for a CPG for locomotion in humans (Clancie et al., 1994). This suggests that an understanding of the CPG in lower mammals including the definition of circuitry and pharmacology may have direct clinical relevance for spinal cord injured patients. In order to begin to understand the CPG, a classification of neurons active during locomotion in the *in vitro* neonatal rat in necessary. For example, in the tetrapod *Necturus,* intracellular recordings from interneurons during locomotion have described 5 electrophysiologically distinct populations (Wheatley et al., 1994). The present study has made an initial characterization of cellular properties in a specific population of neurons during locomotion. Future experiments should begin with the characterization of cells possessing endogenous bursting capabilities similar to those reported by Hochman and colleagues (Hochman et al., 1994a,b) as it is very likely that cells with this attribute play a direct role in the generation of the rhythm of locomotion although the lack of endogenous oscillatory features does not immediately exclude a cell from being a component of the CPG. The location, morphology and neurotransmitter content of the cells can be determined through the use of fluorescent labels and immunohistochemical techniques. The inputs the cells receive from segmental afferents and the MLR may provide a means to further classify cell types. It is important to determine if these segmental inputs are reciprocally organized, as previous research has indicated that this is an important criteria which must be met by a putative component of the CPG. Once this information has been determined in the intact spinal cord preparation it can be utilized in the spinal cord slice preparation to determine functional connections between various classes of interneurons and motoneurons. Such research will allow the proposition of models for the CPG which may suggest to clinicians possible therapeutic interventions for spinal cord injured patients.

This study presents data which are not unexpected, given the results of activity-dependent labeling studies and extracellular recordings during locomotion. However this

work does represent an important advance towards the elucidation of the cellular components comprising the locomotor CPG since it provides the first intracellular recordings from interneurons in the neonatal rat lumbar spinal cord during a monitored motor behavior. The study also puts forth many of the principals and methodological approaches necessary to gain insight into cellular organization of the CPG.

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