Neurochemical Substrates of Locomotor and Non-Locomotor Rhythms in Rat Spinal Cord

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In Partial Fulfillment of the Requirements
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NEUROCHEMICAL SUBSTRATES OF LOCOMOTOR AND NON-LOCOMOTOR RHYTHMS IN RAT SPINAL CORD

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Abstract

It has long been understood that the mammalian spinal cord is capable of generating locomotion. However, the neurochemical and anatomical substrates underlying this rhythmic behaviour remain largely unknown. Mechanisms underlying the generation and coordination of locomotion were investigated in this thesis using an *in vitro* neonatal rat spinal cord preparation.

Part I examines whether ventral root activity reliably indicates the presence of flexor and extensor activity in the *in vitro* rat spinal cord preparation. Ventral root patterns recorded during locomotion and ventral root transection experiments indicated that ankle flexor and extensor nerve activity depended on motor units coursing through common lumbar roots in the majority of animals tested, suggesting that ventral root recordings alone are not a reliable means of monitoring phasic hindlimb flexor and extensor activity during locomotion in this preparation.

Part II compares the different patterns of hindlimb flexor and extensor discharge induced by each of N-methyl-D,L-aspartate (NMA), serotonin and acetylcholine (ACh). These findings demonstrate that exogenously applied neurochemicals induce a variety of *in vitro* motor rhythms although some substances preferentially activate specific patterns. Serotonin was the best single agent for inducing a pattern of flexor-extensor activity consistent with locomotion.

Part III examines the role of inhibitory amino acid receptor mechanisms in the coordination of left-right and flexor-extensor discharge during neurochemically induced motor rhythms. These findings indicated that γ-amino-butyric acid ($GABA_A$) and glycine receptor activation may mediate reciprocal left-right and flexor-extensor phase relationships during locomotion, and that blockade of these receptors facilitates the expression of rhythms dominated by excitatory coupling within the rhythmogenic network.

Part IV investigates the neuroanatomical substrates generating and coordinating motor rhythms induced by different neurochemicals. The results suggest that a serotonin-sensitive oscillatory network capable of generating locomotion is distributed throughout the supralumbar cord whereas NMA- and ACh-activated rhythmogenic elements are distributed throughout the spinal cord. In addition, an extensive propriospinal network of redundantly organized reciprocal excitatory connections exists which may underlie the simultaneous activation of specific combinations of motor groups during locomotion.

The findings of this thesis are summarized, some results reported subsequent to publication of Parts I-IV are discussed, and a possible model of the spinal rhythm-generating network is presented.

List of Abbreviations used in General Introduction and Discussion

CPG	central pattern generator
MLR	mesencephalic locomotor region
CNS	central nervous system
ENG	electroneurogram
EMG	electromyogram
VR	ventral root
Tib	tibial
Per	peroneal
BF	biceps femoris
VL	vastus lateralis
IL	Iliacus
5-HT	serotonin
5-HTP	5-hydroxytryptoaphan, (5-HT precursor that crosses blood brain barrier)
ACh	acetylcholine
EDRO	edrophonium (anticholinesterase)
NE	Norepinephrine
L-Dopa	L-3,4-dihydroxyphenylalanine
NMA	N-methyl-D,L-aspartate
NMDA	N-methyl-D-aspartate
DHK	dihydrokainic acid
CNQX	6-cyano-7-nitroquinoxaline (NMDA receptor antagonist)
EAA	Excitatory amino acid
IAA	Inhibitory amino acid
GABA	gamma amino butyric acid
BIC	bicuculline ($GABA_A$ receptor antagonist)
C	Cervical
T	Thoracic
L	Lumbar
S	Sacral

General Introduction

The act of locomotion is considered one of the simplest rhythmic behaviours controlled within the nervous system, yet it is far from being clearly understood. Increasing our understanding of the spinal control of locomotion will enhance our ability to explain more complex functions of the brain, and represents an essential first step in ameliorating the effects of spinal cord injury and disease. This thesis is an examination of some of the neurochemical mechanisms used by the spinal cord in generating and coordinating locomotion.

In particular, the introduction will briefly describe the pattern of electromyographic activity during mammalian locomotor behaviour, and will review the literature with respect to the neurochemical mechanisms initiating and coordinating locomotion as well as the distribution of neuroanatomical substrates underlying locomotion. An overview of relevant neurotransmitter effects on cells and the anatomical distribution of cells, fibres and receptors that may mediate locomotor activity within the spinal cord is provided. A brief history and description of the advantages and some limitations of using the *in vitro* spinal cord preparation to investigate the neurochemical mechanisms of locomotion will also be presented. The primary focus of the literature review is mammalian locomotion leading up to the publication of the four papers in this thesis. Where appropriate, results of experiments in lower vertebrates are included.

Generation of Locomotion by the Mammalian Spinal Cord

In general, hindlimb stepping or walking consists of alternation between pairs of flexors and extensors acting on each joint within a single limb, coordinated with alternation of the extensors/flexors of the contralateral limb, such that while one limb supports the body (stance phase), the contralateral limb is engaged in swinging forward (swing phase). Generally, functionally defined extensors alternate with the functionally defined flexors of the hip, ankle and foot within each limb (Grillner 1981). In cat, some

muscles (e.g. semitendonosus) deviate from this pattern and show two bursts per step cycle (Engberg & Lundberg 1969; Rasmussen et al. 1978). In contrast to cat, single and reciprocal bursts occur in semitendonosus in adult rat during treadmill stepping (Gruner et al. 1980) but show two bursts per step cycle during swimming (Gruner and Altman 1980). During most slow forms of locomotion, interlimb coordination consists of strict alternation between the two limbs of the same girdle. Thus, if the step cycle starts when one limb touches the ground then the contralateral limb touches the ground when half of the step cycle is complete. It should be noted, however, that even when functionally defined extensors alternate with functionally defined flexors there is a sequence of activation within each functional motor group such that the onset of all flexor (or extensor) activity is not simultaneous. For example, when the onset of activity of four extensors was examined in cat during trotting, Engberg and Lundberg (1969) observed that the onset of the hip extensors (adductor femoris and semimembranosus (femoral part)) preceded the main burst of activity of the knee and ankle extensors (quadriceps (vastus lateralis) and gastrocnemius, respectively). For a review of the biomechanical and electromyographical descriptions during walking and other forms of locomotion in different species in intact and reduced preparations see Grillner (1981).

In 1911 and 1914, T. Graham Brown reported his observations of narcosis progression; that, under certain anaesthetic conditions, spinalized cats walked. Graham Brown's observations of spinalized walking were the same in afferented and de-afferented animals, leading Graham Brown to conclude the act of progression was spinally generated. One implication of Graham Brown's work was to disprove the chain reflex hypothesis for locomotion. The chain reflex hypothesis postulated that contraction of one muscle group resulted in the signal for contraction of another group and so on until the entire step cycle was complete (Philippson, 1905 as referenced in Shik and Orlovsky 1976). Subsequent experimentation was directed at confirming and explaining the 'centrally generated' pattern. For example, electromyogram (EMG) recordings in the 1970's showed that coordinated stepping movements of all four limbs could be generated in the high spinal cat (Miller and van der Meche 1976; Halbertsma et al. 1976). Later, patterns of muscle activity during treadmill stepping in spinal cats were compared to the

intact preparation and found to be similar (Grillner and Zanger 1979; Forssberg et al. 1980 a, b). Another important implication of Graham Brown's work (1914) was that a model to account for the alternation between flexors and extensors (the half-centre model) was put forth (discussed below within *Distribution of cells generating and coordinating...*).

Once it was established that the simple rhythmic motor act of walking could be generated entirely by the spinal cord in the absence of afferent feedback, various methods were employed to study the neurochemical mechanisms used by, and organization of, the spinal neurons generating locomotion. The set of neurons within the spinal cord producing the basic locomotor pattern are functionally defined as the locomotor central pattern generator, or CPG. Although significant work has been done indicating that peripheral afferent and descending input shape the final form of centrally generated patterns of locomotion (e.g. reviewed in Pearson (1995); Grillner and Dubuc 1988), this thesis will focus on the centrally generated locomotor activity.

Overview of Neurotransmitters Inducing Locomotor-like Rhythms in Mammalian Spinal Cord.

Lundberg and coworkers first showed that L-3,4-dihydroxyphenylalanine (L-Dopa), initiated stepping movements in the spinalized cat (Jankowska et al. 1967a). Initially, since noradrenaline is synthesized from dopamine and dopamine is synthesized from L-Dopa, it was unclear whether the effects of L-Dopa were due to activation of noradrenergic or dopaminergic pathways. As reviewed in Baldissera et al. (1981), several lines of evidence suggested the effect of L-Dopa was due to noradrenaline rather than dopamine actions. In particular, the effect of L-Dopa is abolished by α-adrenergic receptor blockade and increased if the degradation of noradrenaline is inhibited. Interestingly, it is thought that L-Dopa acts by forming dopamine, which then displaces noradrenaline from its stores, causing noradrenaline release from synaptic terminals (see references in Baldissera et al. 1981).

In addition to being the first demonstration of neurochemical activation of locomotion, observations of mutually inhibitory interneurons revealed by L-Dopa (Jankowska et al. 1967a) led Lundberg and co-workers to formulate a revised 'half-centre' model to explain reciprocal inhibition between flexors and extensors during locomotion (see below within *Distribution of cells generating and coordinating...*). Although this was the first demonstration of exogenous application of a neurotransmitter leading to locomotor activity, it did not necessarily mean that either of these L-Dopa products were required to produce locomotion. It did, however, begin the next phase of research attempting to identify neurotransmitters that may either initiate locomotion for further study, or have an essential role in generation of the rhythm. Although differences exist between various mammalian preparations, several excitatory neurotransmitters have been implicated in initiating rhythm within the spinal cord, including noradrenaline, dopamine, serotonin, acetylcholine and excitatory amino acids.

Noradrenergic receptor activators have induced locomotion in all mammalian species examined to date. In cat, systemic injection of the noradrenergic precursor L-Dopa or agonist (clonidine) evoked treadmill stepping (Forssberg and Grillner 1973; Barbeau and Rossignol 1991), even in the absence of afferent feedback (Grillner and Zangger 1979). Norepinephrine, when intrathecally administered, induced locomotion in the acute spinal cat (Omeniuk and Jordan, 1982; Kiehn et al. 1992a). In rabbit, systemic L-Dopa also resulted in locomotor activity (Viala and Buser 1969, 1971). Bath application of norepinephrine (NE) induced rhythmic ventral root discharge in the *in vitro* rat spinal cord (Smith et al. 1986).

Serotonin: In rabbit spinalized at high thoracic levels, application of the serotonin precursor 5-hydroxytryptophan (5-HTP) induced locomotion (Viala and Buser 1969, 1971). However, in the acute (Grillner and Shik 1973) or chronic (Barbeau and Rossignol 1991) spinal cat (spinalized at T13), serotonergic drugs did not produce rhythmic locomotor activity. In the rat spinal cord, serotonin (5-HT) initiated rhythmic lumbar ventral root discharge (Cazalets et al. 1990, 1992).

Dopamine induced locomotion (Atsuta et al. 1991) or rhythmic ventral root discharge (Smith et al., 1986) in the neonatal rat spinal cord, but showed either weak facilitory effects when intrathecally administered in the cat (Omeniuk and Jordan 1982). Systemic administration of dopaminergic drugs did not induce coordinated stepping in the cat (Barbeau and Rossignol 1991).

Excitatory amino acids induced locomotion in the mammalian spinal cord. In the *in vitro* rat spinal cord, locomotion has been observed after activation of NMDA receptors (Kudo and Yamada 1987; Atsuta et al. 1991) or application of aspartate (Atsuta et al. 1991). Rhythmic ventral root discharge has been induced by aspartate (Cazalets et al. 1992), glutamate or NMDA (Smith and Feldman 1985; Smith et al. 1986; Cazalets et al. 1990, 1992), as well as kainate and quisqualate (Cazalets et al. 1992) in the *in vitro* rat spinal cord. Intrathecal administration of N-methyl-D-aspartate (NMDA) with the excitatory amino acid uptake inhibitor dihydrokainic acid (DHK) induced locomotion in spinal cat although kainate and quisqualate receptor activation was ineffective (Douglas et al. 1993).

Acetylcholine, in combination with the anticholinesterase inhibitor edrophonium (EDRO) produced rhythmic ventral root discharge in the neonatal rat spinal cord (Smith and Feldman 1985; Smith et al. 1986; Smith and Feldman 1987; Smith et al. 1988). However, when using electromyogram records to monitor activity, Atsuta et al. (1991) reported that acetylcholine (ACh) did not induce locomotion. Instead, increased tonic discharge and co-contractions of all muscles was observed, with no consistent alternation (Atsuta et al. 1991). To date there have been no reports of acetylcholine-induced locomotion in cat or other mammalian preparations. Muscarinic acetylcholine receptor antagonists shortened bouts of fictive swimming in the *Xenopus* brain-spinal cord and acetylcholine increased the duration of swimming in spinal animals (Panchin et al. 1991), suggesting some cholinergic action on the spinal CPG.

Similar to findings in the cat, not all neurotransmitters tested induce locomotion in lower vertebrates, such as the lamprey. In particular, L-Dopa as well as the excitatory

amino acids glutamate and aspartate (Cohen and Wallen 1980; Poon 1980) induced swimming in lamprey spinal cord whereas serotonin (Harris Warrick and Cohen 1985), noradrenaline or α-adrenergic agonist clonidine, and dopamine (Poon 1980) did not.

Anatomy of Putative Locomotion Inducing Neurotransmitters within the Mammalian Spinal Cord

Noradrenaline, serotonin, dopamine, and acetylcholine and excitatory amino acids are present throughout the rostro-caudal extent of the mammalian spinal cord (Dahlström and Fuxe 1964; Carlsson et al. 1964; Steinbush 1981; Mouchet et al. 1992; Shirouzu et al. 1990; Barber et al. 1984; Johnson and Aprison 1971; Patrick et al., 1983). Noradrenaline and dopamine fibres are thought to originate solely from cell bodies within brainstem nuclei (Carlsson et al. 1964; Nygren and Olson 1977; Westlund et al, 1983; Dahlström and Fuxe 1964; Björklund and Skagerberg 1979; Hökfelt et al. 1979 but see Mouchet et al. 1986). Although the main source of serotonin originates in brainstem raphe nuclei (Dahlström and Fuxe 1964), 2-15% of serotonin within the rat spinal cord has been estimated to originate from cell bodies in Rexed's Lamina X and VII in supralumbar regions of the spinal cord (Newton et al. 1986, 1989; Newton and Hamill 1988). Glutamate within the spinal cord may originate from either descending (e.g. Minson et al. 1991) or intraspinal cell bodies (Berger et al. 1977). Evidence reported to date does not support the supraspinal origin of acetylcholine within the spinal cord (Kanazawa et al. 1979; Sherriff et al. 1991). Receptors for subtypes of each of noradrenaline, serotonin, dopamine, acetylcholine and excitatory amino acid neurotransmitters are also present throughout the mammalian spinal cord, each with particular patterns of high density distribution (Roudet et al. 1993, 1994; Marlier et al. 1991; Fischette et al. 1987; van Dijken et al. 1996; Yokoyama et al. 1994; Dubois et al. 1986; Gillberg et al. 1990; Petralia et al. 1994; Valerio et al. 1996; Bonnet et al. 1996). The developmental changes in some of these neurotransmitter systems that relate to the use of the neonatal rat spinal cord preparation to examine mechanisms of locomotion are discussed below (section entitled *The In Vitro Spinal Cord Preparation*).

It is also known that various neurotransmitters co-exist within the same cell in the mammalian spinal cord. For example, both GABA and acetylcholine were observed in cells around the central canal, in 'partition cells' that occur along the border between the dorsal and ventral spinal grey, and within the dorsal horn (Kosaka et al. 1988). GABA and glycine co-exist in axons and cell bodies of rat spinal cord (Chiba and Semba 1991; Todd and Sullivan 1990; Triller et al. 1987). In addition, glutamate, serotonin and substance P were observed in synaptic boutons surrounding motoneuron cell bodies in rat and monkey spinal cord (Nicholas et al. 1992).

Direct effects of the above neurotransmitters have been reported within many cells of the spinal cord. However, most neurotransmitters show different effects on membrane potential, depending upon the area or cell type studied. For example, although the excitatory amino acids glutamate and aspartate are considered excitatory and reported to directly depolarize cells (Curtis et al. 1959; McLennan and Lodge 1979), serotonin, norepinephrine and acetylcholine show mixed effects, with some cells exhibiting direct depolarization, direct hyperpolarization and others showing no direct effect but a modulation of other neurotransmitter effects (5-HT: Neuman 1984; Wang and Dun 1990; Takahashi and Berger 1990; Ziskind-Conhaim et al. 1993, NE: Neuman 1984, ACh: Bordey et al. 1996; Blake et al. 1987). In addition, some neurotransmitters produce more complex actions, such as promoting intrinsic membrane oscillations (NMDA and/or 5-HT: Hochman et al. 1994a,b; MacLean et al. 1998), and plateau potentials (5-HTP, L-Dopa, clonidine: Hounsgaard et al. 1988; Conway et al. 1988).

An Endogenous Role for Putative Locomotion-Inducing Neurotransmitters?

Although many exogenously applied neurotransmitters have been reported to induce locomotor activity within the spinal cord, evidence for an endogenous role for each in initiating locomotion is not as clear. It is possible that exogenous application of neurotransmitters or receptor agonists acts indirectly to initiate locomotion. For example, applied neurotransmitters or receptor agonists may specifically excite spinal neurons that in turn may activate cells within locomotor CPGs. Alternatively, exogenously applied

neurotransmitters or receptor agonists may increase the general excitability of neurons, not specifically related to locomotion, yet be sufficient to activate the locomotor CPG. Blocking endogenous neurotransmission using receptor antagonists provides support for an endogenous role of the neurotransmitter in locomotion (Harris-Warrick 1988).

In the cat, depletion of serotonin and norepinephrine to 10% of control levels did not abolish mesencephalic locomotor region (MLR)-evoked locomotion (Steeves et al. 1980), suggesting that either these neurotransmitters are not essential or that only minimal amounts are required to facilitate locomotion. This issue requires further investigation since more recent evidence from the rat spinal cord suggests serotonin may play an essential role in both the network generation of locomotor activity as well as in membrane oscillatory behaviour of motoneurons (MacLean et al. 1998). The role of excitatory amino acids may also be endogenous, in that NMDA receptor activation may be essential for the production of motor rhythms in both the cat and rat (Douglas et al. 1993; Smith et al. 1988) and preferentially effects the output amplitude of motoneuron discharge before affecting locomotor rhythm in the rabbit (Fenaux et al. 1991) and the rat (Schmidt et al. 1989). Similar evidence has not been observed regarding acetylcholine. In particular, intravenous muscarinic or nicotinic acetylcholine receptor antagonists did not effect MLR-induced treadmill stepping in cat (Noga et al. personal communication, 1993), suggesting acetylcholine is not required for operation of the spinal CPG.

Different Neurotransmitters Induce Distinct Locomotor Patterns?

As noted above, a variety of neurotransmitters or receptor agonists induce locomotor rhythms in different mammalian preparations. Whether these neuroactive substances achieve their effects through excitation of a common locomotor network, or instead activate functionally or anatomically distinct spinal rhythm generating elements is unknown. Different rhythm-inducing substances may be associated with specific actions, such as selectively enhancing flexion phase activity within the locomotor cycle. In the *in vitro* neonatal rat, excitatory amino acid-induced locomotion was associated with enhanced flexion phase activity while dopamine-induced locomotion was associated with

enhanced extension phase activity (Bodine-Fowler et al. 1988). In the chronic spinal cat, apomorphine (dopamine agonist) and L-Dopa specifically enhanced the flexor phase activity (Barbeau and Rossignol 1991). In the acute spinal rabbit, intravenous application of L-Dopa selectively facilitated extension phase activity whereas 5-HTP enhanced the flexion phase (Viala and Buser 1969, 1971).

Thus one of the research goals of this thesis was to determine whether several putative locomotion-inducing substances (N-methyl-D,L-aspartate (NMA), ACh and 5-HT) activate distinct motor patterns.

HYPOTHESIS PART II: **Different neurochemicals produce distinct locomotor patterns in the neonatal rat spinal cord.**

The *In Vitro* Spinal Cord Preparation

Many of the neurotransmitters reported to induce rhythmic locomotor activity (see above) were studied using the *in vitro* neonatal rat spinal cord preparation. The *in vitro* whole spinal cord preparation from neonatal rat was first presented by Otsuka and Konishi (1974) and has become a valuable tool for the study of simple rhythmic behaviours such as respiration and locomotion. Some problems associated with investigation of neurochemical mechanisms of locomotion in traditional *in vivo* models, such as systemic toxicity when delivering neurochemicals to the intact central nervous system (CNS), and long wash-in, wash-out times are overcome by using an *in vitro* spinal cord preparation. Although both *in vitro* and tissue or cell culture preparations offer precise control over the extracellular environment, only a fraction (if any) of a particular neural network is preserved in cell and tissue culture, thus making these preparations generally unsuitable for studies of the locomotor network activity.

Since the *in vitro* spinal cord preparation does not have an intact circulatory system, oxygen is transported to the living cells by diffusion from the oxygenated artificial cerebrospinal fluid surrounding the cord. Thus the concentration of oxygen will be greater in the superficial layers of spinal cord and less in the central regions, and will

diffuse more successfully throughout small pieces of spinal tissue rather than pieces with a large diameter. Smaller pieces of spinal cord will therefore remain viable for longer periods than larger pieces. As a result, successful use of the *in vitro* spinal cord preparation to examine locomotor network behaviour requires that very young animals be used. Thus, in any examination of locomotor activity, consideration must be given to the developmental aspects of the observed results.

During the first two weeks of life, intact rats use mainly their forelimbs for pivoting and crawling; they do not develop sufficient hindlimb weight support for quadruped walking until day 12 - 13 (Altman and Sudarshan 1975). However, coordinated gait, involving all four limbs, is seen on the day of birth during swimming or during L-Dopa-induced air-stepping in suspended rats (Bekoff and Trainer 1979; McCrea et al. 1994; Stenhouwer et al. 1994). During air-stepping and overground walking, forelimb stepping predominates over quadrupedal patterns until after day 5. Electrical stimulation of the brainstem MLR-evoked hindlimb stepping in the *in vitro* rat on the day of birth, indicating descending locomotor pathways are functional at birth (Atsuta et al. 1988, 1990).

Most major descending pathways are present at birth in the neonatal rat (Kudo et, al. 1993; Leong et al. 1984; Shieh et al. 1983). However, the corticospinal tract reaches lumbar levels at post-natal day 6, enters the grey matter about 3 days later and myelination begins about post-natal day 12 (Schreyer and Jones 1982). Acetylcholine (Phelps et al. 1984), norepinephrine (Aramont et al. 1986), serotonin (Rajofetra et al. 1989), GABA (Ma et al. 1992) and glycine (Bruning et al. 1990) are present within the spinal cord of the rat at birth. Although present at birth, glycine receptor levels as well as the concentration of serotonin (Rajofetra et al. 1989), norepinephrine (Aramont et al. 1986), and acetylcholine (Phelps et al. 1984) increase during the first few post-natal weeks to reach adult levels. In contrast, levels of NMDA receptor and the concentration of GABA present in the spinal cord are reported to decrease from birth to adult (Kalb et al. 1992; Ma et al. 1992). Further, specific isoforms of various receptors are expressed only during the neonatal period (e.g. glycine Akagi et al. 1991; Betz 1991) in the rat spinal cord. Neonatal receptor isoforms display different biochemical characteristics than

adult isoforms. For example, the binding affinity of strychnine is lower in neonatal rat glycine isoforms in comparison to the adult (Becker et al. 1988).

Spinal cord cell responses and interactions also change during the neonatal period in rat. In contrast to adult motoneurons, motoneurons in the late embryonic rat spinal cord are depolarized in response to exogenous GABA or glycine (Wu et al. 1992). Electrotonic coupling between synergist or homonymous motoneurons declines during the first two postnatal weeks (Walton and Navarrete 1991). Application of 5-HT excites spinal motoneurons before serotonin fibres grow into the ventral horn (Ziskind-Conheim et al. 1993), suggesting that cellular mechanisms responsive to activation by this neurotransmitter exist before synaptic contact is made. Further, although activation of 5-HT_{1A} receptors excites spinal motoneurons in embryonic (Ziskind-Conheim et al. 1993) or neonatal (2 - 3 day old, Takahashi and Berger 1990) rat spinal cord, 2 - 3 week old rat spinal motoneurons are hyperpolarized (Elliot and Wallis 1992) by 5-HT_{1A} receptor activation. Thus, descending fibres with an anatomical pattern similar to the adult may be present in the neonatal rat spinal cord, yet the nature of synaptic activity observed in the neonatal preparation may not reflect that of the adult.

Subsequent to the initial description of the *in vitro* spinal cord preparation by Otsuka and Konishi (1974), several researchers began using the preparation in attempts to understand the neurochemical mechanisms underlying mammalian locomotion. Most early studies using the *in vitro* rat spinal cord relied on ventral roots to record lumbar motoneuronal activity (Smith and Feldman 1987; Smith et al. 1988; Cazalets et al. 1990, 1992) as opposed to recording directly from hindlimb flexor and extensor muscles (Kudo and Yamada 1987; Atsuta et al. 1991). Although ventral roots were acknowledged to contain a mixture of flexor and extensor motor units (Cazalets et al. 1992; Kiehn et al. 1992b; Smith and Feldman 1987), some reports also suggested that L2 and L3 ventral root discharge was extensor activity and L5 ventral root discharge coincided mainly with flexor activity (Cazalets et al. 1992; Kiehn et al. 1992b). In the rat, anatomical studies indicate that each lumbar ventral root contains a mixture of flexor and extensor motor axons (Nicolopoulos-Stournaras and Iles 1983). These conflicting observations and statements led to the research goal of Part I, that is, to examine whether ventral root

recordings can be used reliably to selectively monitor flexion and extension phases of the step cycle.

> HYPOTHESIS PART I: **Ventral roots contain a mixture of flexor and extensor motor axons and therefore cannot be used reliably to monitor hindlimb locomotion.**

Role of Inhibitory Amino Acid Receptor Mechanisms in the Generation and Coordination of Hindlimb Locomotion

During locomotion, coordination of rhythmic muscle discharge must occur at several levels. Intralimb coordination occurs between muscles acting on a single joint and between muscles acting on different joints within a limb. Interlimb coordination occurs between muscles within different limbs of the same girdle and between muscles of different girdles. As noted above, although overlapping or simultaneous discharge exists, alternation is a consistent feature in many forms of locomotion (see Grillner 1981 for review). This thesis will focus on the neurotransmitter mechanisms mediating hindlimb alternation during locomotion.

Reciprocal inhibition occurs if two neurons, or two groups of neurons, mutually inhibit each other. In many half-centre models proposed to account for alternation between functional antagonist motor groups (e.g. Graham Brown 1911, 1914; Jankowska et al. 1967a; Lundberg 1981), reciprocal inhibition is proposed as the neural mechanism to ensure that when one motor group is active, the functional antagonist motor group is inhibited (see further below in Distribution of Cells Generating and Coordinating...). Due to their direct inhibitory effects on cells, inhibitory amino acids are potential candidates for modulating locomotor networks and mediating reciprocal inhibition during locomotion. Both GABA and glycine receptors have been observed in all laminae throughout the rostrocaudal extent of the mammalian spinal cord (van den Pol and Gorcs 1988). In addition to being present within fibres and cell bodies throughout the spinal cord (McLaughlin et al. 1975), GABAergic neurons project from the brainstem to

terminate around motoneurons and in the intermediate grey of the lumbar spinal cord (Holstege 1991). $GABA_A$, $GABA_B$ receptors also exist throughout the rostrocaudal extent of the spinal cord with a more dense dorsal horn distribution of $GABA_B$ receptors (Bowery et al. 1987).

Within the spinal cord GABA and glycine act at physiologically defined synapses. In particular, glycine mediates inhibition of motoneurons from Ia inhibitory interneurons, Ib inhibitory interneurons, and group II interneurons as well as the Renshaw cell inhibition of Ia interneurons. GABA may contribute to group II and group Ib interneuron-mediated inhibition of motoneurons, and is thought to mediate the inhibition from flexor reflex afferents to Ia interneurons, as well as the presynaptic inhibition of primary afferents (reviewed in Jankowska et al. 1992). In addition, both GABA and glycine are thought to mediate recurrent inhibition from Renshaw cells to motoneurons (Cullheim and Kellerth 1981; Schneider and Fyffe 1992).

GABA agonists abolished locomotor activity in the neonatal rat spinal cord (Atsuta et al. 1991; Cazalets et al. 1994), although it is unknown whether suppression of rhythmic locomotor discharge is due to a general reduction in excitability or due to a direct effect on cells within the locomotor network. An endogenous role for inhibitory amino acids in modifying locomotor networks is suggested by observations that blocking $GABA_A$ receptors facilitated walking in spinal cats (Robinson and Goldberger 1986), and enhanced frequency and amplitude of ventral root discharge in neonatal rat (Cazalets et al. 1994). Antagonism of glycine receptors enhanced walking in spinal dogs (Hart 1971) and suppressed the rhythmic inhibition of motoneurons that occurs during locomotion in the cat (Pratt and Jordan, 1987).

Although the alternating activity of functional antagonists during locomotion is thought to result from reciprocal inhibitory interactions within the spinal rhythm generating network in both mammals (Graham Brown 1911; Jankowska et al. 1967a) and lower vertebrates (Cohen and Harris-Warrick 1984; Dale 1985), direct evidence that inhibitory amino acids mediate these effects arises from work in lower vertebrates. In *Xenopus* and lamprey, application of the glycine receptor antagonist strychnine converted alternating intrasegmental discharge into synchronous activity (Cohen and Harris-

Warrick 1984; Dale 1985), suggesting glycine receptors mediate side-to-side coordination of antagonistic motor groups during swimming. However, glycinergic receptor mechanisms are reported to contribute to, but not be required for, alternation during scratching in the turtle spinal cord (Currie and Lee 1997). In mammals, application of the glycine receptor antagonist to the spinal cord converted alternating left-right ventral root discharge to synchronous bursts in embryonic rat (Kudo et al. 1991), synchronized high frequency flexor-extensor EMG bursts in neonatal mouse (Droge and Tao 1993), and disrupted flexor-extensor coordination during fictive locomotion in cat (Kriellaars et al. 1988; Noga et al. 1993b).

Although glycine receptor mechanisms are implicated in intrasegmental alternation of functional antagonists in lamprey (Cohen and Harris-Warrick 1984), longitudinal intersegmental coordination is unaffected after glycine receptor blockade (Alford and Williams 1989). In addition, GABAergic systems do not appear to mediate intrasegmental alternation (Grillner and Wallen 1980), although $GABA_A$ and $GABA_B$ receptor mechanisms may contribute to longitudinal intersegmental coordination during swimming in lamprey spinal cord (Tegner et al. 1993). In mammals, GABA receptor antagonists were reported to modify frequency and amplitude of rhythmic ventral root discharge but not the pattern of rhythmic ventral root activity (Cazalets et al. 1994).

Thus, glycine receptor mechanisms have been shown to contribute to intrasegmental coordination and GABA receptor mechanisms to intersegmental coordination in lower vertebrates. In addition, GABA and glycine may modulate locomotor activity in mammals, but it is not clear if left-right and flexor-extensor alternation is mediated by inhibitory amino acids. Our third research goal was to examine whether GABAergic and glycinergic mechanisms mediate reciprocal inhibitory interactions during locomotion in the mammalian spinal cord.

HYPOTHESIS PART III: **Inhibitory amino acid receptor mechanisms mediate reciprocal inhibition during locomotion in the *in vitro* neonatal rat spinal cord.**

Distribution of Neuronal Networks Generating and Coordinating Locomotor Activity in the Mammalian Spinal Cord.

In addition to neurotransmitter mechanisms initiating and coordinating locomotion within the mammalian spinal cord, the neuroanatomical substrates underlying these functions are of interest. In simpler experimental systems, such as the lower vertebrate spinal cord, it is thought that a distributed and redundant organization of networks of cells generates locomotion (Grillner et al. 1991; Roberts 1990). For example, locomotion with appropriate rostrocaudal phase lags and left-right alternation in lamprey can be predicted if modeled as two chains of redundant and coupled oscillators, with the two oscillators in each segment being mutually inhibitory (Grillner et al. 1993; Sigvardt 1993; Williams et al. 1990). As noted above, left-right intrasegmental alternation is thought to result from discharge of reciprocally inhibitory cells within each segment. Rostrocaudal phase lags are thought to be mediated by a caudally decreasing gradient of excitatory coupling along the rostrocaudal extent of each side of the spinal cord (reviewed in Tunstall and Sillar 1993; Matsushima and Grillner 1990). Experimental observations support a redundant and distributed system of coupled oscillators in lamprey, *Xenopus*, dogfish, and chick (Cohen and Wallen 1980; Grillner 1974; Khan and Roberts 1982; Ho and O'Donovan 1993). For example, short segments of spinal tissue generate coordinated swimming in lamprey and dogfish, regardless of the rostrocaudal location of the small segments (Cohen and Wallen 1980; Grillner 1974).

In mammals, the original and revised 'half-centre' models (Graham Brown 1911, 1914; Jankowska et al. 1967a; Lundberg 1981) provide a mechanism to account for the alternation underlying functional antagonists at a given joint. A fundamental feature of both models is that inhibitory interneurons act reciprocally such that in the presence of a common excitatory drive, when one 'half-centre' is active, the opposite 'half-centre' is inhibited and alternation produced. Other models of mammalian locomotor activity that focused on alternation of antagonists at a given joint have been described (Pearson and Duysens 1976; Miller and Scott 1977), but subsequent experimental evidence did not support these model predictions (discussed in Jordan 1983). Grillner (1981) proposed

that multiple 'unit burst' generators exist within the hindlimb, each driving a particular group of close synergists acting on a given joint, and acting in concert to provide the appropriate timing of discharge along the hindlimb to produce various locomotor activities. Grillner's model predicts that each unit burst generator along the length of the limb is coupled by inhibitory and excitatory projections. The different phase relationships observed between all joints of the hindlimb for forward and backward alternating, and synchronous gaits, depend on whether the coupling between generators is excitatory or inhibitory. This model predicts that individual unit burst generators are distributed throughout the spinal cord close to the motoneuron populations they drive, and that the networks generating locomotion would be distributed, rather than show a discrete localization.

Similar to a distributed system for lower vertebrate locomotion, experimental evidence suggests that the cells generating scratching are distributed over several spinal cord segments in the turtle (Mortin and Stein 1989) and the cat (Deliagina et al. 1983). However, discrete segments of spinal tissue are sufficient for generating locomotor activity. In particular, L-Dopa induced rhythmic locomotor discharge was observed in both fore- and hindlimb muscle nerves in rabbits spinalized at C1 and T12, demonstrating separate rhythm generating cells exist for the fore- and hindlimbs in the rabbit spinal cord (Viala and Vidal 1978). Further, small segments of caudal spinal tissue can generate rhythmic alternation of ankle flexor and extensor discharge in the cat (L6-S1, Grillner and Zangger 1979) and the rat (L4-L5 hemisegment, Kudo and Yamada 1987) in response to drug application. Recently, single cervical segments were reported to sustain rhythmic activity in elbow flexors and extensors in the mudpuppy (Cheng et al. 1998).

Although discrete caudal spinal segments can generate locomotor activity, a distributed system involving more rostral components of the spinal cord is not precluded. In some experimental systems, a regional hierarchy wherein more rostral lumbar segments have a relatively greater capacity for rhythm generation compared with caudal segments has been observed (e.g. Deliagina et al. 1983; Ho and O'Donovan 1993; Mortin and Stein 1989). In contrast, recent investigations in neonatal rat spinal cord suggest that caudal lumbar segments do not participate in either locomotor rhythm generation or

pattern organization and that these functions are restricted to the L1 and L2 segments (Cazalets et al. 1995, 1996).

Thus, our fourth research goal was to examine whether cells generating locomotor activity are distributed throughout the rostrocaudal extent of the spinal cord or localized to a particular location. We examined the effect of acute transverse and midsagittal lesions on locomotor rhythm generation in the isolated spinal cord of neonatal rats. We were particularly interested in rhythms resembling locomotion but since we have observed that different neurochemicals preferentially induce specific patterns of rhythmic activity (not necessarily locomotor in nature) we were interested in examining whether these different patterns of motor activities are mediated by common neuroanatomical substrate(s).

HYPOTHESIS PART IV: **That the neuronal networks generating locomotor activity exist as a distributed rather than discrete system in the *in vitro* neonatal rat spinal cord and that different forms of rhythmic behaviour are mediated by networks with regionally distinct distributions.**

PART I: Some Limitations of Ventral Root Recordings for Monitoring Locomotion in the In Vitro Neonatal Rat Spinal Cord Preparation

Kristine C. Cowley and Brian J. Schmidt

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Summary

Studies of the *in vitro* neonatal rat spinal cord have used ventral root recordings, among other methods, to monitor locomotion. However, whether ventral root activity reliably indicates the presence of hindlimb stepping has not been established. In the present study, we use an *in vitro* spinal cord-hindlimb preparation to analyse lumbar ventral root recordings obtained while simultaneously observing coordinated stepping movements or rhythmic alternation of ankle flexor and extensor nerve activity. During locomotion ventral root patterns included: tonic activity, rhythmic left-right alternation with in-phase activity of ipsilateral roots, and rhythmic activity that was in-phase both bilaterally and ipsilaterally at different segmental levels. Ventral root transection during rhythmic activity demonstrated that ankle flexor and extensor nerve activity depended on motor units coursing through common lumbar roots, in 31/ 39 hindlimbs. These findings suggest that ventral root recordings alone are not a reliable means of monitoring phasic hindlimb flexor and extensor activity during locomotion in the *in vitro* rat preparation.

Introduction

The *in vitro* neonatal rat brainstem-spinal cord preparation is now recognized as a valuable model for the study of the electrophysiological and neurochemical properties of the mammalian nervous system. In particular, this preparation has been used to investigate neural mechanisms underlying the generation of locomotion (e.g. [1,4,6]). Although previous studies acknowledge that ventral roots contain a mixture of flexor and extensor motor units [1,3,6], some reports also suggest that L2 and L3 ventral root discharge can be used to monitor extensor activity during locomotion while L5 root discharge coincides mainly with flexor activity [1,3]. However, knowing that anatomical studies of the rat are consistent with the presence of a combination of both flexor and extensor motor axons coursing through each lumbar root level [5], raises the question whether ventral root recordings can be used reliably to selectively monitor flexion and extension phases of the step cycle. Furthermore, the occurrence of flexor motor unit discharge alternating with extensor unit activity in the same root predicts that, depending on the relative proportion of the two axon types sampled by the recording electrode, a relatively unmodulated or tonic ventral root discharge might be observed throughout the locomotor cycle. Thus, the present study examines the relationship of lumbar ventral root activity to simultaneously observed hindlimb stepping, and ankle flexor and extensor nerve activity during *in vitro* locomotion.

Methods

Experiments were performed on 55 Sprague-Dawley rats, aged 1 to 7 days. The *in vitro* chamber design and some of the methods have been described previously [6,7]. In brief, following induction of anesthesia with ether the animals were decerebrated and placed in a bath chamber containing 128 mM NaCl, 3.0 mM KCl, 0.5 mM NaH2PO4, 1.5 mM CaCl2, 1.0 mM MgSO4, 21 mM NaHCO3 and 30 mM glucose, equilibrated to pH 7.4 with 95% O2/5% CO2. The spinal cord was transected at the first cervical level and then removed, bilaterally intact, with the pelvis and hindlimbs attached. The pelvis was split along the vertebral bodies in order to expose the spinal roots. In experiments involving electroneurogram (ENG) recordings, the sciatic nerve was dissected along with the peroneal and tibial branches. The hindlimbs were then disarticulated at the pelvis and removed. Ankle extensor activity was recorded from the tibial nerve or one of its branches to the medial or lateral gastrocnemius, soleus or posterior tibial muscles. The peroneal nerve was used to monitor ankle flexor activity. Surgery was performed with cool bath temperatures (5-19oC) while recordings were obtained at 25-27 °C. Extracellular recordings of ventral root and ENG activity were obtained with glass suction electrodes. Records were digitized and stored at 5.5 kHz using a Vetter pulse code modulator videocassette adaptor. A continuous paper copy of the data was produced by an Astromed (model MT9500) oscillographic recorder. Further analysis and display of selected segments of taped data was performed with software developed for use on a Masscomp 5400 computer (sample rate 2 kHz per channel). Rhythmic activity was induced by the bath application of either 5-hydroxytryptamine (5-HT, 25-300 μM, [1]) or acetylcholine hydrochloride (ACh, 3-70 μM) in combination with the acetylcholinesterase inhibitor edrophonium chloride (EDRO, 20-500 μM, [7]). In some examples, dihydrokainic acid (DHK, 200 μM) was added to the bath, since we have observed that DHK can facilitate the development of 5-HT-induced rhythms. The neurochemicals were added directly to the bath solution and the bath chamber was repeatedly rinsed between trials of different agents. All chemicals were obtained from Sigma.

Results and Discussion

We initially examined 22 preparations to determine whether ventral root recordings from specific lumbar levels (L3-5) displayed consistent patterns of activity following the bath application of 5-HT (10 trials) or ACh/EDRO (17 trials). The hindlimbs were left attached in 7 of these preparations (7/10 5-HT trials; 2/17 ACh/EDRO trials). The activity of left and right ventral roots at the same lumbar level and a third ventral root originating from a different lumbar level was monitored in the following combinations: L3 and L4 in 13 trials, L4 and L5 in 7 trials, L3 and L5 in 6 trials. In one trial 3 segmental levels (L3, L4 and L5) were monitored (Fig. 1C).

In 10/10 trials 5-HT induced an initial tonic discharge of all ventral roots under observation. In 8/10 trials, the initial tonic increase of activity was followed by the appearance of 1 of 3 different types of rhythmic patterns within 1 - 3 minutes. The first pattern is shown in Fig. 1A, and consisted of rhythmic activity that was in-phase on all ventral roots including roots from left and right sides (L4 and L5 in 1 trial; L3 and L5 in 1 trial). The second type of rhythm induced by 5-HT consisted of ventral root activity that alternated between left and right sides while root pairs monitored on the same side of the cord were in phase (L3 and L4 in 3 trials; L3 and L5 in 1 trial). The third pattern was characterized by rhythmic activity that alternated between left and right sides as well as between root pairs on the same side of the cord (L4 and L5 in 1 trial, L3 and L5 in 1 trial, Fig. 1B). In 2/10 trials a sustained pattern of rhythmic activity failed to develop following the initial 5-HT-induced tonic discharge. In all 7 hindlimbs-attached preparations, 5-HT induced coordinated stepping movements which alternated between the right and left hindlimbs regardless of the ventral root pattern. Specifically, alternating hindlimb stepping movements occurred despite the presence of: a) tonic activity in 1 trial (L3 and L4 [50 μM 5-HT]); b) alternating left-right discharge but in-phase activity on ipsilateral ventral roots (L3 and L4 in 1 trial [25 μM 5-HT]; L3 and L5 in 1 trial [300 μM 5-HT]); and c) in-phase rhythmic discharge of all monitored ventral roots both bilaterally and at different segmental levels (L4 and L5 in 1 trial [175 μM 5-HT]; L3 and L5 in 1 trial [50 μM 5-HT]).

In 17/17 trials, ACh/EDRO elicited rhythmic discharge that alternated between the left and right sides. In all trials, ventral root pairs on the same side of the cord were in phase regardless of the segmental levels monitored (L3 and L4 in 8 trials; L4 and L5 in 5 trials; L3 and L5 in 3 trials; and L3, L4 and L5 in 1 trial, Fig. 1C). Although this pattern of ventral root activity was similar to one of the patterns of ventral root discharge observed during 5-HT-induced hindlimb stepping, we did not observe stepping in the 2 hindlimbs-attached preparations. Instead, side-to-side alternation of limb stiffening, which appeared to be due to muscle co-contractions, was observed. The possibility that direct cholinergic effects on limb muscles hindered the development of coordinated stepping during ACh/EDRO-induced activity is not excluded.

Because of the failure to obtain consistent ventral root patterns, and the discrepancy between ventral root patterns and the presence of 5-HT-induced hindlimb stepping, we examined ventral root activity in relation to identified flexor and extensor ENGs, in an additional 4 preparations. Ventral roots L3, L4, L5 and L6 were monitored during alternating rhythmic discharge from the ipsilateral tibial (extensor) and peroneal (flexor) nerves (method shown on left in Fig. 2). In 1 preparation, only increased tonic activity was observed on all ventral roots despite rhythmic alternation of the tibial and peroneal ENG activity. In another preparation, the L3 root was rhythmically active (in phase with the peroneal nerve) during reciprocal flexor and extensor ENG bursts, however roots L4, L5 and L6 were poorly modulated (Fig. 2) and demonstrated mainly an increase in tonic activity (not shown by the integrated records in Fig. 2). In 2 preparations only the L3 and L5 ventral roots were rhythmically modulated. In both cases the rhythmic L5 ventral root activity occurred in phase with tibial nerve discharge. Rhythmic L3 activity in 1 of these preparations occurred in phase with peroneal activity and could not be related to either peroneal or tibial discharge in the other preparation. These observations are in contrast to the recent suggestion that L2 and L3 ventral roots contribute mainly to extensor activity and L5 only to flexor activity during *in vitro* locomotion [1]. The occurrence of arrhythmic or weakly modulated ventral root discharge despite well developed alternating flexor and extensor ENG bursts may be

explained by the presence of both flexor and extensor motor unit activity in the same ventral root.

Further evidence that lumbar ventral root activity during locomotion may reflect a combination of flexor and extensor motor unit firing was provided by root sectioning experiments. Based on visual inspection of peroneal and tibial ENGs, we examined the effect of sequentially cutting ventral roots from L3 to L6, or inversely from L6 to L3, during rhythmic ankle flexor and extensor activity in 23 preparations (39 hindlimbs). A ventral root was considered to contribute to a particular ENG if sectioning the root either abolished or caused an unequivocal decrease in subsequent nerve activity (e.g. Fig. 3A). This method may be relatively insensitive for the identification of roots that provide only a small contribution to the total nerve activity. Using this technique, we observed that the rhythmic activity of 46% of tibial nerves and 38% of peroneal nerves depended on motor units supplied by more than one ventral root. Based on the combined results for peroneal and tibial ENGs, summarized in Fig. 3B, it is evident that each segmental level may give rise to either flexor or extensor motor units, or both. In addition, at least part of the activity of peroneal and tibial ENGs was derived from common ventral roots in 79% (31/39) of the hindlimbs examined. In 8 other preparations, the contribution of ventral roots L2-L6 to biceps femoris, iliacus, and vastus lateralis ENG discharge was examined during rhythmic activity. Biceps femoris (a hip extensor [2]) was supplied by L4 and L5 in 2 preparations, by L5 and L6 in 1 preparation, and by L4 alone in 1 preparation. Rhythmic iliacus (a hip flexor [2]) ENG activity derived from L3 alone in 4 preparations and L2 alone in 1 preparation. Vastus lateralis (a knee extensor [2]) received innnervation from L3 alone in 2 preparations and L3 and L4 in 1 preparation. These findings further illustrate that single lumbar roots can contribute to multiple flexor and extensor ENGs.

The use of ventral root recordings for routine monitoring of locomotor rhythms will be further confounded by any variation among animals in the relative endowment of specific flexor or extensor motor units at a particular root level, as well as any variation in the proportion of flexor versus extensor units sampled by the extracellular recording electrode. In addition, the amount of flexor compared to extensor activity recorded from

any particular root might be expected to vary according to the motor behaviour under observation and its method of induction. Although ventral root recordings during locomotion in the *in vitro* rat preparation may be adequate for certain experimental requirements, we conclude that this method does not consistently detect the presence of rhythmic activity, nor do ventral root recordings reliably identify the flexion and extension phases of the step cycle. Electrophysiological characterization of locomotor patterns is best accomplished through the use of either ENG or EMG recordings.

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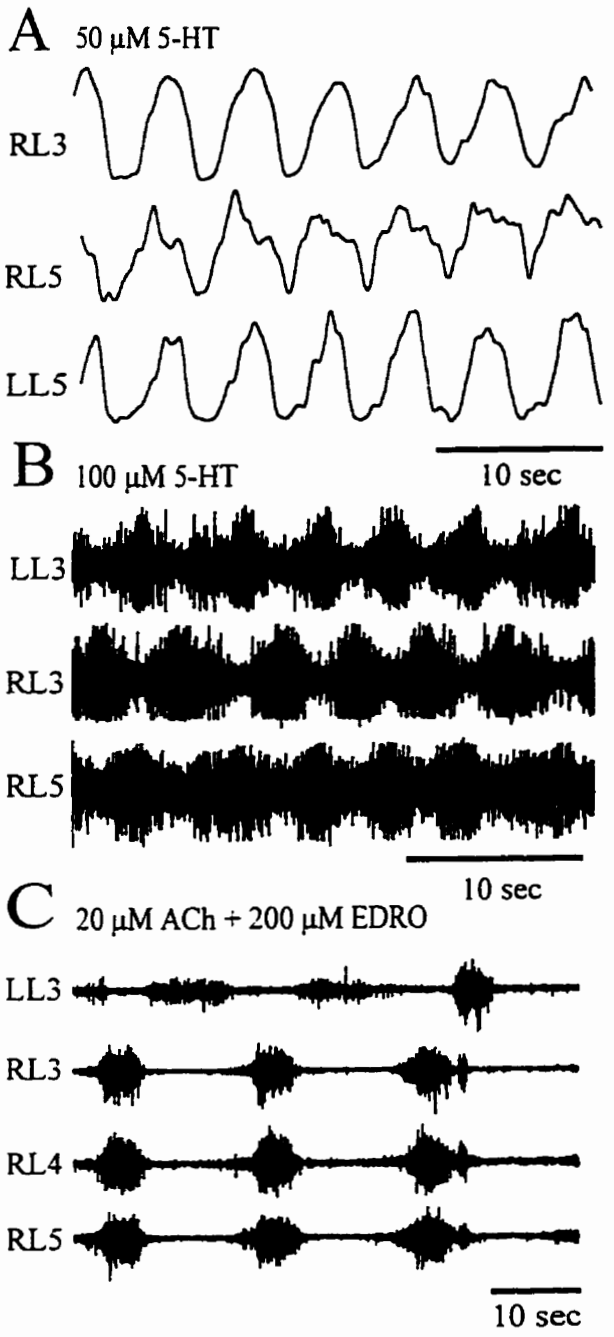

A
50 µM 5-HT
RL3
RL5
LL5
10 sec
B
100 µM 5-HT
LL3
RL3
RL5
10 sec
C
20 µM ACh + 200 µM EDRO
LL3
RL3
RL4
RL5
10 sec

Figure 1

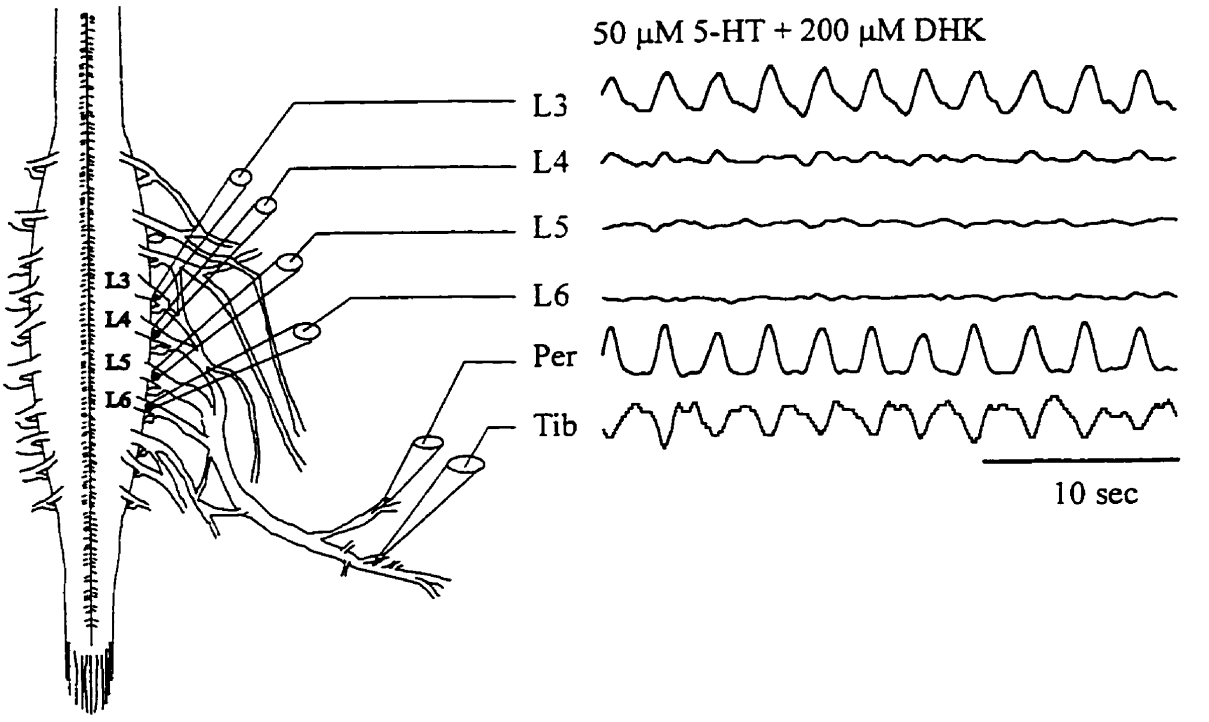
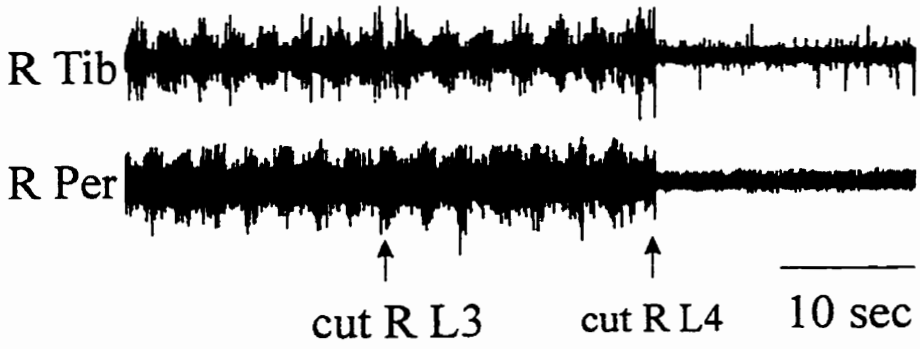

50 μM 5-HT + 200 μM DHK
L3
L4
L5
L6
Per
Tib
10 sec
L3
L4
L5
L6
Ventral Surface

Figure 2

A


R Tib
R Per
cut R L3
cut R L4
10 sec

B

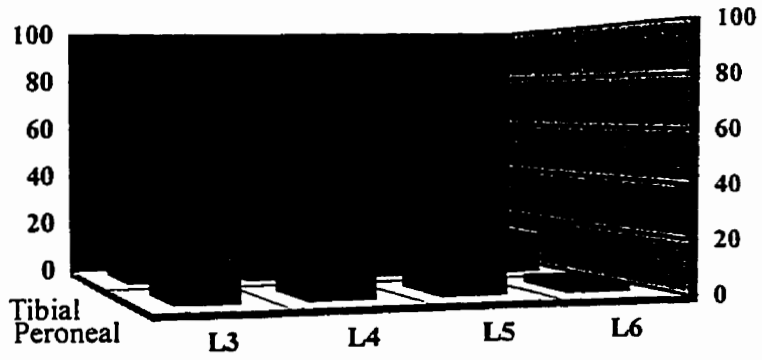

100
80
60
40
20
0
Tibial
Peroneal
L3
L4
L5
L6

Figure 3

Figure Legends

Figure 1. Different patterns of rhythmic ventral root discharge following 5-HT or ACh/EDRO. A. 5-HT-induced activity that was in-phase bilaterally on the L5 ventral roots and ipsilaterally on the right L3 and L5 roots. B. In this example, 5-HT-induced alternating discharge of the right and left L3 ventral roots; the right L3 and L5 root activity was also out of phase. Well coordinated hindlimb stepping movements were observed while recording the ventral root activity shown in A and B. ACh/EDRO-induced alternating activity between left and right L3 ventral roots while the right L3, L4 and L5 roots were active in phase. Waveforms in A were integrated during recording.

Figure 2. Right L3-L6 integrated ventral root activity during alternating rhythmic discharge of the ipsilateral peroneal (flexor) and tibial (extensor) nerves. The L3 ventral root rhythmicity was well developed, while L4, L5 and L6 root activity was not well modulated. Unfiltered records (not shown) of L4, L5, and L6 demonstrated an increase in tonic discharge coincident with the occurrence of rhythmic ENG activity.

Figure 3. A. Cutting R L4 (but not L3, L5, or L6) resulted in abolition of activity in both the tibial and peroneal ENGs indicating that L4 contributed to each of the tibial and peroneal ENG discharge in this example. B. Relative contribution of different lumbar ventral roots to peroneal and tibial ENGs during rhythmic activity in 39 hindlimbs. Each bin indicates the percentage of tibial or peroneal nerves studied that received at least some of their motor units via the indicated root level. For example, 82% of the peroneal nerves examined received at least some of their axons via the L4 ventral root.

PART II: A Comparison of Motor Patterns Induced by N-methyl-D-aspartate, Acetylcholine and Serotonin in the In Vitro Neonatal Rat Spinal Cord.

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Key words: locomotion, spinal cord, NMDA, acetylcholine, serotonin, *in vitro*, CPG, rat

Summary

Using an *in vitro* preparation from neonatal rat spinal cord, we compared the motor patterns induced by three putative locomotion-inducing substances. N-methyl-D,L-aspartate (NMA) induced rhythmic hindlimb nerve activity in 17/20 preparations that was characterized by: a) side-to-side alternation, but co-activated intralimb flexor-extensor pairs in 29%; b) bilateral co-activation of all flexors and extensors in 24%; and c) rhythmic but poorly coordinated activity in 35%. Acetylcholine induced rhythmic activity in 34/35 preparations, which in 68% of animals was characterized by side-to-side alternation of co-activated intralimb flexor-extensor pairs. Only rarely did NMA (2/20 trials) and acetylcholine (1/35 trials) induce sustained ENG patterns compatible with hindlimb stepping. Serotonin, however, induced rhythmic activity in 22/24 preparations that was consistent with locomotion in intact rats in 13/22 (59%). These findings demonstrate that exogenously applied neurochemicals induce a variety of *in vitro* motor rhythms although some substances preferentially activate specific patterns. The results also highlight the importance of monitoring flexor and extensor activity from both hindlimbs in order to distinguish locomotor-like patterns from other types of neurochemically-induced rhythms.

Introduction

Several exogenously administered neuroactive substances have been identified that are capable of inducing locomotor activity in the mammalian lumbar cord, including L-dopa, clonidine, norepinephrine (see [7]), and N-methyl-D-aspartate (NMDA; [4]) in the cat, and 5-hydroxytryptophan (5-HTP) and L-dopa in the rabbit [13]. Glutamate, aspartate, NMDA, kainate, dopamine, serotonin (5-HT), norepinephrine, and acetylcholine (ACh) have been reported to induce locomotor-like rhythmicity in the *in vitro* rat spinal cord (e.g. [1,3,8,11,12]). Whether these neuroactive substances achieve their effects through excitation of a common locomotor network, or instead activate functionally or anatomically distinct spinal rhythm generating elements is unknown.

Evidence that different rhythm-inducing substances may be associated with specific actions is suggested by the observation that some neurochemicals selectively enhance either the flexion or extension phase of the locomotor cycle. For example, excitatory amino acid-induced locomotion in the *in vitro* neonatal rat has been reported to be associated with enhanced flexion phase activity while dopamine-induced rhythmicity was associated with enhanced extension phase activity [2]. In the acute spinal rabbit, intravenously administered L-dopa selectively facilitated extension phase activity whereas 5-HTP enhanced the flexion phase [13]. Further characterization of motor patterns may help clarify the role of these substances, if any, in the endogenous initiation and maintenance of activity in mammalian rhythm generating circuits. In the present study we monitor bilateral hindlimb flexor and extensor nerve activity in the *in vitro* neonatal rat spinal cord preparation, to determine whether several putative locomotion-inducing substances (NMA, 5-HT and ACh) activate distinct motor patterns.

Methods

Experiments were performed on 37 Sprague-Dawley rats (1 - 7 days). The *in vitro* chamber design and methods used are similar to those described previously [10,11]. In brief, following the induction of anesthesia with ether the animals were decerebrated and placed in a bath chamber containing 128 mM NaCL, 3.0 mM KCl, 0.5 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 21 mM $NaHCO_3$ and 30 mM glucose, equilibrated to pH 7.4 with 95% O_2/5% CO_2. The spinal cord was transected at the first cervical level and then removed, bilaterally intact, with the hindlimbs attached. The pelvis was split along the vertebral bodies to expose the lumbosacral cord and spinal roots to the bath solution. Dissection of the peroneal and tibial branches of the sciatic nerve was followed by disarticulation and removal of each hindlimb at the pelvis. Dorsal roots were left intact and the pelvis was stabilized to prevent movement from occurring during the application of neurochemicals. The peroneal nerve was used to monitor ankle flexor activity. Ankle extensor activity was monitored using the tibial nerve or one of its branches to the medial or lateral gastrocnemius, soleus or posterior tibial muscles. Surgery was performed with cool bath temperatures (5-19 °C) while recordings were obtained at 25-27 °C. Electroneurograms (ENGs) were obtained using glass suction electrodes. Records were digitized and stored at 5.5 kHz using a Vetter pulse code modulator videocassette adaptor. Further analysis and display of selected segments of taped data was performed using software developed for use on a Masscomp 5400 computer (sample rate 2 kHz per channel). Pharmacological substances were added directly to the bath solution and the bath chamber was rinsed repeatedly between trials of different substances. The effects of the following neurochemicals were examined: N-methyl-D,L-aspartate (NMA; 7-16 μM); acetylcholine (ACh; 10-80 μM) in combination with the acetylcholinesterase inhibitor edrophonium (EDRO; 100-300 μM); and serotonin (5-HT; 50-200 μM). All chemicals were obtained from Sigma. No relation was observed between the concentration of applied rhythm-inducing neurochemical and type

of pattern induced. All three neurochemicals were tested in the same animal in 10 of the 37 experiments.

Results and Discussion

ACh (10-80 μM, mean 38 μM) in combination with EDRO (100-300 μM, mean 205 μM) induced rhythmic alternation of left and right hindlimb ENGs (0.04-0.41 Hz, mean 0.10 Hz) associated with in-phase activation of flexor and extensor nerves within each hindlimb, in 23/35 preparations (Fig. 1A). In another 5/35 preparations ACh (10-60 μM, mean 36 μM) in combination with EDRO (100-200 μM, mean 180 μM) initially induced rhythmic flexor-extensor alternation within one hindlimb that was coupled to extensor-flexor alternation in the contralateral hindlimb (0.06-0.37 Hz, mean 0.22 Hz), compatible with an overground locomotor pattern in the adult intact rat [6]. This pattern was sustained in only 1 of the 5 preparations. The pattern changed within 5 minutes in the other 4 preparations, becoming either uncoordinated or similar to that shown in Fig. 1A. In 4/35 experiments ACh (10-30 μM, mean 18 μM) with EDRO (200 μM) induced rhythmic activity that displayed no clear pattern of flexor-extensor or left-right relationship. In 2/35 preparations flexor and extensor ENG activity in one hindlimb was in-phase, while ENGs on the contralateral side showed alternating discharge of the flexor-extensor pair during application of ACh (10 and 40 μM) and EDRO (200 μM). One preparation displayed only a transient (2 minute) increase in tonic flexor and extensor activity during application of 20 μM ACh and 200 μM EDRO.

5-HT was applied to 24 preparations, all of which showed an immediate increase in tonic ENG activity. In 22 of the 24 preparations, initial tonic activity was followed within 1 to 3 minutes by the appearance of rhythmic modulation of ENG discharge. In 13 of the 22 preparations 5-HT (50-200 μM, mean 92 μM) induced a sustained pattern of ENG activity (0.07-0.37 Hz, mean 0.26 Hz) compatible with locomotion in intact rats, as shown by the example in Fig. 1B. In 3/22 preparations 5-HT (75-150 μM, mean 108 μM) induced rhythmic alternation of left and right hindlimbs (0.05-0.22 Hz, mean 0.13 Hz) with in-phase intralimb flexor and extensor discharge, similar to the ACh/EDRO-induced example shown in Fig 1A. In 4/22 preparations 5-HT (75-150 μM, mean 100 μM) induced rhythmic activity of only one ENG in each hindlimb which alternated

between the left and right sides (0.25-0.34 Hz, mean 0.29 Hz), while the other ENG from each hindlimb pair was tonically active. 5-HT (75 and 50 μM) induced rhythmic, but poorly coordinated, activity with no clear pattern of nerve discharge in 2/22 preparations.

In all preparations tested, NMA induced an initial tonic increase of ENG activity, that in 17 of 20 experiments was replaced by rhythmic modulation of activity after several minutes. In 5 of the 17 rhythmically active preparations, NMA (7-16 μM, mean 10 μM) induced left-right alternation (0.11-0.73 Hz, mean 0.33 Hz) but co-activation of intralimb flexor-extensor ENG pairs (e.g. Fig. 1C), similar to the pattern induced by ACh/EDRO shown in Fig. 1A. In another 4/17 preparations NMA (9-12 μM, mean 11 μM) induced rhythmic co-activation of all ENGs (0.05-0.20 Hz, mean 0.12 Hz; Fig. 2B). NMA (10 and 12 μM) induced rhythmic activity consistent with locomotion (0.57-0.71 Hz) in only 2/17 preparations. In contrast to 5-HT-induced activity, NMA-induced rhythms were often interrupted by periods of reduced ENG amplitude or complete cessation of rhythmicity lasting 10 to 60 seconds. In 6/17 rhythmically active preparations the patterns were poorly sustained and variable during the application of NMA (10-16 μM, mean 13 μM; e.g. Fig. 2).

The occurrence of rhythmic discharge that was bilaterally synchronous among all flexor and extensor ENGs was unique to NMA-induced trials. Among rhythmically active preparations, an alternating side-to-side pattern associated with co-activation of flexor and extensor ENGs within each limb was observed in 68% of ACh/EDRO trials, 29% of NMA trials, and 14% of 5-HT trials. Neither of these 2 patterns, that include co-activation of intralimb antagonists, resemble the sequence of hindlimb flexor and extensor activity seen during locomotion in intact rats [6].

A previous study of the *in vitro* rat reported that NMDA receptor activation induced an alternating pattern of lumbar ventral root activity on the left and right sides in 53% of animals; although, with time some with time some preparations showed "desynchronization of the contralateral activities" [3]. One reason for the low incidence (12%) of NMA-induced locomotor-like patterns observed in the present study may be

related to our use of bilateral hindlimb flexor and extensor ENGs rather than ventral root recordings. Thus, although 85% of our NMA-induced preparations were rhythmically active, only a more detailed analysis of the timing of bilateral flexor and extensor activity demonstrated that many of these patterns were incompatible with locomotor activity. Another study described the occurrence of alternating ankle flexor and extensor EMG activity from both hindlimbs in 84% of NMA-induced *in vitro* rat preparations [8]. ENG recordings should provide similar information about rhythmic efferent activity to that obtained using EMGs. However, if phasic afferent feedback from muscle receptors has any influence in determining the rhythmic pattern induced by NMA, this effect will be absent in our hindlimbs-detached preparation. The infrequent occurrence of NMA-induced locomotion we observed is not likely due to technical limitations of ENG recordings, since 5-HT alone induced a locomotor-like rhythm in 9 of the 11 preparations tested separately with both NMA and 5-HT (NMA induced a locomotor pattern in only 1 of these examples).

5-HT was the best single agent for the induction of a locomotor-like pattern in this series. The occurrence of locomotion in 57% of the trials is similar to the 41% incidence of stable 5-HT-induced ventral root rhythmicity previously observed in this preparation [3]. These findings suggest an important role for 5-HT in the endogenous activation or operation of mammalian locomotor networks, as supported by evidence from previous studies. (reviewed in [3]).

Initial studies of locomotion using *in vitro* rat preparations suggested a possible role for cholinergic mechanisms in the induction of rhythmic spinal cord activity [10,11]; and a recent study of the Xenopus spinal cord has also implicated cholinergic mechanisms in the operation of the central pattern generator [9]. Our results confirm that cholinergic receptor activation regularly induces rhythmic activity in this preparation (97% of trials). However, unlike previous reports [10,11], the present study systematically monitored bilateral flexor and extensor ENG activity and demonstrated a stable locomotor-like sequence of ENG activation in only 1/35 experiments. Experimental conditions alone do not appear to account for the rare occurrence of

ACh/EDRO-induced locomotor patterns in this series, since 52% of the 23 preparations tested with ACh/EDRO displayed locomotor patterns when separately tested with 5-HT. Although ACh/EDRO commonly established rhythmic alternation of left-right activity (83%), it appears that bath applied cholinergic agonists fail to promote reciprocal interactions between flexor-extensor antagonist pairs of the same hindlimb.

Several factors may account for the failure of exogenously applied neurochemicals to induce consistent patterns of motor activity. For instance, the time course of action of an endogenously released neurotransmitter and its specific site of action within the spinal cord circuitry cannot be mimicked by the exogenous application of the substance to the bath. In addition, the administration of single neurochemicals may not activate network or cellular properties that normally require the combined actions of more than one neuroactive substance. For example, in other experiments we have noted that the excitatory amino acid uptake inhibitor, dihydrokainic acid, regularly facilitates the development of locomotor patterns during 5-HT application. Finally, different behaviours may be generated by functionally and anatomically overlapping neural substrates, as illustrated by the common mechanism thought to be involved in the generation of both scratching and locomotor rhythms in cats (eg. [5]). Therefore, although activation of a particular receptor such as the NMDA receptor may be essential for the generation of rhythmic activity, the specific motor pattern that emerges from a network likely depends on other neural control mechanisms or conditions. Because the motor patterns activated by exogenously applied substances are unpredictable, it seems prudent to monitor flexor and extensor activity from both hindlimbs to distinguish locomotion from other types of neurochemically-induced rhythms. Although the analysis of ankle flexor and extensor ENG patterns permits the distinction of several types of motor patterns, future studies that include simultaneous recordings of multiple nerves or muscles throughout the hindlimb would be of value in further characterizing the effects of neuroactive substances.

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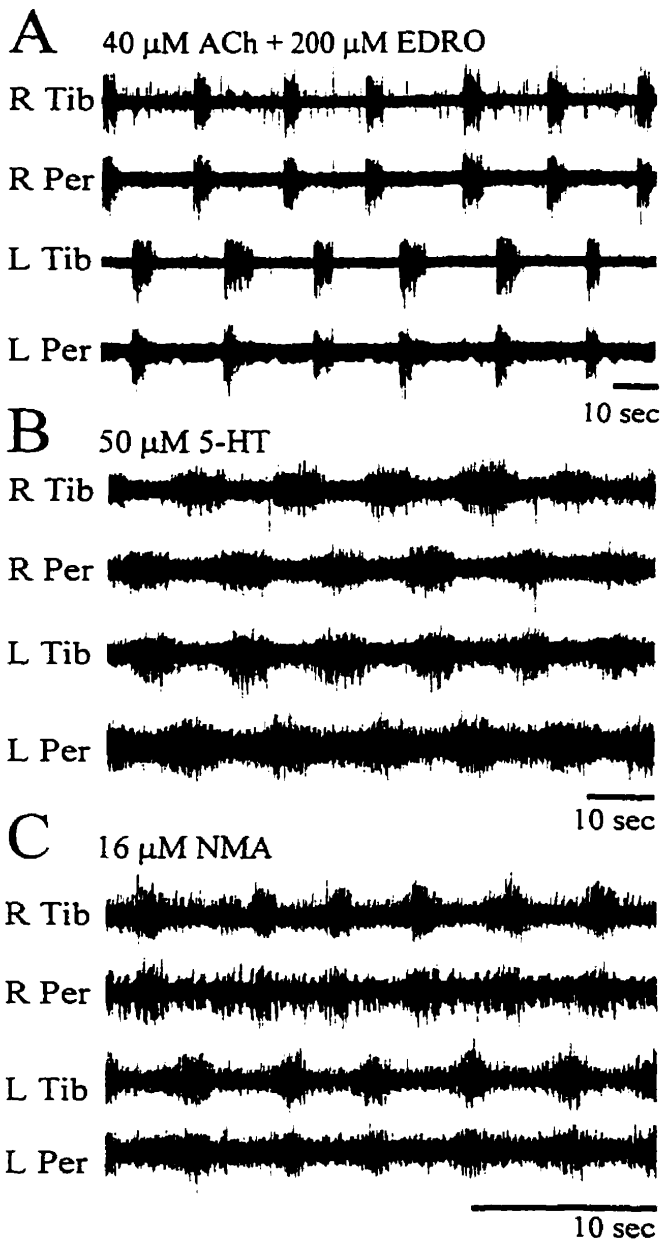
A
40 μM ACh + 200 μM EDRO
R Tib
R Per
L Tib
L Per
10 sec
B
50 μM 5-HT
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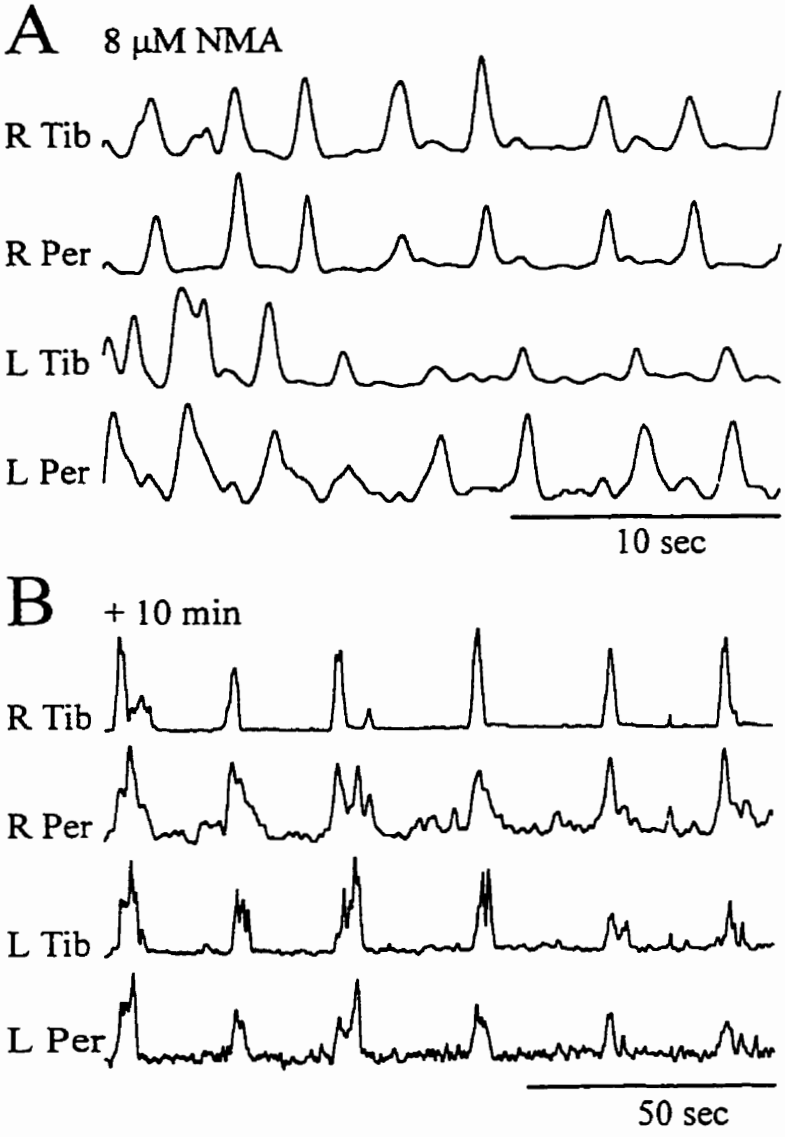
Figure 1


A
8 μM NMA
R Tib
R Per
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L Per
10 sec
B
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Figure 2

Figure Legends

Figure 1. Examples of ankle flexor and extensor ENG patterns during bath application of ACh/EDRO, 5-HT and NMA. **A:** ACh/EDRO induced rhythmic alternation of left and right hindlimbs; however, the flexor-extensor pair in each hindlimb showed in-phase activity. **B**: 5-HT induced rhythmic activity characterized by co-activation of the right flexor and left extensor nerves alternating with right extensor and left flexor nerve co-activation. This sequence of ENG activity is compatible with patterns expected during locomotion in intact rats. **C**: In this example the NMA induced rhythm was similar to the pattern frequently induced by ACh/EDRO (e.g. Fig. **1A**). Rhythmic ENG activity in **B** and **C** was superimposed on increased tonic background discharge.

Figure 2. Two patterns of motor rhythm during the same trial of NMA-induced activity. The rhythm shown in **A** consisted of in-phase flexor-extensor activity on one side alternating with in-phase activity of the ENG pair on the contralateral side. This pattern was replaced by a period of poorly coordinated ENG activity and then (10 minutes later in **B**) by rhythmic co-activation of all ENGs. Waveforms were rectified and integrated.

PART III: Effects of Inhibitory Amino Acid Antagonists on Reciprocal Inhibitory Interactions During Rhythmic Motor Activity in the In Vitro Neonatal Rat Spinal Cord

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Abbreviated title: Inhibitory neurotransmission during rhythmic activity

Summary and Conclusions

1. The role of inhibitory amino acid transmission in the coordination and generation of rhythmic motor activity was examined with the use of an *in vitro* neonatal rat spinal cord preparation. Before adding γ-aminobutyric acid (GABA) or glycine receptor agonists and antagonists, rhythmic motor activity was induced by bath application of acetylcholine (ACh), N-methyl-D,L-aspartate (NMA), or serotonin (5-HT) while monitoring bilateral ankle flexor and extensor electroneurograms (ENGs). The timing of rhythmic flexor and extensor discharge was consistent with that seen during overground locomotion in 27% of 84 bath applications of these substances (n = 65 preparations).

2. Subsequent addition of the $GABA_A$ receptor agonist muscimol, the $GABA_B$ receptor agonist baclofen, or glycine, abolished rhythmic activity in 95% of the tested applications.

3. $GABA_B$ receptor blockade did not disrupt alternating patterns of ENG discharge. However, addition of the $GABA_A$ receptor antagonist bicuculline, or the glycine receptor antagonist strychnine, transformed alternating flexor-extensor and left-right activity into patterns characterized by bilaterally synchronous rhythmic activation of all hindlimb ENGs. The onset of individual ENG bursts was more abrupt following bicuculline or strychnine. Strychnine also synchronized high frequency (4-8 Hz) packets of rhythmic discharge within ENG bursts.

4. Some preparations developed synchronous, but unstable, rhythmic activity in the presence of bicuculline or strychnine alone. However, NMA, 5-HT, or ACh was usually required in addition to these antagonists to promote sustained rhythmic activity.

5. The results suggest that 1) $GABA_A$ and glycine receptor activation may mediate reciprocal left-right and flexor-extensor phase relationships during locomotion, 2) blockade of these receptors facilitates the expression of rhythms dominated by excitatory coupling within the rhythmogenic network, and 3) during rhythmic activity, inhibitory amino acid transmission may influence phasic discharge characteristics but is not essential for generation of the rhythm.

Introduction

It has been proposed that the alternating activation of functional antagonists during locomotion is coordinated by reciprocal inhibitory interactions within the spinal rhythm generating network (Cohen and Harris-Warrick 1984; Dale 1985; Jankowska et al. 1967; Lundberg 1981). Previous studies of various vertebrate preparations suggest that inhibitory amino acid neurotransmitters may mediate these interactions. For example, in the *Xenopus* and lamprey spinal cord, glycinergic receptors mediate side-to-side co-ordination of antagonistic motor groups during swimming (e.g. Cohen and Harris-Warrick 1984; Dale 1985). Although GABAergic systems do not appear to be involved in these reciprocal interactions, $GABA_A$ and $GABA_B$ mediated mechanisms contribute to longitudinal intersegmental co-ordination in the lamprey spinal cord (Alford et al. 1990; Matsushima et al. 1993; Tegnér et al. 1993).

Inhibitory amino acid transmitters may also modify the operation of mammalian locomotor circuits. For example, walking is facilitated in chronic spinal cats treated with the $GABA_A$ receptor antagonist bicuculline (Robinson and Goldberger 1986). In the *in vitro* neonatal rat spinal cord preparation, GABA abolishes locomotor activity (Atsuta et al. 1991; Cazalets et al. 1994), whereas GABA receptor antagonists modify the frequency and amplitude of rhythmic motor bursts (Cazalets et al. 1994). Similarly, glycinergic mechanisms may be involved in mammalian locomotor systems. Walking is facilitated in spinal dogs treated with the glycine receptor antagonist strychnine (Hart 1971), and rhythmic inhibition of cat motoneurons during locomotion is suppressed following intravenous strychnine (Pratt and Jordan 1987).

Although these studies suggest that inhibitory amino acid transmission may modulate overall activity of mammalian locomotor networks, the precise role, if any, of these neurotransmitters in determining the pattern of motor output remains is unknown. However, several lines of evidence suggest a potential role for glycine. Strychnine converts alternating left-right ventral root discharge into bilaterally synchronous activity in fetal rats (Kudo et al. 1991) and synchronizes high frequency flexor-extensor EMG bursts in the neonatal mouse (Droge and Tao 1993). Strychnine also disrupts flexor-

extensor co-ordination during fictive locomotion in the cat (Kriellaars et al. 1988; Noga et al 1993).

In the present study, we examine whether GABAergic and glycinergic mechanisms mediate reciprocal inhibitory interactions during locomotion in postnatal mammals, with the use of an *in vitro* neonatal rat preparation. Because some models of locomotor network rhythmogenesis include an essential requirement for inhibitory connections between circuit elements (e.g. Friesen and Stent 1977; Kling and Szekely 1968), these experiments also examined whether rhythm generation itself depends on inhibitory amino acid transmission.

A preliminary report of this work appeared in abstract form (Harder and Schmidt 1992).

Methods

Experiments were performed on 65 Sprague-Dawley rats, 1 to 7 days old. The *in vitro* chamber design and methods used are similar to those described previously (Smith and Feldman 1987). Briefly, after induction of anesthesia with ether, the animals were decerebrated and placed in a 50 ml bath chamber containing (in mM): 128 NaCl, 3.0 KCl, 0.5 NaH_2PO_4, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 21 $NaHCO_3$, and 30 glucose, equilibrated with 95% O_2-5% CO_2, pH 7.4. The spinal cord was transected at the first cervical level and then removed, bilaterally intact, with the pelvis and hindlimbs attached. The pelvis was split along the vertebral bodies in order to expose the lumbosacral cord and spinal roots to the bath solution. After dissection of the sciatic nerve along with its tibial and peroneal branches, the hindlimbs were disarticulated at the pelvis and removed. The peroneal nerve was used to monitor ankle flexor activity. Ankle extensor activity was monitored with the use of the tibial nerve or one of its branches to the medial or lateral gastrocnemius, soleus or posterior tibial muscles. Surgery was performed with cool bath temperatures (5 - 19 ^{0}C) whereas recordings were obtained at 25 - 27 ^{0}C.

Electroneurograms (ENGs) were obtained with the use of glass suction electrodes. Records were digitized at 5.5 kHz and stored using a Vetter pulse code modulator videocassette adaptor. A continuous paper copy of the data was produced by an Astromed (model MT9500) oscillographic recorder. Further analysis and display of selected segments of taped data was performed on a Masscomp 5400 computer (sample rate 2 kHz per channel).

All neurochemicals used in this series were initially dissolved in distilled water and stored as millimolar stock solutions. With the use of a micropipette, the selected volume of concentrated stock solution was then slowly applied in fractionated amounts at the periphery of the chamber, during vigorous bubbling, in order to obtain the desired final concentration of the bath solution. All neurochemical concentrations, including ranges, specified in this report refer to the final bath concentration. For the purposes of this study, a 'trial' begins with application of one or more substances to the bath and ends after washout of the added neurochemicals. Recordings were obtained after waiting 2 to

10 minutes for a stable pattern of rhythmic activity to emerge. Stable patterns were monitored for 5 to 15 minutes before adding further neurochemical agents. The recording chamber was flushed with normal bath solution repeatedly between trials. Rhythmic activity was induced by bath application of either serotonin (5-hydroxytryptamine, 5-HT; 50-200 μM, mean 120 μM, 17 trials); acetylcholine hydrochloride (ACh, 10-80 μM, mean 40 μM, 36 trials) with the acetylcholinesterase inhibitor edrophonium chloride (EDRO, 100-300 μM, mean 200 μM, 36 trials); or N-methyl-D,L-aspartate (NMA, 7-21 μM, mean 13 μM, 19 trials). 5-HT (5-75 μM, mean 40 μM) was used in combination with dihydrokainic acid (DHK, 200 μM) in 5 other trials, since DHK enhances the development of 5-HT-induced rhythms (personal observations). In an additional seven trials, various combinations of these neuroactive agents were used to induce rhythmic activity. Once a stable pattern of rhythmic activity was established, the effects of the following inhibitory amino acid agonists and antagonists were tested: muscimol, baclofen, glycine, bicuculline methiodide (BIC), 2-hydroxysaclofen, phaclofen, and strychnine hydrochloride. All drugs used in this series were obtained from Sigma, with the exception of 2-hydroxysaclofen and phaclofen, which were obtained from Research Biochemicals International.

Results

Before the addition of inhibitory amino acid agonists or antagonists several patterns of neurochemically-induced rhythmic activity were observed during 84 trials. These patterns included: 1) rhythmic co-activation of intralimb flexor-extensor ENG pairs but alternating activity between the left and right sides (52% of trials), 2) a locomotor pattern (27% of trials, e.g. Figs. 1 and 2, *A1* and *B1*), and 3) simultaneous rhythmic activation of all flexor and extensor ENGs (6% of trials, e.g. Fig. 3*A*). The locomotor pattern was characterized by alternating flexor-extensor activity in one hindlimb coupled with extensor-flexor alternation in the contralateral hindlimb, similar to that reported in adult intact rats during locomotion (Gruner et al. 1980). ACh in combination with EDRO (ACh/EDRO) commonly elicited the first type of pattern (31/36 ACh/EDRO trials). Simultaneous rhythmic discharge of all flexors and extensors bilaterally was observed only during NMA-induced activity (5/19 NMA trials). 5-HT most commonly produced an ENG discharge sequence consistent with locomotion (14/17 5-HT trials), although this pattern was also induced by NMA (1/19 NMA trials) and ACh/EDRO (1/36 ACh/EDRO trials). The occurrence of these different patterns of ankle flexor and extensor activity is consistent with observations we have detailed elsewhere (Cowley and Schmidt 1994). The effect of adding inhibitory amino acid receptor agonists and antagonists in these experiments was independent of the initial pattern of induced rhythmic activity.

Effects of inhibitory amino acid receptor agonists on locomotor rhythms

The effect of inhibitory amino acid receptor agonists on neurochemically-induced rhythmic activity was examined in 60 trials (38 preparations). Rhythmic ENG discharge was induced with NMA in 15 trials, ACh/EDRO in 28 trials; and 5-HT in 12 trials. Various combinations of these neurochemicals were used to elicit rhythmic ENG activity in the 5 other trials.

The $GABA_A$ receptor agonist muscimol (2-10 μM, mean 8 μM) abolished rhythmic ENG discharge in 11/11 trials (11 preparations), and the $GABA_B$ receptor agonist baclofen (1-16 μM, mean 4 μM) abolished rhythmic activity in 20/22 trials (15 preparations), consistent with the results by Cazalets et al (1994). Similar to the effect of GABA receptor agonists, we observed that glycine (400-6000 μM, mean 1200 μM) abolished locomotor rhythmicity in 26/27 trials (12 preparations; e.g., Fig. 1).

Effects of $GABA_A$ and glycine receptor antagonists on motor patterns

In six of six preparations, initial out-of-phase rhythmic flexor and extensor ENG discharge became synchronous following addition of the $GABA_A$ receptor antagonist bicuculline to the bath (e.g. Fig. 2, *A1* and *A2*). The final bath concentration of bicuculline ranged from 10-25 μM (mean 18 μM). The pattern of rhythmic discharge observed before bicuculline-induced synchronization was consistent with locomotion in two of the six preparations, whereas, in four of the six preparations, left-right alternation was present, but intralimb flexor and extensor discharge was overlapping (but not synchronous in onset).

The effect of the glycine receptor antagonist strychnine on rhythmic discharge was similar to the effect of bicuculline. Rhythmic out-of-phase discharge became synchronous following the addition of strychnine (6-20 μM, mean 16 μM) in six of six experiments (e.g. Fig. 2, *B1* and *B2*). The ENG pattern recorded before addition of strychnine was compatible with locomotion in five of these experiments.

In addition to synchronizing hindlimb nerve activity, bicuculline and strychnine altered the shape of individual ENG discharge envelopes. Both antagonists caused a more abrupt onset of ENG bursts. Strychnine also produced an abrupt termination of discharge in all trials (e.g. Fig. 2*B2*). The amplitude of bicuculline-induced synchronous ENG discharge envelopes tapered more gradually than that observed in response to strychnine (e.g. Fig 2*A2*), although, in one example (shown in Fig. 5) relatively abrupt termination of bursts was noted.

Strychnine also had synchronizing effects on motor-unit activity within individual ENG bursts. For example, in one preparation the initial NMA-induced pattern consisted of simultaneous activation of flexors and extensors, bilaterally (Fig. 3*A1*). Following strychnine (18 μM), not only was the onset and termination of each ENG burst more abrupt (Fig. 3*B1*), but strychnine also promoted synchronous high frequency (4-8 Hz) packets of discharge within each ENG burst, as shown on the expanded time scale in Fig. 3*B2*. ENG bursts in some NMA, 5-HT, and ACh/EDRO-induced trials displayed 4-8 Hz packets of discharge before the addition of strychnine. However, these packets were predominantly asynchronous and only after the application of strychnine did multiple ENGs display in-phase discharge of the type shown in Fig. 3*B2*. ENG bursts composed of synchronous 4-8 Hz packets of discharge were observed in 80% of the strychnine-treated preparations and during none of the bicuculline trials.

Because our interest was to examine the effect of maximal blockade of inhibitory amino acid receptors on the co-ordination of alternating rhythmic activity, we did not systematically test the effects of low or threshold concentrations of antagonists on rhythm frequency and amplitude. The initial bath concentration of bicuculline was <5 μM in only a few trials. However, immediately following the addition of bicuculline, the amplitude and frequency of alternating rhythmic activity increased in four of six trials before converting to a synchronized pattern. Increased amplitude and frequency of alternating ventral root discharge in the presence of low concentrations of bicuculline (<5 μM) was reported by Cazalets et al. (1994). In the present series, strychnine also produced a transient increase in the frequency and amplitude of the alternating rhythm in 2 trials before establishing a synchronous pattern. In other preparations the effect of bicuculline and strychnine on alternating rhythm could not be analyzed because the transition from an alternating to a synchronous pattern was dominated by a period of disorganized or tonic bursting, lasting up to several minutes. Once established, however, the synchronous rhythms were slower than the initial alternating patterns in four of six bicuculline and five of six strychnine trials (e.g. Fig. 2).

The synchronizing effect of bicuculline on flexor and extensor activity was completely reversible after washout. However, despite repeated washout of strychnine-containing bath solution, the rhythmic discharge evoked by re-application of NMA, 5-HT or ACh/EDRO remained in-phase.

In 14 trials, NMA, EDRO/ACh or 5-HT failed to elicit stable rhythmic activity. However, the subsequent addition of bicuculline (8-30 μM; mean 18 μM) in nine trials, and strychnine (8-30 μM; mean 18 μM) in five trials induced well developed synchronous rhythmic activity in each instance.

Effect of $GABA_B$ receptor antagonists on motor patterns

In contrast to the effect of $GABA_A$ and glycine receptor antagonists, synchronous rhythmic discharge was not observed following the application of $GABA_B$ antagonists. In particular, neither phaclofen (4 preparations; 100-2000 μM, mean 1,800 μM) nor 2-hydroxysaclofen (6 preparations; 200-1,500 μM, mean 800 μM) disturbed the phase relationship of hindlimb flexor-extensor discharge, regardless of the initial ENG pattern or neurochemical used to induce activity.

Because $GABA_B$ receptor antagonists appeared to have no effect on the timing of rhythmic flexor and extensor discharge, we tried to establish whether sufficient concentrations of the antagonists had been used. Therefore in three preparations tested with phaclofen and in two preparations tested with 2-hydroxysaclofen, we also determined the concentration of antagonist required to block baclofen-mediated ($GABA_B$ receptor-activated) suppression of neurochemically-induced rhythmic activity. Following baclofen-induced ENG silence, rhythmic activity was restored by phaclofen and 2-hydroxysaclofen at bath concentrations that were 15-53% and 20-30%, respectively, of the final concentrations used to test the effect of these antagonists on motor patterns in the same preparations in the absence of baclofen. Thus the failure of phaclofen and 2-hydroxysaclofen to alter neurochemically induced alternating rhythms in these experiments was not likely due to inadequate concentrations of these agents.

Effects of $GABA_A$ and glycine receptor antagonists in the absence of other neuropharmacological agents

We examined whether the bath application of bicuculline or strychnine alone could induce rhythmic ENG discharge. In 5/13 bicuculline trials (12 preparations; 10-80 μM, mean 42 μM) and 10/15 strychnine trials (14 preparations; 8-32 μM, mean 14 μM) neither phasic nor tonic ENG activity was induced. In 8/13 bicuculline trials (10-50 μM, mean 28 μM) and 3/15 strychnine trials (8-18 μM, mean 14 μM), sporadic synchronous bursting was observed. Typically these rhythms were poorly developed (e.g. Fig. 4*A1*) compared to those induced by ACh/EDRO, 5-HT or NMA combined with the antagonist (e.g. Fig. 4*A2*). However, sustained synchronous rhythms were observed during 2 of the 12 trials with the use of strychnine (8 and 24 μM respectively) alone.

Combined blockade of $GABA_A$, $GABA_B$, and glycine receptors during neuropharmacologically induced motor rhythms

In two of two preparations, the effect on the rhythm of simultaneous blockade of $GABA_A$ and $GABA_B$ receptors by bicuculline in combination with either 2-hydroxysaclofen (400 μM) or phaclofen (2,000 μM) was similar to the effect of bicuculline alone (10 and 15 μM, respectively). In three other neurochemically activated preparations, the pattern recorded during combined $GABA_A$ and glycine receptor blockade, using bicuculline (10-30 μM, mean 22 μM) and strychnine (10-15 μM, mean 12 μM) respectively, was similar to the synchronous rhythmic pattern associated with either antagonist alone. However, addition of strychnine resulted in more abrupt termination of synchronous ENG bursts in bicuculline treated preparations. In one preparation, the addition of 2-hydroxysaclofen (500 μM) and bicuculline (30 μM, respectively) to a strychnine-induced (15 μM) synchronous pattern had no further effect on the rhythm characteristics.

Relationship of synchronous rhythm frequency to NMA, 5-HT, and ACh concentration

Previous studies using this preparation reported that the frequency of NMA-induced (Cazalets et al. 1992; Kudo et al. 1991; Smith et al. 1988) and 5-HT-induced (Cazalets et al. 1992) *in vitro* rhythmic activity is directly related to the concentration of these neurochemicals in the bath. In 15 preparations we examined whether there was also a relationship of synchronous rhythm frequency to the bath concentration of NMA (6 trials), 5-HT (8 trials), and ACh (6 trials). Higher concentrations of NMA produced higher frequencies of synchronous rhythmic discharge (Fig. 5*A2*). NMA concentrations >25-30 μM were associated with a loss of phasic activity resulting in tonic discharge only. The relationship of synchronous rhythm frequency to concentration of NMA or 5-HT, in the presence of either bicuculline or strychnine, is shown in Fig. 5, *B* and *C*. Increasing the bath concentration of 5-HT from 20 to 100 μM produced slowing of synchronous activity (Fig. 5*C*), similar to the effect of 5-HT on alternating rhythm frequency when applied in the absence of inhibitory amino acid antagonists (personal observations; 9/10 trials). Although the latter observation stands in contrast to the direct relationship between alternating rhythm frequency and 5-HT concentration previously described (Cazalets et al. 1992), this finding may be of relevance to other reports indicating that serotoninergic system activation decreases the frequency of locomotion in the lamprey (Christenson et al. 1989; Harris-Warrick and Cohen 1985) and the chronic spinal cat (Barbeau and Rossignol 1990). The frequency of ACh-induced synchronous rhythms tended to range from ~0.07 to 0.10 Hz regardless of concentration >20 μM.

Discussion

The main findings of this study are that blockade of either $GABA_A$ or glycine receptors in the neonatal rat spinal cord transforms alternating motor rhythms into synchronous patterns, and that inhibitory amino acid transmission is not essential for the generation of rhythmic activity in the spinal cord. Whether the initial nonlocomotor and locomotor-like patterns of neurochemically-induced activity observed in these experiments were produced by separate or shared neural substrates is unknown. However, it is likely that our observations are of relevance to the functional organization of locomotor networks, because each class of inhibitory amino acid agonist and antagonist was applied during at least one or more locomotor-like sequences of ENG activity, and the results were similar regardless of the initial pattern of neurochemically-induced rhythm.

The possibility that bicuculline or strychnine may have produced direct non-synaptic actions on spinal cord neurons in these experiments, independent of receptor blockade, is not excluded (e.g., Heyer et al. 1981). However, the transformation of reciprocally alternating rhythms into synchronous patterns is more likely due to a modification of network synaptic interactions, which is presumably related to inhibitory amino acid receptor blockade rather than non-synaptic actions on neurons.

GABA immunoreactivity in the ventral horn, intermediate gray, and deeper layers of the dorsal horn of the rat spinal cord declines during the neonatal period (Ma et al. 1992). This evidence of transient expression of GABA in specific regions of the gray matter raises the possibility that the effects of GABA receptor blockade on motor pattern observed in the present series may be developmentally-related. Further studies which systematically examine the effect of GABA receptor antagonists on motor patterns in preparations of different ages would help clarify this issue.

Previous studies have concluded that glycine-mediated reciprocal inhibition is not essential for the generation of locomotor rhythmicity (Cohen and Harris-Warrick 1984; Hagevik and McClellan 1994; Kudo et al. 1991). Similarly, the present observation that

rhythmic discharge persists despite combined $GABA_A$, $GABA_B$, and glycine receptor blockade is incompatible with network models that require inhibitory interconnections for the generation of periodic activity (Kling and Szekely 1968; Friesen and Stent 1977). In particular, our results suggest that the termination of individual ENG bursts does not depend critically on inhibitory input from an antagonist component of the locomotor network. However, this interpretation does not preclude involvement of inhibitory amino acid transmission in shaping phasic discharge during rhythmic activity. In fact, such a role for inhibitory amino acids is suggested by the observation that the onset, and sometimes the termination, of individual ENG bursts was more abrupt following the administration of bicuculline or strychnine. In addition, this study does not exclude the possibility that rhythmogenesis in the spinal cord may depend on inhibitory neurochemicals other than GABA and glycine.

The synchronizing effect of strychnine and bicuculline on hindlimb motor patterns suggests that maintenance of side-to-side alternation normally requires inhibitory amino acid receptor-sensitive mechanisms. This conclusion is consistent with previous studies showing strychnine-induced synchrony of left-right discharge during locomotor activity in the fetal rat (Kudo et al. 1991) and lamprey (Cohen and Harris-Warrick 1984; Hagevik and McClellan 1994). In addition, the present data demonstrate that intralimb flexor-extensor alternation during locomotion depends on inhibitory amino acid receptor activation, compatible with the observation that strychnine synchronizes high frequency flexor-extensor EMG bursts in the neonatal mouse (Droge and Tao 1993). Similarly, during fictive locomotion in the decerebrate cat, we observed that intrathecal infusion of strychnine promotes bilaterally synchronous rhythmic discharge of hindlimb flexor and extensor ENGs (Noga et al. 1993). Therefore the combined evidence suggests that inhibitory amino acid receptor-mediated reciprocal inhibition of antagonist motor nuclei may be a common feature of locomotor network operation in mammals as well as lower vertebrates.

In contrast to the effects of $GABA_A$ and glycine receptor antagonists, evidence that $GABA_B$ receptors mediate reciprocal interactions between functional antagonists was

not found. However, the efficacy of the $GABA_B$ receptor agonist muscimol in abolishing rhythmic activity was similar to $GABA_A$ and glycine receptor agonists. This suppressive effect may reflect a role for some or all of these receptor types in the overall endogenous modulation of locomotor network activity. Alternatively, bath application of these agonists may promote relatively non-specific inhibition of neuronal, and therefore network, excitability in the spinal cord.

The occurrence of synchronous, rather than independent, rhythmic activity on multiple ENGs suggests that bicuculline and strychnine either unmask a single source of network oscillation, or promote in-phase coupling of separate rhythmogenic elements within the network. These experiments do not provide direct evidence of either mechanism, however, the first alternative seems less likely. In particular, although a network model featuring a common source of rhythmic excitation for functionally distinct motor nuclei might explain ENG patterns observed in the presence of $GABA_A$ or glycine receptor blockade, it would not account for the generation of alternating discharge under normal conditions, and such a model would be incompatible with experimental evidence of multiple rhythmogenic elements within the locomotor network (Grillner 1981). The second mechanism implies that reciprocal inhibitory links normally dominate the interaction between antagonist centers, the suppression of which unmasks excitatory connections that produce in-phase coupling of rhythmogenic centers, as proposed for left-right alternation in the fetal rat (Kudo et al. 1991) and lamprey (Cohen and Harris-Warrick 1984; Hagevik and McClellan 1994).

Also compatible with a network model composed of parallel reciprocal inhibitory and excitatory coupling are the effects of strychnine and bicuculline on rhythm frequency. Immediately after addition of strychnine and bicuculline to the bath, a transient increase in the frequency of the alternating pattern was observed before converting to a synchronous pattern. Once the synchronous pattern was established the frequency in most trials was slower than the frequency of the initial alternating pattern. In the lamprey spinal cord, a progressive increase in strychnine concentration produces a

similar bi-phasic effect on frequency (Hagevik and McClellan 1994). Furthermore, computer modeling of the lamprey locomotor system, based on parallel reciprocal inhibitory and excitatory coupling of unit oscillators, predicts that as the strength of reciprocal inhibition is gradually reduced in favor of excitation, the frequency of alternating patterns should increase; then as inhibition is further blocked, synchronous rhythms with slower frequencies should emerge, as was observed experimentally in the lamprey (Hagevik and McClellan 1994) and in the present study.

It is of interest that a similar disturbance of motor pattern results from blocking either one of two distinct inhibitory transmitter systems. In retinal ganglion cells strychnine (20 μM) blocks GABA receptors (Tauck et al. 1988). Therefore some of the effects of strychnine we observed at higher concentrations may have been due to non-specific blockade of both glycine and GABA receptors. However, we also observed strychnine-induced synchronization of motor patterns with initial bath concentrations as low as 1 μM (strychnine concentrations up to 5 to 10 μM have been shown to have no effect on the GABAergic system, Tauck et al. 1988; Wu et al. 1992), and, unlike bicuculline, the strychnine effect persists after washout of strychnine from the bath solution. Conversely, glycine receptors are insensitive to bicuculline concentrations as high as 50 μM in the *in vitro* rat spinal cord preparation (Wu et al. 1992).

Alternatively, the comparable effects of bicuculline and strychnine on motor pattern may be due to an interaction between GABAergic and glycinergic systems, rather than non-specificity of antagonist action. Anatomical studies have shown GABA and glycine co-existence in axons and cell bodies in the rat spinal cord (e.g. Chiba and Semba 1991; Triller et al. 1987; Todd and Sullivan 1990), and co-localization of $GABA_A$ and glycine receptors at GABAergic synaptic contacts (Bohlhalter et al. 1994). The ultrastructural evidence of a close association between GABA and glycine receptors suggests these receptors may functionally interact (Bohlhalter et al. 1994), consistent with the observation that both GABAergic and glycinergic mechanisms contribute to Renshaw cell-mediated recurrent inhibition of lumbar motoneurons (Cullheim and

Kellerth 1981; Schneider and Fyffe 1992). Therefore, the similar effect of $GABA_A$ and glycine receptor antagonists in the present study may represent an example, during behavior, of the functional interaction predicted by ultrastructural studies.

Rhythmic high frequency (4-8 Hz) packets of ENG discharge were observed during some trials, before the addition of strychnine. These asynchronous bursts resemble the neurochemically-induced fast rhythms reported by others using *in vitro* preparations (Cazalets et al. 1990; Hernandez et al. 1991). In addition, the synchronization of high frequency rhythms by strychnine in the present experiments is consistent with the effects of strychnine on motor discharge recently observed in the *in vitro* mouse spinal cord (Droge and Tao 1993). The neural origin and functional significance of these high frequency oscillations is unknown. However, our recent observations of similar superimposed 4-8 Hz packets of synchronous flexor and extensor ENG discharge in the adult cat, following intrathecal strychnine administration (Noga et al. 1993), suggests the phenomenon is not restricted to the immature rat spinal cord. The fast rhythms may be related to synaptically-mediated 4- to 5-Hz oscillations that appear in motoneurons following the application of strychnine to rat spinal cord slice cultures (Streit 1993). Of the possible cellular mechanisms involved, low-threshold calcium currents are of particular interest because they have been associated with spontaneous 3.5- to 8-Hz oscillations in motoneurons in the *in vitro rat* spinal cord (Walton and Llinas 1986), as well as the 6- to 9-Hz oscillations observed in habenular, inferior olivary and thalamic nuclei (e.g. Gutnick and Yarom 1989).

In summary, the results suggest that inhibitory amino acid transmission is not essential for rhythmogenesis in the spinal cord, although $GABA_A$ergic and glycinergic mechanisms may be critically important in determining the co-ordination of hindlimb side-to-side and flexor-extensor activity. The results also imply that functionally independent motor centers within the pattern generating network are linked by both reciprocal inhibitory and excitatory pathways. In keeping with Grillner's (1981) hypothesis of locomotor network organization, the existence of both inhibitory and excitatory links between "unit burst generators", in conjunction with the capacity to

selectively modulate the efficacy of transmission in these pathways, would provide the central nervous system with the flexibility it requires to generate a wide range of locomotor and other rhythmic patterns.

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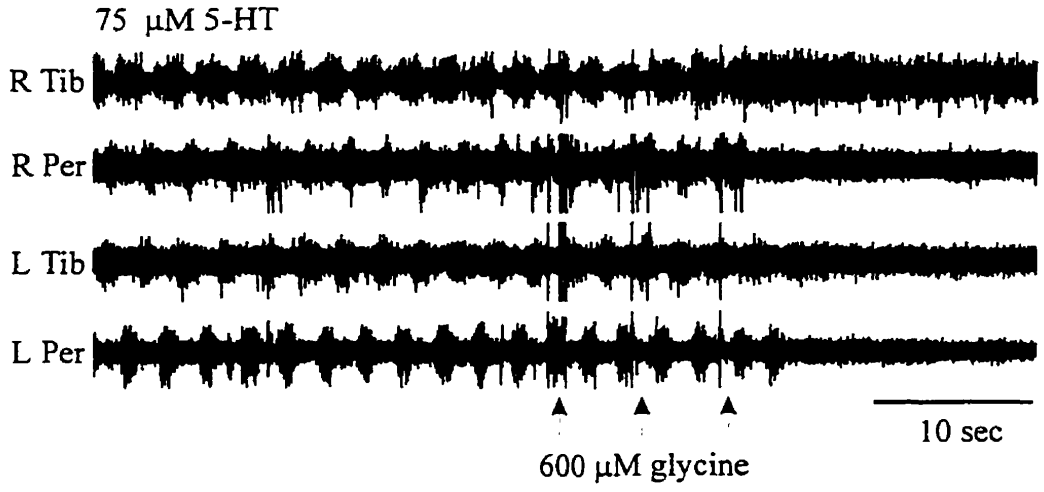

Figure 1

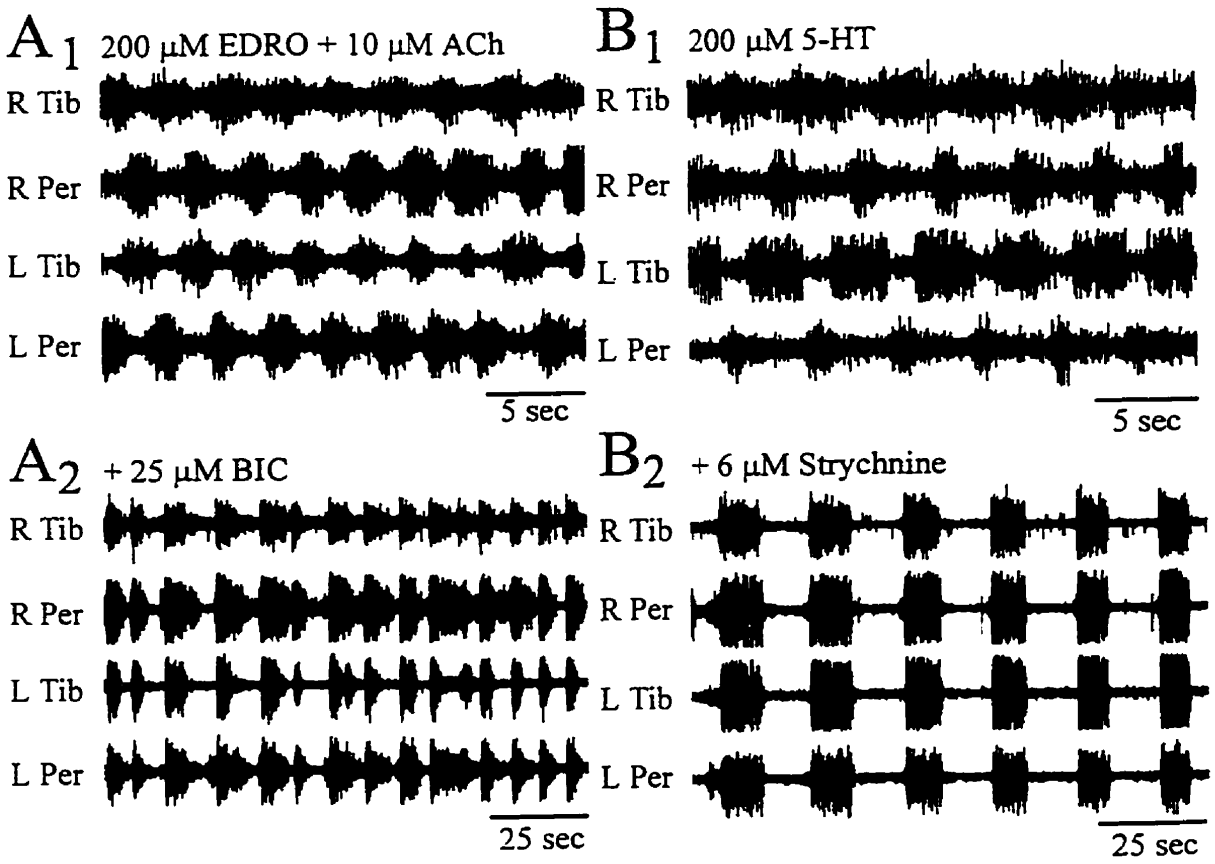
A1
200 μM EDRO + 10 μM ACh
R Tib
R Per
L Tib
L Per
5 sec
B1
200 μM 5-HT
R Tib
R Per
L Tib
L Per
5 sec
A2
+ 25 μM BIC
R Tib
R Per
L Tib
L Per
25 sec
B2
+ 6 μM Strychnine
R Tib
R Per
L Tib
L Per
25 sec

Figure 2

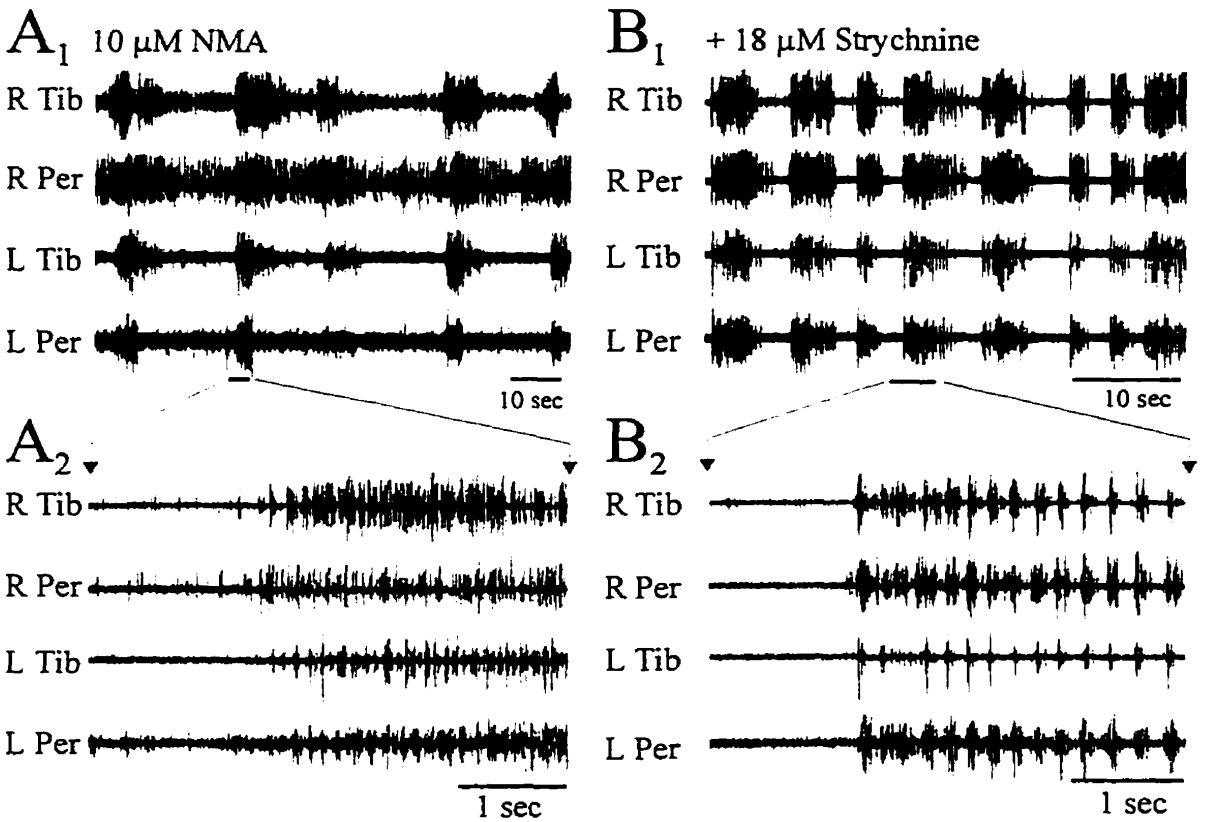
A1 10 μM NMA
R Tib
R Per
L Tib
L Per
10 sec
A2
R Tib
R Per
L Tib
L Per
1 sec
B1 + 18 μM Strychnine
R Tib
R Per
L Tib
L Per
10 sec
B2
R Tib
R Per
L Tib
L Per
1 sec

Figure 3

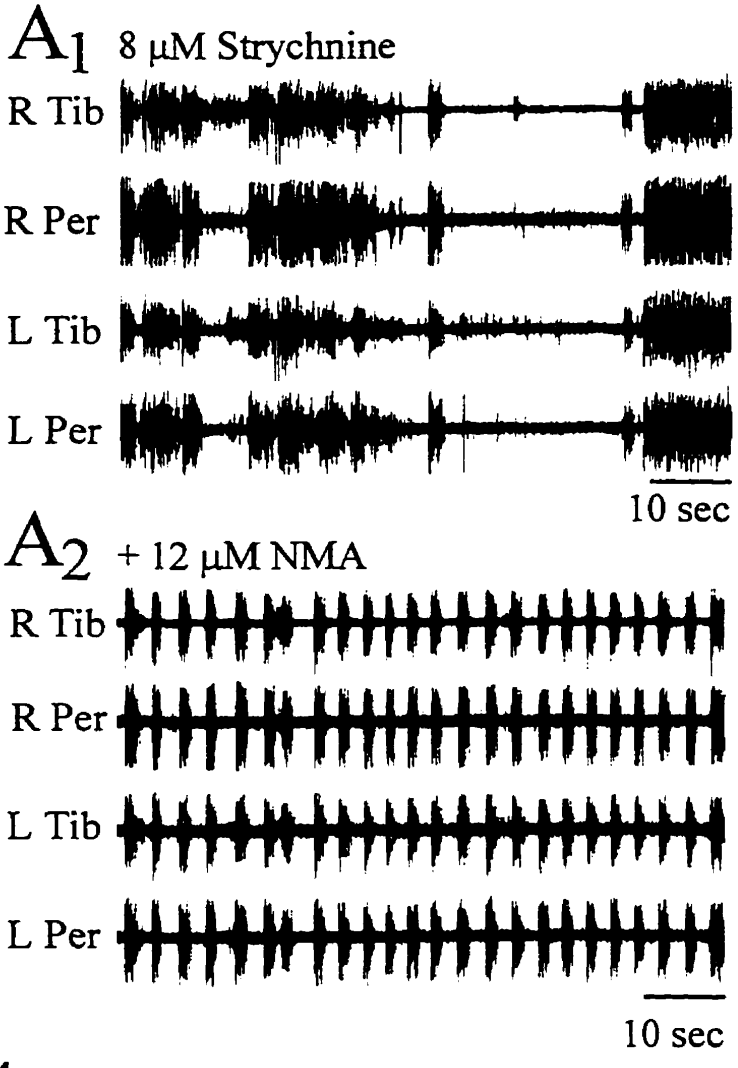
A1
8 μM Strychnine
R Tib
R Per
L Tib
L Per
10 sec
A2
+ 12 μM NMA
R Tib
R Per
L Tib
L Per
10 sec

Figure 4

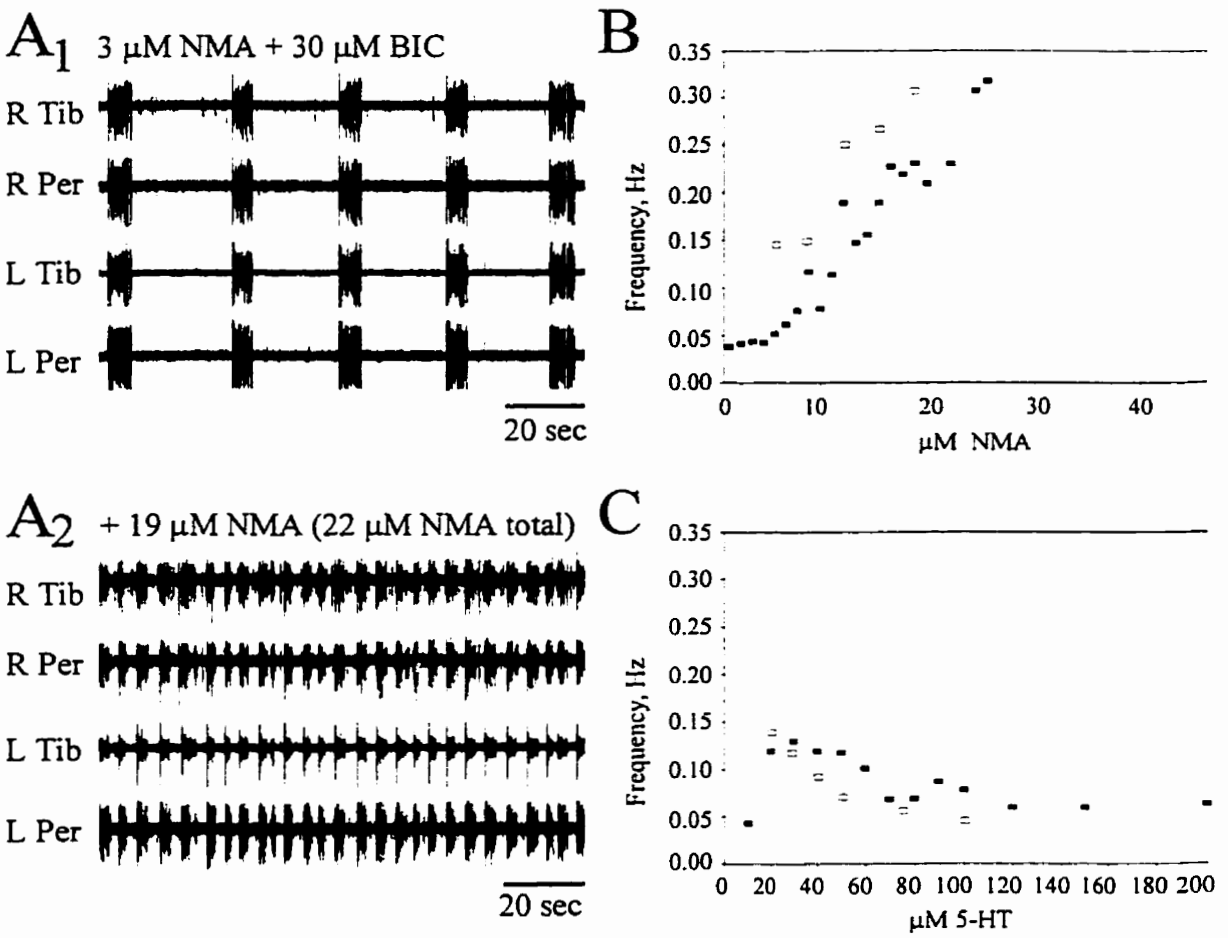
A1
3 μM NMA + 30 μM BIC
R Tib
R Per
L Tib
L Per
20 sec
B
Frequency, Hz
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
μM NMA
A2
+ 19 μM NMA (22 μM NMA total)
R Tib
R Per
L Tib
L Per
20 sec
C
Frequency, Hz
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 20 40 60 80 100 120 140 160 180 200
μM 5-HT

Figure 5

Figure Legends

Figure 1. Suppression of 5-hydroxytryptamine (5-HT)-induced locomotor-like hindlimb flexor (peroneal; Per) and extensor (tibial; Tib) electroneurogram (ENG) activity by 600 μM glycine (bath concentration increased by 200 μM at each arrow). The residual tonic activity, best seen in the right Tib recording, completely disappeared after 60 s (not shown).

Figure 2. Effect of γ-aminobutyric acid-A ($GABA_A$) and glycine receptor antagonists on locomotor-like ENG discharge. *A1*: the initial acetylcholine [ACh; in combination with edrophonium chloride (EDRO)] -induced pattern consisted of alternating flexor-extensor and left-right ENG activity. *A2*: rhythmic discharge became synchronous following addition of the $GABA_A$ receptor antagonist bicuculline (BIC; bath concentration 25 μM). *B1*: in another preparation, the 5-HT-induced initial pattern also consisted of alternating flexor-extensor and left-right ENG activity. *B2*: rhythmic discharge became synchronous after addition of the glycine receptor antagonist strychnine (bath concentration 6 μM). In both examples, addition of antagonists resulted in more abrupt onset of discharge; strychnine also produced a more abrupt termination of each ENG burst.

Figure 3. Effects of strychnine on N-methyl-D,L-aspartate (NMA)-induced ENG activity. *A1*: in this preparation, NMA alone induced simultaneous rhythmic discharge of the flexor and extensor ENGs bilaterally. *B1*: after the addition of strychnine (bath concentration 18 μM), the onset and termination of each ENG burst was more abrupt and the rhythm frequency increased. *A2* and *B2*: expanded time scale shows synchronization of high frequency (4 HZ) packets of discharge after the application of strychnine.

Figure 4. Effect of NMA on strychnine-induced ENG activity. *A1*: synchronous, but irregular, bursts of ENG discharge were induced by the application of strychnine alone. *A2*: subsequent application NMA (bath concentration 12 μM) induced sustained rhythmic discharge.

Figure 5. Effect of NMA and 5-HT concentration on synchronous rhythm frequency. *A1*: the initial synchronous rhythm was established with 3 μM NMA and 30 μM bicuculline (BIC). *A2*: increasing the NMA concentration in the bath to a total of 22 μM was associated with an increased frequency of synchronous rhythm. *B*. and *C*: filled and empty squares in each graph represent the data obtained from strychnine and bicuculline trials, respectively. *B*: increasing the NMA concentration in the bath produced an increased frequency of synchronous rhythms. *C*: increasing the bath 5-HT concentration from 20 to 100 μM was associated with a decrease in rhythm frequency.

Part IV: Regional Distribution of the Locomotor Pattern-Generating Network in the Neonatal Rat Spinal Cord

K. C. Cowley and B.J. Schmidt

Part IV is reprinted here as it appears in the
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Running title: Distribution of the locomotor network

Summary

The regional distribution of spinal cord networks producing locomotor-like, as well as non-locomotor-like, activity was studied with the use of an *in vitro* neonatal rat preparation. Rhythmic activity was induced by bath application of either serotonin (5-HT), acetylcholine (ACh), N-methyl-D,L-aspartate (NMA), or combined 5-HT/NMA and was monitored via hindlimb flexor (peroneal) and extensor (tibial) electroneurograms (ENGs) or ventral root recordings. In some experiments, synchronous patterns were produced by the addition of inhibitory amino acid (IAA) receptor antagonists. Selective application of 5-HT to cervical and thoracic cord regions induced rhythmic activity in these segments but failed to evoke hindlimb ENG discharge. Exposure of the isolated lumbar region to 5-HT produced tonic activity only. Application of 5-HT to the whole cord produced bilaterally coordinated locomotor-like activity in hindlimb flexor and extensor nerves which persisted after midsagittal section of the spinal cord from the conus to the thoracolumbar junction. In other experiments, transverse hemisection of the rostral lumbar cord during whole cord exposure to 5-HT abolished rhythmic activity in ipsilateral hindlimb ENGs, suggesting that under these conditions rhythmic activity on one side of the lumbar cord may be insufficient to maintain rhythmic activity on the contralateral side. Selective application of NMA or ACh to cervical and/or thoracic spinal cord regions evoked rhythmic activity in these supralumbar segments, as well as rhythmic, but non-locomotor-like, activity in the lumbar region. In contrast to the effect of 5-HT, both NMA and ACh evoked rhythmic activity when applied solely to the lumbar region, and the side-to-side alternation produced by whole cord ACh application was uncoupled by midsagittal lesions of the lumbar region. In the presence of IAA antagonists, the side-to-side coupling of bilaterally synchronous rhythms was maintained despite extensive midsagittal lesions leaving all but one or two segments of either cervical, thoracic, or lumbar cord bilaterally intact, and rhythmic activity could be maintained even in single isolated hemisegments. The effects of 5-HT/NMA were similar to those observed using 5-HT alone, although 5-HT/NMA induced rhythmic activity in hindlimb ENGs when applied selectively to supralumbar regions. The results suggest that 1) a 5-HT-sensitive oscillatory network, capable of producing a locomotor-

like pattern of activity, is distributed throughout the supralumbar region of the spinal cord and mediates descending rhythmic drive to lumbar motor centers; 2) NMA- and ACh-sensitive rhythmogenic elements are distributed throughout the spinal cord, including the lumbar region; and 3) the spinal cord contains an extensive propriospinal network of reciprocal inhibitory and excitatory connections characterized by redundantly organized side-to-side projections.

Introduction

It is well established that the mammalian spinal cord contains the neural circuitry required to generate a variety of rhythmic behaviors, including locomotion. Grillner (1981) proposed that the network producing rhythmic limb movements is composed of multiple "unit burst" generators, each driving a particular group of close synergists acting on a given joint. This concept implies that individual unit burst generators are distributed throughout the spinal cord close to the motoneuron populations they drive. Indeed, there is evidence of a distributed organization of unit generators in the locomotor systems of a number of experimental preparations including the dogfish (Grillner 1974), lamprey (Cohen and Wallen 1980), frog embryo (Khan and Roberts 1982), and embryonic chick (Ho and O'Donovan 1993). Similarly, the neural mechanism generating scratching is dispersed over multiple segments in the cat (Deliagina et al. 1983) and turtle (Mortin and Stein 1989). Some of these systems have also been characterized by a regional hierarchy wherein more rostral lumbar segments have a relatively greater capacity for rhythm generation compared with caudal segments (eg. Deliagina et al. 1983; Ho and O'Donovan 1993; Mortin and Stein 1989).

Despite the evidence of distributed networks for locomotion in lower animals, and for scratching in the cat, it is unclear whether locomotor pattern generating circuitry in mammals is also regionally dispersed. Several studies have documented that relatively caudal segments of the lumbar cord are capable of generating rhythmic locomotor activity. For instance, intravenous L-DOPA induces fictive alternation of ankle flexor and extensor activity in cats following acute isolation of the L_6-S_1 segments of lumbar cord, suggesting that at least some components of the locomotor pattern generator are located caudal to the L_5 level (Grillner and Zangger 1979). A small portion of chronically isolated cat lumbar hemicord (hemisected at the L_3 level combined with midsagittal sectioning from L_3 to S_1) is capable of generating locomotor activity in the ipsilateral hindlimb, although rhythm generation in this preparation may depend, at least in part, on phasic afferent impulses (Kato 1990). In the presence of bath-applied N-methyl-D-aspartate (NMDA), alternating ankle flexor and extensor muscle activity has

been documented following acute hemisection and isolation of the L_4-L_5 segment of the *in vitro* neonatal rat lumbar cord (Kudo and Yamada 1987). These studies, which focused on the rhythm generating potential of middle and caudal lumbar segments, do not exclude the possibility of a distributed system which could include more rostral portions of the spinal cord. In contrast, however, recent investigations of the neonatal rat spinal cord preparation suggest that caudal lumbar segments participate in neither locomotor rhythm generation nor pattern organization; it was concluded these functions are localized in the L_1 and L_2 segments (Cazalets et al. 1995, 1996). This major disparity in the literature highlights the need for further investigation of the regional organization of mammalian locomotor pattern generating networks.

In the present study, we examine the effect of acute transverse and midsagittal lesions on locomotor rhythm generation in the isolated spinal cord of neonatal rats. In addition, because different neurochemicals preferentially induce specific patterns of rhythmic activity, not all of which are locomotor-like in nature (Cowley and Schmidt 1994b, 1995), we test the hypothesis that different forms of rhythmic behavior are mediated by networks with regionally distinct distributions. The evidence we provide in support of this hypothesis may account for some of the inconsistencies noted in the literature.

Preliminary findings of this work have appeared in abstract form (Cowley and Schmidt 1993; Harder and Schmidt 1992).

Methods

Experiments were performed on 77 Sprague-Dawley rats (0-7 days). The *in vitro* bath system, artificial cerebrospinal fluid composition, and the method of isolating the intact spinal cord with hindlimb nerves attached has been described previously (Cowley and Schmidt 1994a,b; 1995). All preparations initially involved the use of a bilaterally intact spinal cord from C_1 to the conus medullaris (the cone shaped caudal-most portion of the spinal cord). Specific segmental levels were identified by counting spinal roots starting with C_1 and proceeding caudally. Peroneal nerve recordings were used to monitor ankle flexor activity. Ankle extensor activity was monitored with the use of the tibial nerve or one of the branches to the gastrocnemius, soleus or posterior tibial muscles. In a few preparations, hip flexor (iliacus muscle) activity was monitored. In other experiments ventral root recordings from cervical, thoracic and/or lumbar segments were obtained.

Electroneurograms (ENGs) and ventral root recordings were obtained with the use of glass suction electrodes. Electromyograms (EMGs) were obtained using 0.015-in. diam resin-coated wire. Records were digitized and stored at 5.5 kHz with the use of a Vetter pulse code modulator videocassette adaptor. Further analysis and display of selected segments of taped data was performed using software developed for use on a Masscomp 5400 computer (sample rate 2kHz per channel).

With the aid of a surgical dissecting microscope, fine insect pins were used to separate the left and right halves of spinal cord along the midsagittal plane. Transverse and hemi-transverse cord lesions were made with iridectomy scissors. The completeness of the lesions was readily confirmed by separating the sectioned tissue such that an unobstructed view of the black Sylgard base of the chamber was obtained. In some experiments, the bath was partitioned by a barrier made of acetate film sealed at the chamber and cord contact edges with petroleum jelly. Absence of barrier leakage was confirmed at the beginning and end of experiments by draining one side of the barrier and using the dissecting microscope to look for fluid seepage across the partition. As an additional precaution, a higher fluid

level (and therefore greater hydrostatic pressure) was maintained on the side of the partition not receiving the added neurochemicals.

All neurochemicals were initially dissolved in distilled water and stored as millimolar stock solutions. To obtain the selected final bath concentration of a neurochemical substance, stock solution was directly added in fractionated amounts, with the use of a micropipette, at the periphery of the chamber during vigorous bubbling. All concentrations, including ranges, specified in this report refer to the final bath concentration. Recordings were started once stable patterns of activity had emerged. If the discharge pattern was abolished or disturbed in response to making a spinal cord lesion, the neurograms were then monitored for at least 30-60 minutes (and up to 3 h) to confirm that prelesion activity did not return; during this time repeated attempts were also made to induce rhythmic activity by reapplying the same neurochemical. Using a syringe, the bath solution was exchanged repeatedly with normal solution between tests of different substances. Rhythmic activity was induced with the use of either serotonin (5-HT; 10-125 μM), N-methyl-D,L-aspartate (NMA, 4-18 μM), or acetylcholine (ACh 10-100 μM, in combination with the acetylcholinesterase inhibitor edrophonium EDRO 100-300 μM). In some experiments, the effect of 5HT applied in combination with NMA was examined. Synchronous rhythms were obtained by adding strychnine (8 - 30 μM) or bicuculline (14-70 μM) to the bath in combination with either 5-HT, ACh/EDRO or NMA, as detailed previously (Cowley and Schmidt 1995). All chemicals were obtained from Sigma.

Results

Previously we documented that flexor and extensor hindlimb ENG activity generated by the *in vitro* neonatal rat spinal cord preparation was usually locomotor-like in response to bath applied 5-HT, whereas non-locomotor-like patterns were more typical of NMDA- and ACh-induced rhythms (Cowley and Schmidt 1994b). In keeping with our earlier work, the use of the term "locomotor-like" in the present paper is restricted to ENG patterns characterized by alternation of ankle flexor-extensor activity in one hindlimb coupled with extensor-flexor alternation in the contralateral hindlimb (e.g. Fig. 1A). This pattern is similar to that reported for stepping in the adult rat *in vivo* (Gruner et al. 1980). However, it should be noted that alternation of ankle flexor-extensor activity alone, as monitored in these experiments, does not allow distinction between steppinglike and swimminglike patterns of locomotion (Gruner and Altman. 1980). Furthermore, it remains to be shown whether or not the networks that generate other patterns of rhythmic discharge (such as side-to-side alternation of coactivated intralimb flexor-extensor pairs, or rhythmic coactivation of all flexor and extensor ENGs bilaterally) share components of locomotor pattern generating circuitry. However, for the purposes of this study, these other patterns of hindlimb flexor and extensor activation are referred to as "non-locomotor-like".

Because different substances preferentially activate different patterns of activity (Cowley and Schmidt 1994b, Kiehn and Kjaerulff, 1996), we first compared the effects of spinal cord lesions and bath partitioning on rhythms activated by single neurochemicals (5-HT, NMA, and ACh). As a means of examining the regional distribution of reciprocal excitatory connections within rhythmogenic circuitry, we used similar lesioning and bath partition methods in other experiments, in which synchronous rhythms were evoked using 5-HT, NMA, or ACh in the presence of inhibitory amino acid receptor antagonists (Cowley and Schmidt 1995). Finally, because 5-HT combined with NMA or the excitatory amino acid uptake inhibitor dihydrokainic acid (DHK) may produce more stable locomotor rhythms than obtained with either substance alone (Cowley and Schmidt 1994a; Kjaerulff et al. 1994; Sqalli-Houssaini et al. 1993), we investigated the regional distribution of the network underlying combined 5-HT/NMA-mediated rhythmic activity.

Rostral-caudal distribution of the locomotor-like network activated by 5-HT

The minimal substrate required for 5-HT-induced locomotor-like patterns was examined first by completely transecting the spinal cord at several levels starting rostrally and proceeding caudally (n = 7 preparations). Application of 5-HT to the bath solution induced rhythmic locomotor-like discharge in the intact spinal cord. In the example shown in Figure 1A, locomotor-like activity resumed twenty seconds after transecting the spinal cord between the T_4 and T_5 segments (↓), indicating that the cervical enlargement and rostral thoracic cord were not essential for generating locomotor activity in the lumbar segments. Similarly, after transverse section between T_{12} and T_{13} (performed before the onset of the recording shown in Fig. 1B) rhythmic activity transiently ceased and then reappeared again within 4 min, as shown in Fig. 1B. However, after transection between T_{13} and L_1 (Fig. 1B, ↓), 5-HT-mediated rhythmic hindlimb ENG activity terminated permanently (observed for up to 2.5 h in some preparations). Repeated attempts to establish rhythmic discharge with the use of progressively higher 5-HT concentrations (from 10-100 μM) produced only tonic activity. In contrast ACh (Fig. 1D) and NMA were capable of eliciting rhythmic activity in the same isolated lumbar cords (see below), suggesting that the lack of 5-HT effect was unrelated to any non-specific depression of neural activity that might result from an acute cord lesion. Similar results were obtained in all seven preparations. In two of the preparations, cervical ventral roots were also monitored. These roots showed continued rhythmic discharge after transection between T_{13} and L_1, suggesting that the failure of 5-HT to induce rhythmic activity in the isolated lumbar cord cannot be accounted for by inadequate concentrations of 5-HT in the bath.

As illustrated in Fig. 1, A and B, the frequency of 5-HT-induced locomotion decreased during the serial transections. The frequency decreased by ~10% after lesioning between T_4 and T_5, and by 25% after transection between T_{12} and T_{13} (Fig. 1C, ■). Similar decreases in frequency were observed in all seven preparations. However, it should be noted that 5-HT-induced rhythms in unlesioned control preparations also

displayed a decline in frequency with time. For instance, the rhythm frequency in the control preparation shown in Fig. 1C (unfilled square) decreased by 25% 8 min after the onset of 5-HT-induced locomotion, and continued at this frequency for the remainder of the 30- min observation period. In two other unlesioned preparations, 5-HT-induced locomotor rhythm frequency decreased by 20 and 40%, respectively, after five min of observation; the latter preparation showed a further decline to 25% of the original frequency at the end of 30 min. Because rhythm frequency spontaneously decreased in intact control preparations, we are unable to conclude that the decline in frequency observed during the lesioning experiments was specifically related to the effects of acute spinal cord transection and/or a reduction in the size of the rhythm generating network.

In three other experiments a barrier was placed between T_{13} and L_1 to partition the bath chamber into two compartments. Addition of 5-HT to the rostral side of the partition induced rhythmic activity in cervical segments but not in lumbar segments. Application of 5-HT in graded concentrations (from 10 to 100 μM) to the caudal compartment alone induced tonic discharge in hindlimb ENGs but no activity on the rostral side of the partition. The latter observation is consistent with the effect of 5-HT on the lumbar cord isolated by means of complete transection at the T_{13}/L_1 level (see above). Only when 5-HT was added to both the rostral and caudal compartments did rhythmic hindlimb ENG activity develop, in contrast to the effects of ACh and NMA (see below).

The above results suggested that supralumbar regions of the spinal cord may contain the rhythmogenic circuitry activated by 5-HT that is required for hindlimb locomotor output. Alternatively, the lumbo-sacral cord may also contain part of the 5-HT-sensitive circuitry, but a critical mass of intact network in this region is required for successful activation. In this case, residual network elements present in the isolated lumbo-sacral cord, after a T_{13}/L_1 transection, may be insufficient for rhythm generation. Two sets of experiments were performed to address this issue. First, the caudal portion of the spinal cord, and therefore any rhythmogenic neural substrate contained therein, was removed by transecting between L_5 and L_6 in one experiment, and between L_6 and S_1 in a second preparation. Rostral transections were then started at the T_{10} level and continued caudally one segment at a time. If the caudal transection removed critical network mass, then one would predict that the

rostral limit of lumbar cord, which contained the minimal amount of network required for activation, should shift above the T_{13}/L_1 junction. However, as was the case in preparations with intact caudal lumbo-sacral cord, 5-HT-induced locomotor activity was still maintained in both preparations until the descending series of rostral transections reached the T_{13}/L_1 junction.

It is possible that the $L_5/_6$ and L_6/S_1 transections had no effect on critical network mass in the lumbo-sacral cord simply because the caudal segments isolated by these transections did not contain 5-HT-sensitive rhythmogenic circuitry. Therefore, in a second set of experiments we examined the rhythmogenic responsiveness of small portions of rostral lumbar cord to 5-HT. Motor axons to the iliacus muscle (hip flexor) exit the spinal cord via L_2 and L_3 ventral roots, whereas tibial and peroneal motor axons exit predominantly via the L_4 and L_5 ventral roots (Cowley and Schmidt 1994a). Therefore we used the iliacus electromyogram in combination with peroneal and tibial ENG recordings (Fig. 2A, prelesion) to monitor the effect of mid-lumbar transverse lesions in two experiments. In both preparations, transection between L_3 and L_4 terminated 5-HT-induced activity in the tibial and peroneal nerves, as expected, whereas rhythmic iliacus muscle activity continued. Rhythmic activity also persisted in the iliacus muscle after subsequent transection between T_{12} and T_{13} left only four cord segments intact (T_{13}-L_3 inclusive, as shown in Fig. 2B, left). Thus just a few segments of lumbar cord were capable of developing rhythmic activity provided continuity was maintained with the supralumbar region (T_{13} segment in this case), even though 5-HT application to the entire lumbosacral cord, transected or partitioned at the T_{13}/L_1 junction, failed to generate rhythmic activity. After transection between T_{13} and L_1 (Fig. 2B, ↓) rhythmic activity terminated permanently. Repeated applications of 5-HT also failed to produce rhythmic activity in the isolated L_1-L_3 segment. Therefore, this evidence does not favor critical mass of lumbar circuitry as the major factor accounting for the results of the T_{13}/L_1-level transection and bath partition results. Instead, the observations suggest that successful induction of rhythmic hindlimb activity in response to 5-HT requires continuity of lumbar segments with an activated supralumbar network.

We then examined whether the essential rhythmogenic elements in the supralumbar region were localized to the caudal thoracic cord specifically (e.g. T_{13}), or whether a more diffuse system, distributed throughout the cervicothoracic cord, mediated the rhythmogenic influence on lumbar motor regions. Therefore in two preparations the effect of 5-HT application to cervical and lumbar regions using double bath partitions (at the T_9/T_{10} and L_2/L_3 levels) was examined. Similar results were obtained in both experiments. The addition of 5-HT to the rostral bath (C_1-T_9) produced rhythmic activity in cervical and thoracic segments, but not in the lumbar region (L_5 ventral root monitored). The addition of 5-HT to the caudal bath alone (L_3-conus) produced tonic activity in the L_5 segment, and no activity in the cervico-thoracic region. These observations are consistent with the results of experiments in which the T_{13}/L_1 bath partition was used, described above. However, simultaneous exposure of the rostral and caudal baths to 5-HT produced rhythmic activity of cervical, thoracic and lumbar regions, despite the absence of 5-HT in the middle bath (T_{10}-L_2). During the latter experiments, the middle bath solution completely exchanged every 20 s, as an added precaution against any undetected partition leakage. Therefore, these observations support the concept of a distributed organization of 5-HT-sensitive rhythmogenic circuitry within the cervicothoracic cord, rather than a mechanism localized to the most caudal region of the thoracic cord, or restricted to the rostral lumbar segments as suggested by Cazalets et al. (1995).

Effect of midsagittal spinal cord lesions on 5-HT-induced locomotor-like activity

To examine whether bilaterally distributed components are essential for generating and co-ordinating 5-HT-induced rhythms, the left and right sides of the spinal cord were separated along the midsagittal plane. The example in Fig. 3 shows that flexor-extensor and left-right relationships were maintained after midsagittal section from the conus to L_1 inclusive. Left-right, and intralimb flexor-extensor coordination was also maintained in eight other preparations after midsagittal section from the conus to the thoraco-lumbar junction region (L_1/L_2, n=2; T_{13}/L_1, n=3; and T_{12}/T_{13}, n=3). Attempts to extend the midsagittal section more rostrally in these experiments resulted in an abrupt loss of 5-HT-

induced rhythms. Repeated applications of 5-HT failed to re-establish rhythmic activity. Two of the preparations with midsagittal lesions of the lumbo-sacral spinal cord (extending to T_{13}/L_1) were also transected in the thoracic region, at the T_4/T_5 and T_{12}/T_{13} junctions, respectively, without effect on flexor-extensor and left-right discharge in the hindlimb nerves. Thus, even one bilaterally intact segment (T_{13}) was capable of maintaining left-right coordination in the lumbar region. When midsagittal separation was started at C_1 and extended caudally, the 5-HT-induced pattern, including the left-right phase relationship, was preserved until the lesion reached the T_{13}/L_1 junction (n=2), at which point the rhythmic activity stopped.

The observation that 5-HT-induced rhythms were abolished by midsagittal lesions that extended into the thoracolumbar junction, regardless of whether the lesion originated from a rostral or caudal approach, suggested that cross projections in this region may be necessary to maintain locomotor network oscillatory activity. Therefore, the effect of midsagittal section restricted to the thoraco-lumbar region (T_{10}-L_4, T_8-L_1, and T_{12}-L_1 inclusive) was examined in three preparations. These lesions disrupted neither hindlimb rhythm production nor coordination (Fig. 4). Attempts to extend the midsagittal lesion further rostrally, in the T_{10}-L_4 and T_{12}-L_1 preparations, resulted in the loss of ENG activity on one side. However, the remaining unilateral rhythmic activity (flexor-extensor alternating) continued until the midsagittal separation reached the T_5 and C_8 levels, respectively, in these two preparations. The midsagittal lesioning results are compatible with a distributed and redundant network organization, in which cross projections in the thoracolumbar region are not essential for rhythmogenesis, provided similar connections are preserved in the lumbosacral and cervicothoracic regions.

The results of the spinal cord transection (e.g. Figs. 1 and 2) and bath partition experiments suggest that 5-HT-induced locomotor rhythmogenesis in the hindlimbs depends on network activation in the supralumbar region. However, the combined results of the midsagittal section experiments (e.g. Figs. 3 and 4) suggest that cross connections throughout the spinal cord, including the lumbar segments, contribute to locomotor network operation. That is, locomotion was obtained in the presence of midsagittal lesions in the thoracolumbar junction region only if spinal cord segments both rostral and caudal to the

lesions remained bilaterally intact. However, the nature of the apparent permissive effect due to preserving cross connections in the lumbar cord was unclear. In particular, we examined whether rhythmic activity in one half of the lumbar spinal cord could phasically drive the contralateral side in the absence of ipsilateral descending excitation from supralumbar regions. Therefore transverse hemisections were made in the rostral lumbar region of three otherwise bilaterally intact spinal cords exposed to 5-HT. In two preparations, hemisection between T_{13} and L_1 had no effect on left and right hindlimb ENG activity. However, in all three preparations, hemisection of the spinal cord one segment caudal to the thoracolumbar junction (between L_1 and L_2) abolished hindlimb ENG activity on the ipsilateral side, whereas the capacity for alternating tibial and peroneal nerve activity was preserved on the contralateral side (Fig. 5). Once again, the observations are consistent with 5-HT activation of a predominately supralumbar network that generates descending (ipsilateral) drive to the lumbar cord. In contrast, any excitation that might be transmitted by segmental cross connections from the contralateral lumbar cord seems insufficient to activate or maintain rhythmic activity on the opposite side.

Effect of spinal cord lesions and bath partition on ACh- and NMA-induced motor rhythms

In contrast to 5-HT-evoked activity, rhythms activated by NMA or ACh are often non-locomotor-like in pattern (Cowley and Schmidt 1994b). Therefore, it was of interest to examine whether the neural substrate(s) underlying NMA- and ACh-activated rhythms have the same, or a distinct, distribution compared to the network activated by 5-HT. After 5-HT-induced locomotor rhythms were abolished by complete spinal cord transections between the T_{13}/L_1 segments, and 5-HT was washed out from the bath, application of either ACh (in combination with EDRO) or NMA induced rhythmic hindlimb discharge in the isolated lumbosacral cord of all seven preparations tested. The pattern however, was not locomotor-like in quality (e.g. Fig. 1D). In two other preparations, with transverse hemisections at the L_1/L_2 level, endogenous excitatory amino acid transmission was enhanced by the bath application of the uptake inhibitor DHK. Consistent with the effect of NMA on the bilaterally intact lumbar cord, DHK restored rhythmic activity in ipsilateral

hindlimb ENGs, despite the same transverse hemisection having previously abolished 5-HT-induced activity (as shown in Fig. 5). Complete transections were made at a more caudal level (L_3/L_4 junction) in eight other preparations. In each case, the transection failed to abolish NMA-induced rhythmic activity, as monitored by lumbar ventral root recordings caudal to the transection (Fig. 6).

In all five preparations examined, selective NMA or ACh application to supralumbar cord regions (bath partitioned at C_8/T_1, n=1; T_9/T_{10}, n=1; and T_{13}/L_1, n=3) elicited rhythmic activity in the cervicothoracic segments, as well as rhythmic, but non-locomotor-like, activity in the hindlimb ENGs. This observation contrasts with the failure to activate hindlimb rhythmicity by applying 5-HT to the supralumbar cord alone (see above). We also observed that application of either ACh or NMA to the lumbar cord alone (bath partitioned at the T_{13}/L_1 level, n=3) induced rhythmic hindlimb ENG activity. Thus, the lesioning and bath partition experiments both indicate that the isolated lumbar cord has the inherent capacity to generate rhythmic activity in response to NMA or ACh, but not 5-HT.

Left-right coordination of ACh-induced rhythmic hindlimb ENG activity was examined before and after isolating the lumbosacral enlargement (transected at T_{13}/L_1, n=3). In each instance the side-to-side relationship became uncoupled after the transection, suggesting that cross connections in supralumbar regions contribute to maintaining side-to-side relationships during ACh-induced activity. Compatible with the results obtained in these three T_{13}/L_1 transected preparations was another experiment in which rostrocaudal midsagittal section from C_1 to T_{13} inclusive uncoupled the left-right coordination of ACh/EDRO-induced hindlimb rhythmic activity. We did not examine coupling between hindlimbs for NMA-induced rhythms since left-right phase relationship tend to be labile during trials of NMA application, as previously reported (Cowley and Schmidt 1994b).

ACh/EDRO-induced rhythmic hindlimb ENG activity persisted after separation of the left and right halves of the lumbosacral enlargement (midsagittal lesion from the conus to the T_{12}/T_{13} junction) in seven of seven preparations. In four experiments side-to-side coordination was recorded both before and after the lesion. One of the four preparations showed bilateral rhythmic activity, but without a consistent side-to-side phase relationship,

even before the lesion was made. Three of the four preparations demonstrated a left-right phase relationship before the lesion, but this activity became uncoupled after midsagittal sectioning. The unlesioned preparation shown in Fig. 7A generated phase-related left-right alternation, but intralimb flexor-extensor coactivation, typical of ACh-induced non-locomotor-like patterns (Cowley and Schmidt 1994b). The same unlesioned preparation also demonstrated a locomotor-like pattern in response to 5-HT (Fig. 7B). After midsagittal separation of left and right halves of the spinal cord, from the conus to T_{13} inclusive, ACh/EDRO-induced rhythm generation was preserved, but at a much slower frequency (Fig. 7C). However, the left-right phase relationship was abolished. Before the midsagittal lesion was made the onset of left tibial ENG discharge regularly occurred after ~60% of the cycle period had elapsed (as measured from the onset of one right tibial ENG burst to the next, Fig. 7D). After the lesion, the onset of rhythmic discharge in the left tibial nerve occurred with no consistent phase relationship to right tibial ENG discharge (Fig. 7D).

Subsequent transection of one side of the split lumbosacral cord, at the rostral lumbar level, completely isolated the corresponding hemicord from the rest of the nervous system. ACh-induced rhythmic activity continued in lumbar hemicord that remained in continuity with the supralumbar spinal cord, but ceased in the completely isolated contralateral hemicord, in three of the four preparations tested. Thus, although the isolated bilaterally intact lumbosacral cord can regularly generate rhythmic activity in response to ACh application, the isolated lumbosacral hemicord has less capacity for ACh-induced rhythmogenesis. Once interconnections with the contralateral lumbar cord are disrupted, descending drive may be required to support rhythm production in the lumbosacral hemicord. Further experiments demonstrated that midsagittal lesions limited to the thoracolumbar region (T_{13}-L_1 inclusive; n = 3) also uncoupled left-right hindlimb coordination during ACh-induced activity. In one of these experiments, 5-HT was applied after washout of ACh; phase-related side-to-side ENG discharge compatible with locomotion was produced. This observation was consistent with the effects of 5-HT in three other preparations with midsagittal sections limited to the thoracolumbar junction region (see above and Fig. 4). In summary, these findings suggest that cross connections located throughout the rostro-caudal axis of the spinal cord may be critical for

maintaining left-right relationships within the ACh-sensitive network; in contrast, a relatively redundant system of cross projections appears to organize side-to-side coordination during 5-HT-induced rhythms.

Effect of transverse and midsagittal lesions on synchronous motor rhythms

Rhythmic patterns elicited by application of NMA, ACh or 5-HT become synchronous during γ-aminobutyric acid-A ($GABA_A$) or glycine receptor blockade (Cowley and Schmidt, 1995). Strong and mutually excitatory links among functionally and regionally distinct motoneuron populations are likely to underlie this highly characteristic and reproducible pattern of neural rhythm activation. In an effort to learn more about the distribution of these excitatory interconnections, experiments involving the application of bicuculline and strychnine were performed.

We first examined whether synchronous rhythms could be generated within small portions of spinal cord that included the ankle flexor and extensor motoneurons in particular. Bilaterally synchronous hindlimb ENG activity, produced by the glycine receptor antagonist strychnine (n=1), or the $GABA_A$ receptor antagonist bicuculline (n=2), in combination with NMA, persisted after transection at the L_3/L_4 junction. Additional lesions in the lumbar regions demonstrated that just two segments of isolated tissue (L_4-L_5 inclusive) readily maintained synchronous hindlimb ENG discharge (e.g., Fig. 8A). Even single hemisegments of spinal tissue (L_4 and L_5) generated rhythmic discharge in the presence of inhibitory amino acid antagonists and NMA (Fig. 8B). Similarly, short lengths of cervical (C_4-C_8 inclusive), thoracic (T_6-T_{10}), and rostral lumbar (L_1-L_3) spinal cord were capable of producing synchronous rhythmic activity. Thus, during inhibitory amino acid receptor blockade, the synchronous rhythmic activity produced in the hindlimbs does not require contributions from supralumbar spinal tissue, in contrast to 5-HT-induced locomotor-like patterns.

We then examined the distribution of the cross connections that mediate side-to-side coupling of synchronous rhythms. In particular, we wished to determine whether any specific rostrocaudal level of the cord contained cross projections that were essential for

maintaining left-right synchrony in the hindlimbs. Thus, the effect of midsagittal lesions at various levels was examined in 20 preparations, treated with either strychnine (n = 7), bicuculline (n = 12) or both strychnine and bicuculline (n = 1). Rhythmic activity was produced by either 5-HT (n = 5), ACh (n = 6), NMA (n = 8), or strychnine alone (n = 1). Bilateral synchrony of hindlimb rhythmic activity was maintained in five of five preparations after midsagittal lesions split the entire spinal cord except for one or two thoracic cord segments at various levels (e.g. Fig. 9, A1 and A2). After complete anatomic separation of the left and right sides of the spinal cord, rhythmic activity as well as intralimb flexor-extensor synchrony was maintained (Fig. 9B1), although side-to-side synchrony was abolished, as expected (Fig. 9B2).

We also determined whether progressive midsagittal separation of the cord, starting at the rostral or caudal end, was associated with a progressive decline in the ability of the network to maintain synchrony between left and right hindlimb motor nuclei (n = 13). A progressive out-of-phase shift of left-right discharge would imply the presence of a dominating or "leading" region within this rostrocaudally distributed system. However, as shown in Figure 9C, no evidence of a shift in the left-right phase coupling of hindlimb ENGs was observed during rostral extension of cord separation, which started at the conus and ended when C_1-C_2 were the only segments still bilaterally intact (Fig. 9C). In two preparations, midsagittal lesions starting at C_1 extended caudally into the lumbar segments. These lesions also failed to disrupt the left-right phase relationship of synchronous hindlimb activity (e.g. Fig. 9D).

Thus it appears that excitatory cross projections within just one or two segments at virtually any rostrocaudal level of the cord can mediate the synchronous coupling of side-to-side activity. This data, together with the results of the experiments in which isolated hemisegments were used, described above, suggest that the generation of rhythmic synchronous activity within each hemicord can occur independent of any connections with the contralateral side of the network.

Effect of spinal cord lesions and bath partition on combined 5-HT/NMA-induced rhythms

Application of 5-HT combined with NMA (or DHK) has proven to be a useful means of establishing locomotor-like patterns in the *in vitro* neonatal rat whole spinal cord preparation (e.g. Cowley and Schmidt 1994a; Kjaerulff et al. 1994; Sqalli-Houssaini et al. 1993). Moreover, Cazalets et al. (1995) recently examined the effects of application of the 5-HT/NMA combination to specific segments of the lumbar cord in this preparation (see Discussion). However, selective application of these neurochemicals to supralumbar regions has not yet been reported. Therefore, it was of interest to investigate the distribution of the rhythmogenic network activated by 5-HT/NMA in the present series, and to compare the results to observations obtained using 5-HT alone.

Although NMA alone elicited rhythmic activity in caudal lumbar segments, isolated by transection at the L_3/L_4 junction (Fig 6), subsequent addition of 5-HT (10-125 μM) to the NMA containing bath solution was associated with loss of rhythmicity caudal to the transection (n = 5). Instead, the NMA-induced rhythmic activity was replaced by tonic discharge, similar to the effect of 5-HT alone on the isolated lumbar cord. However, rostral to the transection, rhythmic activity monitored on cervical, thoracic and rostral lumbar (e.g. L_2) ventral roots continued. Further transection at T_{13}/L_1 in one preparation, and T_{12}/T_{13} in another, resulted in the loss of rhythmic activity on the L_2 ventral root, but not on cervical and thoracic roots. In one preparation additional serial transections yielded short lengths of isolated cervical (C_3-C_8 inclusive) and thoracic (T_5-T_{10} inclusive) cord that were still capable of generating rhythmic activity in the presence of 5-HT and NMA. Consistent with the effect of 5-HT alone, rhythmic lumbar activity evoked by the 5-HT/NMA combination persisted after midsagittal lesions through the lumbar enlargement (conus to T_{13}, n=1; or conus to L_1, n=1). In summary, comparison of these results with those obtained using 5-HT or NMA alone suggests that rhythmogenic network activated by the 5-HT/NMA combination shares more features in common with the 5-HT-sensitive network, than with circuitry activated by NMA alone.

Application of 5-HT/NMA to the rostral side of baths partitioned at the T_9/T_{10} (n=2) or C_8/T_1 (n=2) produced rhythmic activity throughout the spinal cord as monitored via cervical, midthoracic, rostral and caudal lumbar ventral root records (e.g. Fig 10). The addition of 5-HT/NMA caudal to partitions established at the L_3/L_4 level produced only tonic activity, consistent with the effect of 5-HT/NMA application to the transected (at L_3/L_4) spinal cord (see above). It appears that the network activated by the 5-HT/NMA combination is distributed throughout the supralumbar region, similar to the distribution of the network induced by 5-HT alone.

Discussion

These results demonstrate the distributed nature of networks generating motor rhythms, including locomotion, in the mammalian spinal cord. In addition, the data suggest that different patterns of discharge, activated by specific neurochemicals, are mediated by neural circuits with heterogeneous regional distributions.

Locomotor network is distributed in the supralumbar region of the spinal cord

Previous work comparing the effects of bath-applied 5-HT, NMA and ACh to the entire spinal cord indicated that a locomotor-like pattern of flexor and extensor activity is most commonly elicited by 5-HT (Cowley and Schmidt 1994b). In the present series, components of the 5-HT-sensitive network producing a locomotor-like pattern of hindlimb flexor and extensor activity were found to be distributed throughout the supralumbar region of the spinal cord. The lumbar cord itself displayed no inherent rhythmogenic response to 5-HT application. Supralumbar circuitry not only generated oscillatory drive, but also coordinated left-right interactions for more caudal (lumbar) spinal cord regions, as demonstrated by the results of midsagittal spinal cord sectioning experiments.

Our combined results, derived from a variety of transection and bath partition experiments (n = 14 total), suggest that the caudal boundary of the 5-HT-sensitive distributed network is located near the T_{13}/L_1 junction. Similarly, we found no evidence of rhythm production following application of the 5-HT/NMA combination to lumbar segments, although our 5-HT/NMA results are based on a smaller number of observations compared with the 5-HT data. It should be noted, that Cazalets et al. (1995) obtained rhythmic activity in response to 5-HT/NMA application to the rostral lumbar segments, as did Kjaerulff and Kiehn (1994). Therefore, despite our negative results, we hesitate to exclude the possibility that rhythmogenic circuitry responsive to the 5-HT/NMA combination may extend caudally into the rostral lumbar region. Possible reasons for the discrepancy between our results and those reported previously are discussed below.

Regardless of the exact spinal cord level containing the caudal limit of the distributed network, our observation that caudal lumbar segments fail to generate rhythmic activity upon exposure to 5-HT/NMA is consistent with the results of Cazalets et al. (1995) who reported no evidence of inherent rhythm-generating capacity in segments caudal to the L_2 level. Similarly, Kjaerulff and Kiehn (1994) found no rhythmic activity, or only slow low amplitude modulation, in isolated caudal lumbar segments exposed to the 5-HT/NMA combination. These combined observations are compatible with studies of locomotion in the chick embryo (Ho and O'Donovan 1993), and scratching in the cat (Deliagina et al. 1983) and turtle (Mortin and Stein 1989), which have shown dominance of the rhythmogenic capacity of rostral segments over more caudal regions.

The concept that the central pattern generator for locomotion is "not segmentally distributed but is restricted" to the L_1 and L_2 segments (Cazalets et al. 1995) is incompatible with the present results. In contrast to the study reported by Cazalets et al. 1995, we applied neurochemicals to supralumbar portions of the spinal cord, in addition to testing their direct effects on the lumbar cord. Thus rhythmic activity was induced in the cervical and thoracic spinal cord when 5-HT was applied to the isolated cervicothoracic region. Lumbar rhythmicity failed to occur after selective application of 5-HT to the lumbosacral cord (which included the L_1 and L_2 segments), but was evoked in response to application of 5-HT to the entire spinal cord excluding the T_{10}-L_2 region. These observation not only suggest that the network is distributed in supralumbar regions, but also indicate that activation of rostral lumbar cord segments by the applied neurochemicals is not critical for rhythm generation. We also demonstrated combined 5-HT/NMA effects which are incompatible with a restricted L_1/L_2 localization for the locomotor network oscillator. As was observed in the presence of 5-HT alone, 5-HT/NMA elicited rhythmic activity in cervical and thoracic segments despite isolation from more caudal regions (including L_1 and L_2) by bath partition or cord transection. The development of rhythmic activity in the lumbar cord in response to selective application of 5-HT/NMA to the cervical or cervicothoracic segments further argues against a model characterized by hindlimb rhythm generators strictly localized to the L_1/L_2 segments. In

addition, L_2 ventral root rhythmic activity was abolished by transections at the T_{12}/T_{13} or T_{13}/L_1 junctions despite continued exposure of the spinal cord below the lesion to 5-HT/NMA. Finally, midsagittal lesions from the conus through the L_1 segment inclusive, or restricted to the thoracolumbar region in particular (e.g. T_{10}-L_4) had no effect on hindlimb rhythm generation or coordination. In summary, these observations strongly favor a system wherein hindlimb locomotor output is under the influence of a distributed and predominantly supralumbar network. An anatomically dispersed organization of this type is compatible with the multiple "unit burst" concept of Grillner (1981) and is well suited to integrate forelimb and hindlimb rhythmic activity. In addition, this model can readily accommodate thoracic oscillatory mechanisms, as required for the generation of the rhythmic activity which occurs in axial muscles during locomotion (Ho and O'Donovan 1993; Koehler et al. 1984).

Although application of 5-HT to the whole cord produced locomotor-like activity in the hindlimb ENGs, addition of 5-HT to the cervicothoracic cord alone induced rhythmic activity only in supralumbar regions. Selective exposure of the lumbar region to 5-HT produced only tonic activity on hindlimb ENGs. Therefore, relatively non-specific background excitation of hindlimb motor centers, provided in this case by direct actions of 5-HT on the lumbar cord, may be required to bring lumbar circuitry above threshold for responding to the descending rhythmic drive (provided by the supralumbar oscillatory network). Similarly, Cazalets et al. (1995) reported that when 5-HT/NMA application to the L_1/L_2 segments failed to induce ventral root activity in the caudal lumbar region, nonspecific electrical stimulation of the coccygeal spinal cord brought L_5 motoneurons to threshold for rhythmic firing. Because exposure of the cervicothoracic spinal cord to a combination of 5-HT and NMA can induce hindlimb rhythmic activity in the absence of direct neurochemical excitation of the lumbar cord (e.g. Fig 10), the 5-HT/NMA combination may be a more potent activator of supralumbar rhythmogenic circuitry, and its associated descending drive, than is 5-HT alone. This hypothesis is supported by the observation that locomotor-like rhythms induced by 5-HT, combined with NMA or DHK, are often better developed and more sustained than those induced using either substance alone (Cowley and Schmidt 1994a; Sqalli-Houssaini et al. 1993).

Why 5-HT fails to induce rhythmic activity when applied directly to the lumbar spinal cord of the neonatal rat is unclear. Locomotor circuitry in the *Xenopus* displays a rostrocaudal gradient of development and sensitivity to 5-HT in the early post-embryonic stage, corresponding with the caudal growth of 5-HT containing raphe projections (Sillar et al. 1992). In the rat, rhythmogenic circuitry is substantially reorganized by embryonic day 18 (Kudo et al. 1991) and 5-HT-induced patterns of locomotion remain stable in the immediate postnatal period (postnatal days 0 - 4) (Kiehn and Kjaerulff 1996). Immunohistochemical studies in rat indicate that descending 5-HT fibers enter the ventral and intermediate gray of the lumbar cord at approximately embryonic day 18 (Rajaofetra et al. 1989; Ziskind-Conhaim et al. 1993) and show close apposition to motoneurons by postnatal day 1 (Tanaka et al. 1992; Ziskind-Conhaim et al. 1993). However, the adult pattern of serotonergic innervation is not reached until 3 wk postnatally (Rajaofetra et al. 1989). During the first two weeks of life, intact rats use mainly their forelimbs for pivoting and crawling; they do not develop sufficient hindlimb weight support for quadruped walking until day 12 - 13 (Altman and Sudarshan 1975). Although analysis of L-DOPA-induced air-stepping in suspended rats demonstrated coordinated gait, involving all four limbs on the day of birth, forelimb stepping predominated over quadrupedal patterns until after day 5 (McCrea et al. 1994; Stehouwer et al. 1994). Thus, the possibility that the rostrocaudal gradient of spinal cord sensitivity to 5-HT observed in the present study corresponds, at least in part, with developmental factors, cannot be excluded. A further consideration is the distribution of intraspinal 5-HT-containing neurons. In addition to descending 5-HT projections, intraspinal 5-HT-containing neurons contribute 2 - 15% of the total 5-HT content in the rat spinal cord (Newton and Hamill 1988). Although these neurons may be anatomically related to the autonomic nervous system (Newton and Hamill, 1988; Newton et al. 1989), clarification of their targets and functional role remains to be accomplished. In view of the present results, it is of interest that intraspinal 5-HT neurons are located primarily in the thoracic region and L_1 segment, while the L_2 - L_6 segments contain none (Newton and Hamill 1988).

Intravenous injection of noradrenergic precursors (L-DOPA) or agonists (clonidine) induce locomotor activity in acutely spinalized cats (Barbeau and Rossignol 1991; Forssberg and Grillner 1973; Grillner and Zangger 1979). However, attempts to elicit locomotion in similar preparations using serotonergic drugs has been unsuccessful (Barbeau and Rossignol 1991; Grillner and Shik 1973). The present results, demonstrating a 5-HT-sensitive oscillatory network distributed rostral to the thoraco-lumbar junction, suggest that the failure of 5-HT to activate locomotion in previous cat experiments may have been related to the use of low (T_{13}) spinal preparations (Barbeau and Rossignol 1991; Grillner and Shik 1973). In support of this possibility is the observation that systemic administration of the serotonergic precursor 5-hydroxytryptophan evokes locomotion in spinalized rabbits in which some of the thoracic cord had been retained (Viala and Buser 1971). However, this explanation for the varied effects of 5-HT reported in the literature is based upon the unproven assumption that a 5-HT-sensitive oscillatory network exists and has a similar regional distribution in these different species. Obviously, further experiments are necessary to clarify whether interspecies differences exist.

Different neurochemicals activate different rhythmogenic substrates

The present results support our earlier suggestion that different neurochemicals preferentially activate different rhythmogenic substrates (Cowley and Schmidt 1994b), and may also explain certain inconsistencies in the literature. For instance, although Cazalets et al. (1995) found no rhythmogenic properties caudal to the L_2 level, Kudo and Yamada (1987) observed that even isolated L_4 - L_5 hemisegments generated alternating activity in the ipsilateral hindlimb. This discrepancy may be accounted for by the fact that Cazalets et al. (1995) used 5-HT/NMA in their study, whereas Kudo and Yamada (1987) applied NMA alone. In the present study, we showed that application of NMA alone to the lumbar region induces rhythmic activity in those segments, whereas exposure of the same lumbar tissue to 5-HT or 5-HT/NMA evokes tonic activity only. Why exposure of the lumbar cord to combined 5-HT/NMA should produce only tonic activity

while NMA alone induces rhythmic activity is unclear. Possibly the discharge behavior of lumbar interneurons and/or motoneurons is dominated by tonic or excessive excitation during combined 5-HT/NMA exposure, in which case the successful induction of rhythmic network activity may require a careful balancing of the 5-HT and NMA concentrations. This may account for certain discrepancies in the literature including, in the present study, the failure to induce rhythmic activity in rostral lumbar cord segments using 5-HT/NMA, in contrast to the results of others (Cazalets et al. 1995; Kjaerulff and Kiehn 1994). Regardless of the exact explanation for conflicting observations in the literature, the present findings suggest that investigations of spinal cord rhythmogenesis need to consider the particular activating substance(s) employed as well as the type of motor pattern examined.

The data suggest that the spinal cord has a greater inherent capacity to develop rhythmic activity in response to application of NMA than 5-HT, at least in the lumbar region. However, this may not be entirely unexpected. NMDA receptor activation is known to generate intrinsic oscillatory behavior in synaptically isolated spinal cord interneurons and motoneurons (Hochman et al. 1994a,b). Possibly, then, the requirement for NMDA receptor activation in spinal cord rhythmogenesis pertains mainly to the induction of membrane voltage bistability and/or oscillatory activity, whereas other neuromodulators or activators such as 5-HT have a greater role in organizing specific patterns of behavior, such as locomotion, at a network level. This may explain why the pattern of NMA-induced rhythmic activity we observed in the present and previous (Cowley and Schmidt 1994b) studies is often labile and non-locomotor-like in quality (although see Kudo and Yamada 1987) in contrast to the rhythms produced in the presence of 5-HT.

Although it was reported that ACh activates locomotor circuitry in the neonatal rat spinal cord (Smith et al. 1988), we rarely observe a locomotor-like sequence of ENG activity in response to this substance; more commonly side-to-side alternation of coactivated intralimb flexor-extensor pairs occurs (Cowley and Schmidt 1994b). The extent to which the distinct patterns of rhythmic activity evoked by 5-HT and ACh are mediated through differential modulation of common network components, as opposed to activation of

separate neural substrates, is unknown. However, the present study demonstrated that ACh-sensitive rhythmogenic circuitry exists within the (bilaterally intact) lumbar cord, in contrast to the distribution of the 5-HT-sensitive network. Also in contrast to the results of 5-HT application, ACh-induced alternating left-right hindlimb phase relationships could be uncoupled by midsagittal lesions made at a variety of rostrocaudal levels of the spinal cord. The latter finding, in conjunction with the observation that ACh failed to induced rhythmic activity in the isolated lumbo-sacral hemicord in three out of four preparations, suggests that bilaterally distributed components are of particular importance for the activation and organization of the ACh-sensitive network. Similarly, evidence of an important contribution from contralateral spinal cord circuitry was recently shown for the central pattern generator for scratching in the turtle (Stein et al. 1995). In summary, although a more complete description of 5-HT- and ACh-sensitive circuits is awaited, this study suggests that these networks can be characterized, at least in part, by regionally and anatomically distinct elements.

Side-to-side phase relationships are mediated by distributed systems of cross connections.

Cazalets et al. (1995) demonstrated that separation of the left and right halves of the lumbo-sacral spinal cord, up to the L_2/L_3 level, fails to disrupt the side-to-side relationship of 5-HT/NMA-induced rhythmic activity in the lumbar cord. Similarly, chronic midsagittal separation of the cord between L_2 and S_1 had no effect on left-right hindlimb coordination during walking in cats (Kato 1988). In the present study, we observed that midsagittal lesions extending from the conus to the thoracolumbar junction had no effect on 5-HT-induced locomotor-like patterns in the hindlimbs. Thus it appears that in the presence of a bilaterally intact supralumbar spinal cord, reciprocal interconnections in the lumbar region are not essential for interlimb coordination. Hindlimb locomotor-like activity was also preserved in preparations with extensive midsagittal separation of the cervicothoracic spinal cord or more localized midsagittal lesions through the thoracolumbar junction. Therefore, it appears that no single region of

the spinal cord is critical for the maintenance of 5-HT-induced reciprocal inhibitory interactions among the hindlimbs, provided, other regions of the cord are preserved bilaterally intact. These observations are compatible with a widely distributed and redundantly organized system of reciprocal cross projections in the spinal cord. Presumably some of these interconnections are inhibitory in nature and help ensure an alternating pattern of left-right activation.

Similarly, the present results imply the existence of an extensively distributed and redundantly organized system of reciprocal excitatory cross projections. These pathways, which are unmasked by the blockade of inhibitory amino acid receptors, synchronize rhythmic activity among functional antagonists (Cowley and Schmidt 1995), and therefore may be well-suited to mediate coactivation of selected motor populations during locomotion and other behaviors. Although inter- and intralimb synchrony was generated by small portions of lumbar tissue, consistent with reciprocal excitatory connections at the segmental level, the present study also showed that synchronous activity was preserved despite almost complete midsagittal separation of the two halves of the spinal cord (i.e., sparing only a few segments of residual bilaterally intact cord). The location of the preserved residual cross connections along the rostrocaudal axis of the spinal cord was not important, compatible with a redundantly organized system of cross links.

Relevance to mammalian locomotion

We have described the regional distribution of circuitry activated in response to several neurochemicals applied to selected regions of the cord, or the entire spinal cord in the presence of specific lesions. However, identification of the endogenous substances that activate and modulate locomotor networks in intact mammals, as well as the site and temporal pattern of release of those substances, awaits further study. Neuromodulatory systems not yet examined or identified may elicit locomotor behavior through activation of networks with anatomical distributions that are distinct from those characterized in the present study. In addition, it may be discovered that different neuromodulators elicit

specific behaviors through functional reconfiguration of the same anatomical network, as has been demonstrated in lower animals (for review, see Harris-Warrick and Marder 1991). Further investigation of these issues is clearly needed.

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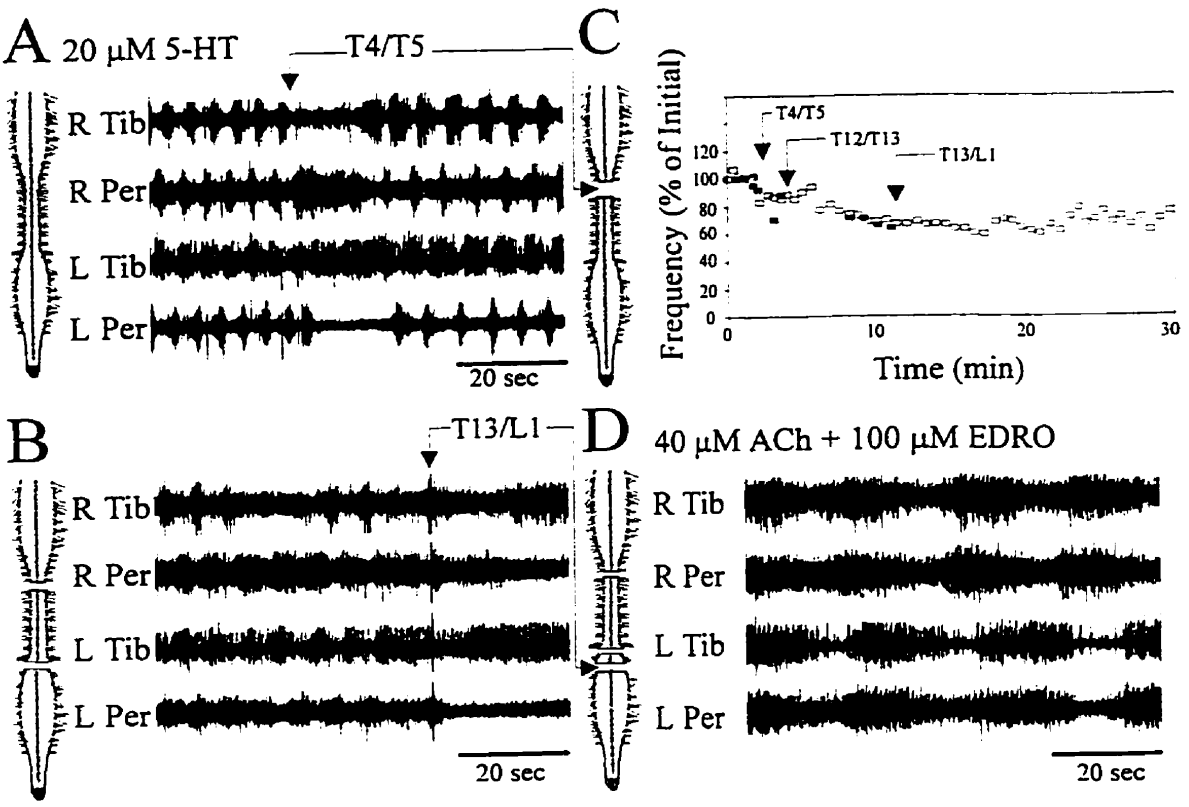

A
20 μM 5-HT
T4/T5
R Tib
R Per
L Tib
L Per
20 sec
C
Frequency (% of Initial)
T4/T5
T12/T13
T13/L1
120
100
80
60
40
20
0
0
10
20
30
Time (min)
B
T13/L1
R Tib
R Per
L Tib
L Per
20 sec
D
40 μM ACh + 100 μM EDRO
R Tib
R Per
L Tib
L Per
20 sec

Figure 1

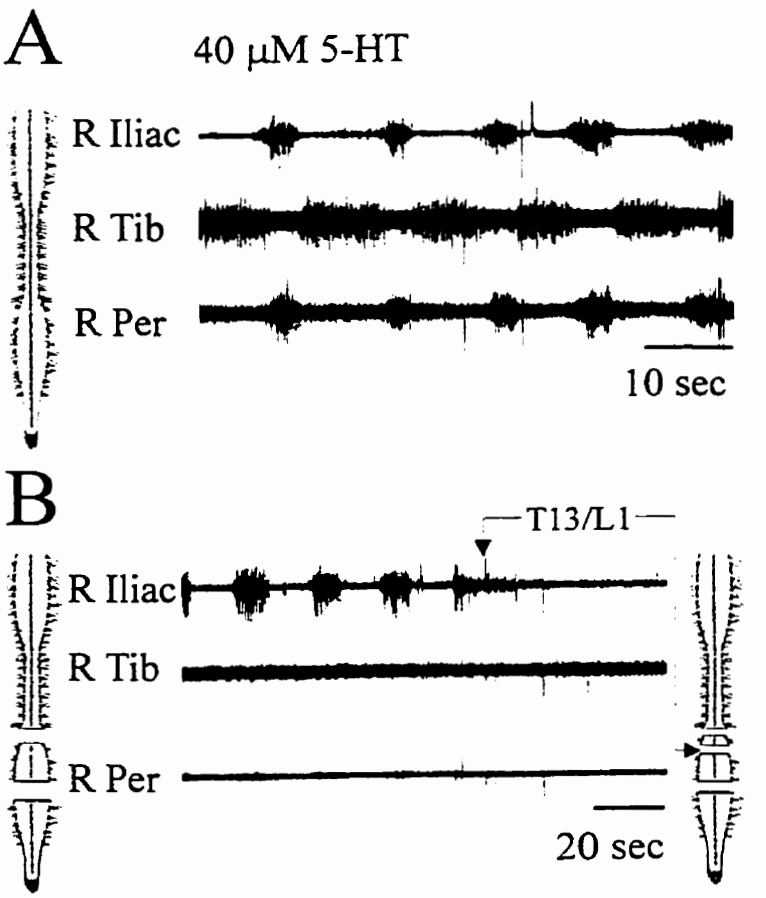

A
40 μM 5-HT
R Iliac
R Tib
R Per
10 sec
B
T13/L1
R Iliac
R Tib
R Per
20 sec

Figure 2

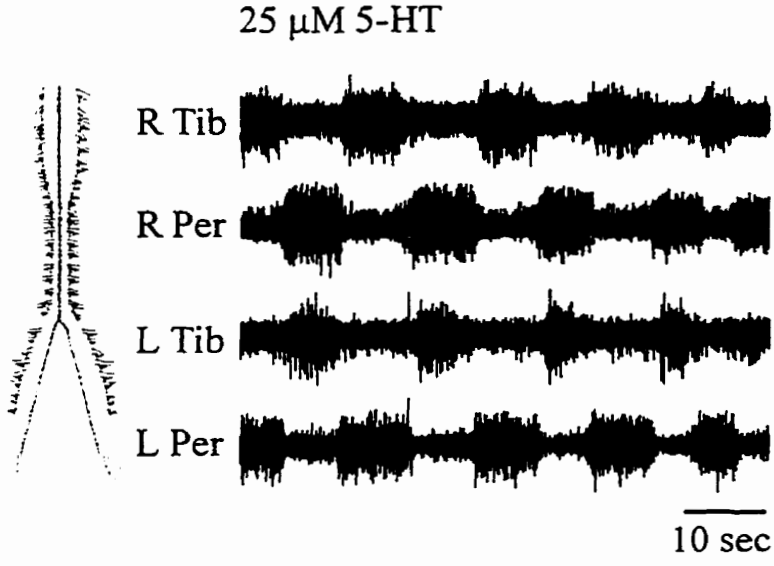


Figure 3

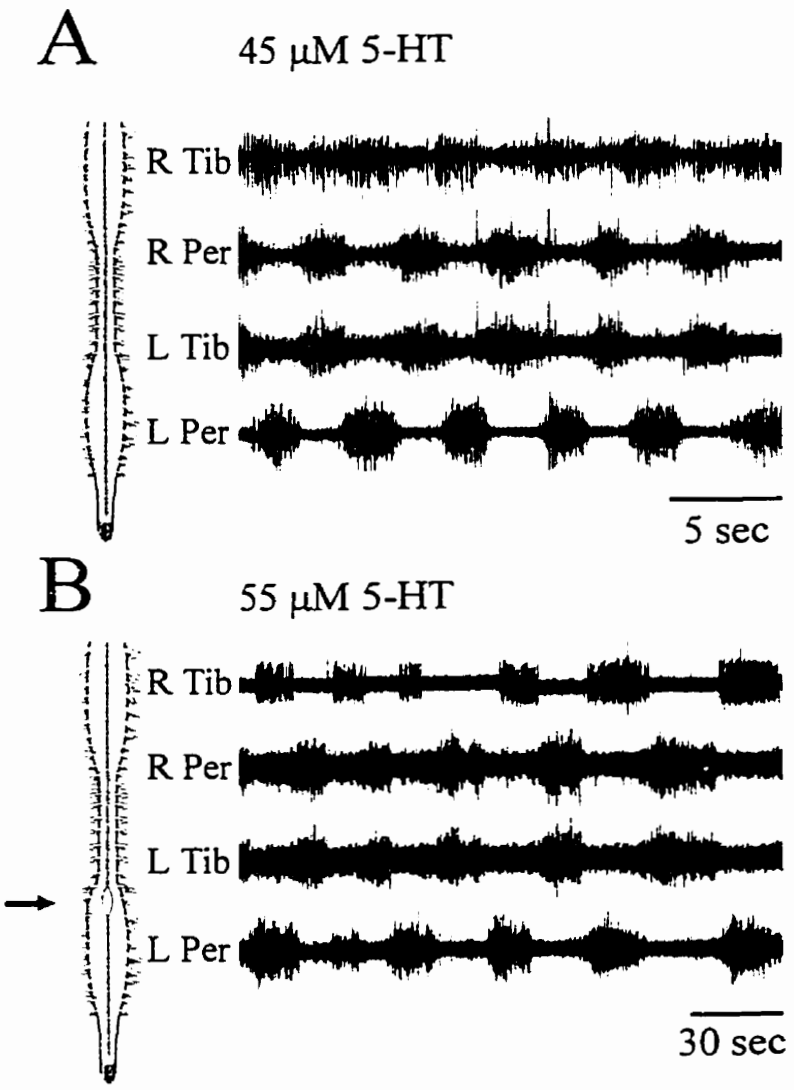

A
45 μM 5-HT
R Tib
R Per
L Tib
L Per
5 sec
B
55 μM 5-HT
R Tib
R Per
L Tib
L Per
30 sec

Figure 4

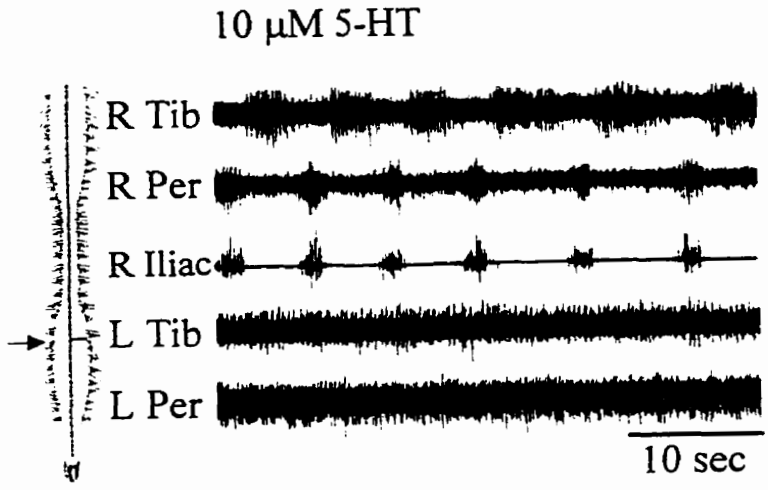


Figure 5

Figure 6

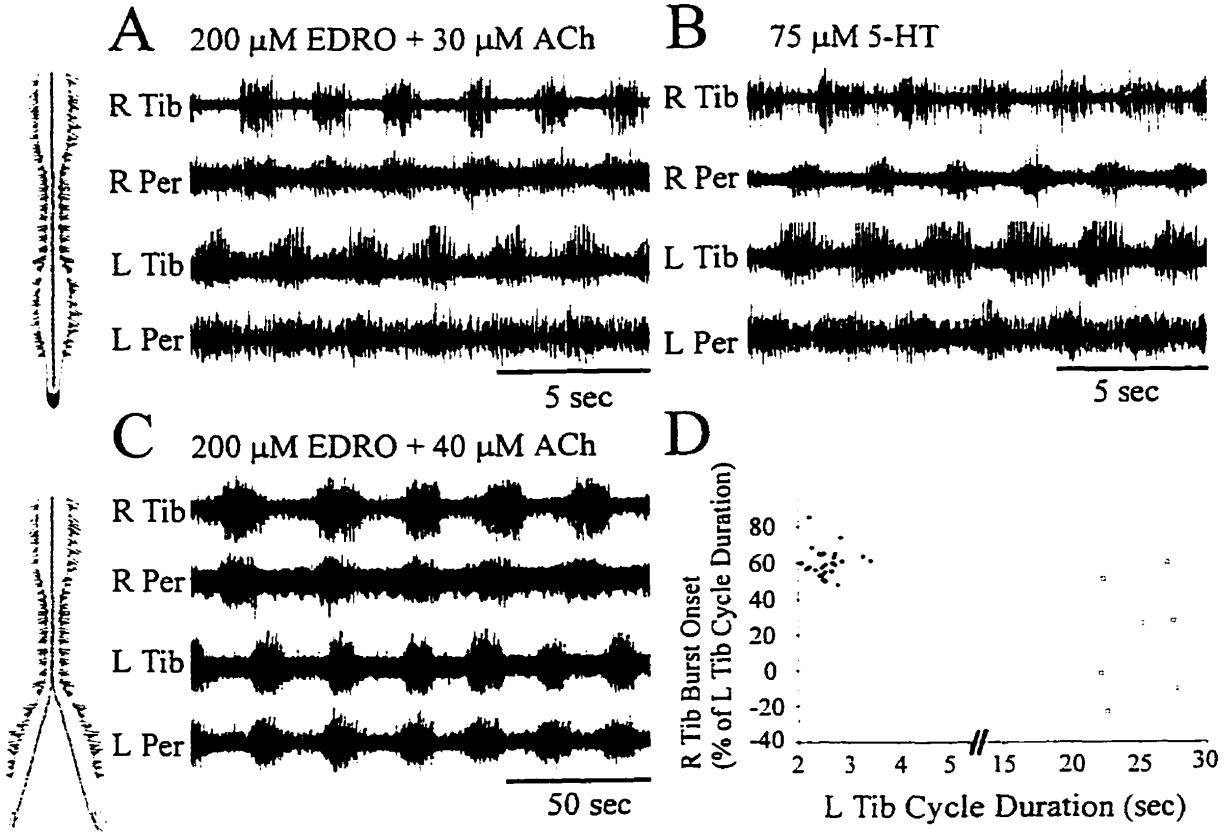

A
200 μM EDRO + 30 μM ACh
R Tib
R Per
L Tib
L Per
5 sec
B
75 μM 5-HT
R Tib
R Per
L Tib
L Per
5 sec
C
200 μM EDRO + 40 μM ACh
R Tib
R Per
L Tib
L Per
50 sec
D
R Tib Burst Onset
(% of L Tib Cycle Duration)
80
60
40
20
0
-20
-40
2
3
4
5
15
20
25
30
L Tib Cycle Duration (sec)

Figure 7

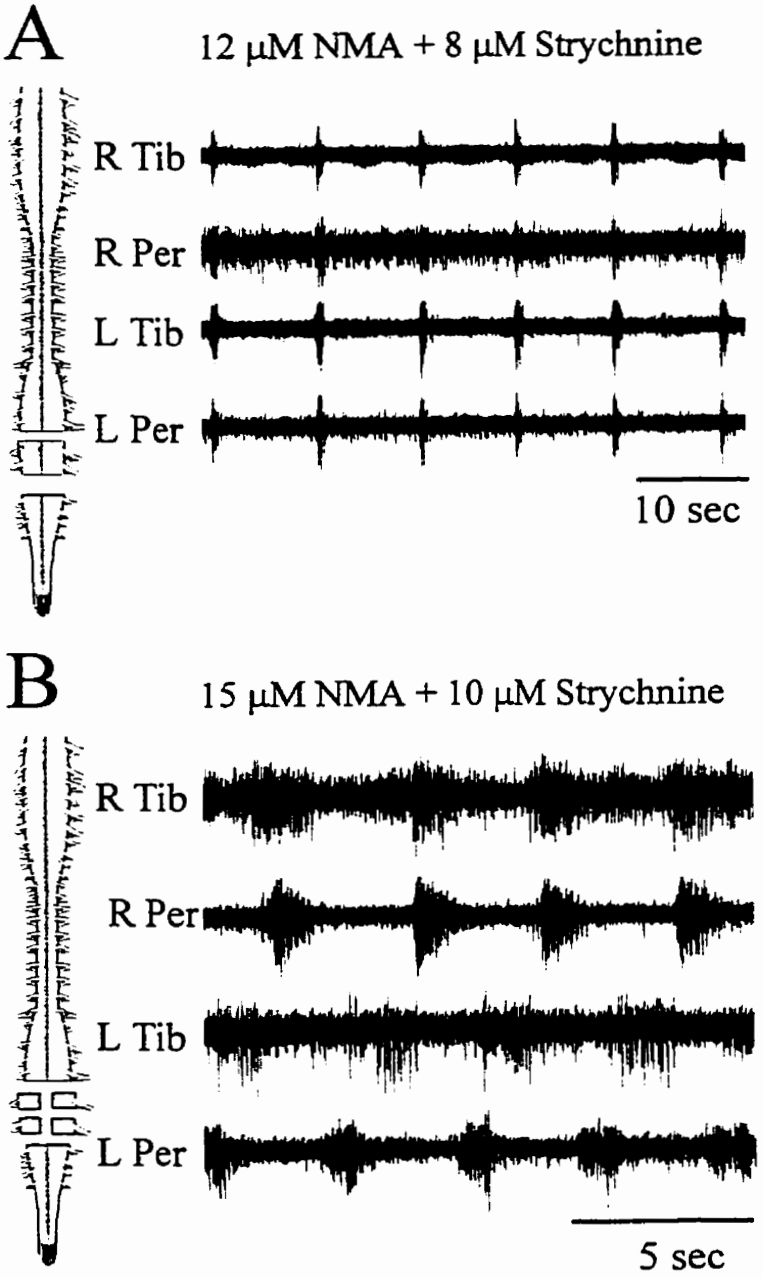

A
12 μM NMA + 8 μM Strychnine
R Tib
R Per
L Tib
L Per
10 sec
B
15 μM NMA + 10 μM Strychnine
R Tib
R Per
L Tib
L Per
5 sec

Figure 8

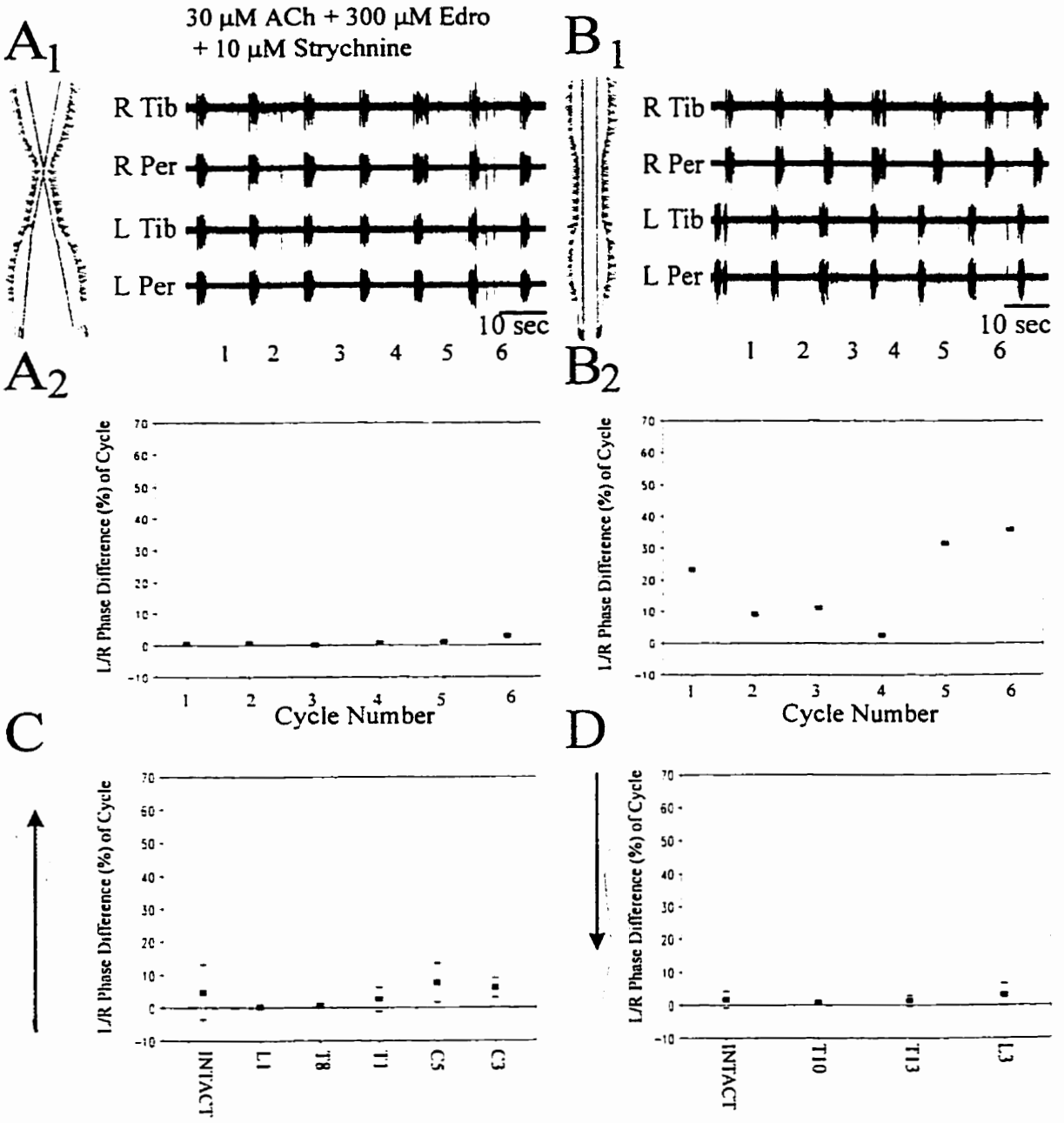

A1
30 μM ACh + 300 μM Edro
+ 10 μM Strychnine
R Tib
R Per
L Tib
L Per
10 sec
1 2 3 4 5 6
B1
R Tib
R Per
L Tib
L Per
10 sec
1 2 3 4 5 6
A2
L/R Phase Difference (%) of Cycle
Cycle Number
B2
L/R Phase Difference (%) of Cycle
Cycle Number
C
L/R Phase Difference (%) of Cycle
INTACT
L1
T8
T1
C5
C3
D
L/R Phase Difference (%) of Cycle
INTACT
T10
T13
L3

Figure 9

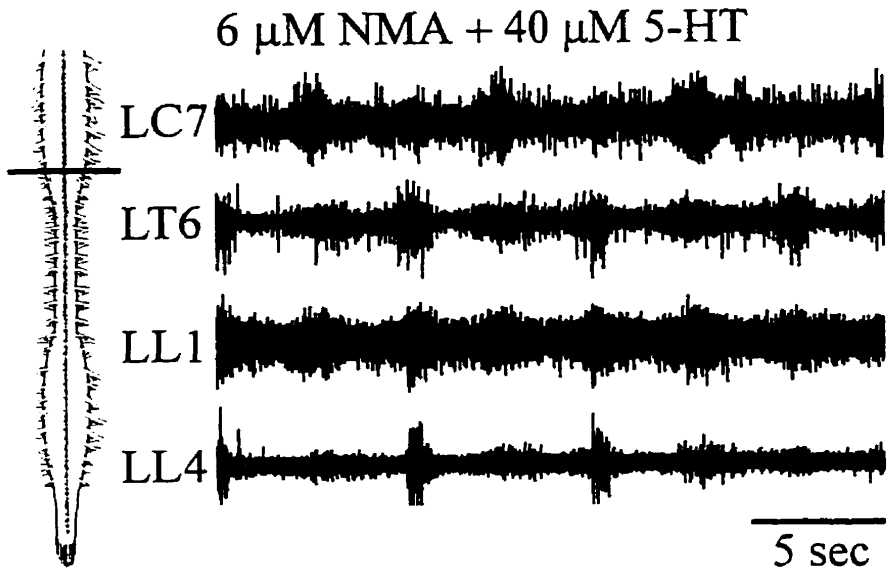


Figure 10

Figure Legends

Figure 1. Effect of complete transverse spinal cord lesions on serotonin (5-HT)-induced locomotion. **A:** locomotor-like pattern of alternating tibial (Tib) and peroneal (Per) nerve activity, produced by bath application of 5-HT, continued after transection through the T_4/T_5 junction (↓). **B:** locomotion also continued after transection at the $T_{12}/_{13}$ junction (performed before the beginning of record shown in **B**), but was permanently abolished after transection at the T_{13}/L_1 level (↓). The increased tonic discharge observed in response to the latter transection completely subsided after several minutes (not shown). **C:** frequency of 5-HT-induced rhythmic activity was plotted as a function of time for an unlesioned preparation (unfilled square) as well as for the lesioned preparation shown in **A** and **B** (■). Values are expressed as a percentage of the rhythm frequency observed at the onset of the recording. **D:** after the T_{13}/L_1 transection acetylcholine (ACh)/edrophonium (EDRO) elicited rhythmic activity despite the failure of 3 further applications of 5-HT.

Figure 2. Essential role of supralumbar cord in generating 5-HT-induced locomotion. **A:** 5-HT-induced rhythmic bilateral hindlimb electroneurogram (ENG) activity (tibial and peroneal, right side only shown) and iliacus (Iliac) EMG activity in this intact preparation. **B:** spinal cord was then transected at the T_{12}/T_{13} and L_3/L_4 junctions (shown in the schematic at left). Rhythmic activity continued at a slower frequency in the iliacus muscle, but ceased completely in the tibial and peroneal nerves. Subsequent transection at the T_{13}/L_1 junction (↓), shown in the schematic on the right, permanently abolished iliacus activity.

Figure 3. Effect of midsagittal section of the lumbo-sacral spinal cord on 5-HT-induced locomotion. A rhythmic locomotor-like pattern of flexor-extensor and left-right alternation persisted despite midsagittal separation of the left and right sides of the cord from the T_{13}/L_1 junction through to the conus inclusive.

Figure 4. Cross connections in the caudal thoraco-rostral lumbar segments were not essential for the generation of a coordinated locomotor-like pattern in response to 5-HT. **A:** 5-HT-induced a locomotor-like pattern of activity in the intact spinal cord. **B:** after a midsagittal section extending from the middle of the T_{12} segment through to the middle of the L_1 segment the locomotor-like pattern persisted, although the frequency was slower and the rhythm was slightly less regular.

Figure 5. Transverse hemisection of the rostral lumbar cord, at the left L_1/L_2 level, abolished 5-HT-induced rhythmic activity in ipsilateral (left), but not contralateral, segments caudal to the lesion. Note the left side of the cord is on the right side of the drawing (cord is depicted ventral side up).

Figure 6. In contrast to the effect of 5-HT, N-methyl-D,L-aspartate (NMA) induced rhythmic activity in spinal cord segments caudal to transection at the L_3/L_4 junction. The variability in pattern of left-right alternation from one moment to the next is not uncommon in response to NMA alone (Cowley and Schmidt 1994b).

Figure 7. Effect of midsagittal sectioning of the spinal cord on ACh-induced rhythmic activity. **A:** in the intact spinal cord, ACh/EDRO-induced rhythmic activity consisting of alternating left-right discharge and intralimb flexor-extensor coactivation. **B:** 5-HT-induced a locomotor-like pattern of activity in the same preparation. **C:** After midsagittal section from the T_{12}/T_{13} junction to the conus inclusive (schematic on left), the spinal cord remained responsive to ACh/EDRO (although the rhythm frequency was slower). **D:** left-right phase relation was calculated for consecutive cycles of ACh/EDRO-induced rhythmic activity induced before (n = 27 cycles) and after (n = 7 cycles) the lesion shown in **C**. Before the lesion, the right tibial nerve discharge occurred after ~60% of the cycle had lapsed (◆). After the midsagittal lesion the right tibial nerve discharge occurred variably throughout the step cycle (unfilled square).

Figure 8. Effect of spinal cord lesions on synchronous rhythms. **A**: bilaterally synchronous activity was induced by NMA, in the presence of strychnine, after isolation of the L_4-L_5 segment (shown schematically on left). All ventral roots except L_4 and L_5 have been cut. **B**. rhythmic activity was also generated by isolated single hemisegments. Because the L_4 and L_5 segments both contribute axons to the peroneal and tibial nerves (Cowley and Schmidt 1994b), the phasic activity displayed by any given nerve in this recording may have originated from rhythm generators in one or both of the isolated hemisegments supplying that nerve.

Figure 9. Effect of midsagittal lesions on synchronous rhythms. **A1:** extensive midsagittal section, sparing only the T_6 segment, had no effect on left-right and flexor-extensor synchrony. **A2:** phase difference between the onset of left and right peroneal nerve discharge, expressed as a percentage of the cycle duration, is shown for each cycle numbered in **A1**. **B1:** after complete separation of left and right halves of the spinal cord intralimb flexor-extensor synchrony was maintained. **B2:** examination of the phase relationship between discharge in the peroneal nerves on the left and right sides (for each cycle numbered in **B1**) shows uncoupling of the two sides. **C:** in another preparation, the effect of a progressively ascending midsagittal section on synchronous rhythms was monitored. The phase difference (mean $\pm$ SD) between the onset of left and right tibial nerve discharge is expressed as a percentage of the cycle duration, and is shown for the intact spinal cord as well as after midsagittal lesions extending rostrally to the levels indicated. Left-right synchrony persisted after lesions from the conus to C_3 inclusive, (i.e. only C_1 and C_2 bilaterally intact). **D:** in another preparation, the effect of progressive midsagittal section in the rostrocaudal direction was monitored. The phase difference (mean $\pm$ SD) between left and right sides remained near zero, even after separating C_1 to L_3 inclusive.

Figure 10: Selective application of 5-HT combined with NMA to the cervical side of a bath partitioned at the C_7/C_8 junction induced rhythmic activity throughout the spinal cord including the lumbar region.

General Discussion

This thesis describes investigations of the neurochemical mechanisms generating and coordinating locomotion, as studied using the *in vitro* neonatal rat spinal cord preparation. The discussion will focus on the following four main findings, as related to the four papers comprising this thesis. First, the use of ventral roots may be unreliable for monitoring hindlimb flexor and extensor discharge during locomotion. Second, exogenous application of different neurochemicals preferentially induces distinct patterns of rhythmic hindlimb flexor and extensor activity. Third, glycine and $GABA_A$ receptor mechanisms both play a role in mediating left-right and intralimb flexor-extensor alternation. Finally, the networks generating rhythmic motor activities, including locomotion, are distributed in nature and motor activities induced by different neurochemicals may be mediated in part by distinct neuroanatomical substrates. For additional discussion the reader is referred to the relevant paper. Reports published after, and relating to, the four papers of this thesis will also be discussed. Finally, a model to account for the findings within this thesis is presented.

Part I: Some limitations of ventral root recordings for monitoring locomotion in the *in vitro* neonatal rat spinal cord preparation.

In part I (Cowley and Schmidt 1994a) we examined hindlimb electroneurogram (ENG) activity and ventral root activity during rhythmic motor behaviours in the neonatal rat spinal cord preparation to determine if ventral root recordings could reliably reflect the underlying flexor-extensor activity. Additionally, we examined the contribution of lumbar ventral roots to the activity of various hindlimb flexor and extensor nerves to demonstrate that ventral root discharge during rhythmic activity was composed of a mixture of flexor and extensor motor axon activity. Based on our observations, we

concluded that using ventral root recordings to observe underlying hindlimb flexor and extensor activity may be unreliable.

In particular, we observed several different patterns of ventral root discharge during hindlimb stepping in hindlimbs-attached preparations. Ventral root discharge during hindlimb stepping included, tonic activity only (on all ventral roots monitored), alternating left-right discharge but in-phase activity on ipsilateral ventral roots (including L3 and L5 in n = 1), and in-phase rhythmic activity of all monitored ventral roots both bilaterally and at different segmental levels (including L3 and L5 in n = 1). Simultaneous monitoring of L3, L4, L5 and L6 ventral root discharge during alternating rhythmic activity from the ipsilateral hindlimb ankle flexor (peroneal, Per) and extensor (tibial, Tib) nerves in four preparations did not show consistent patterns of ventral root discharge. Of particular interest was the relationship between L3 and L5 ventral root discharge and flexor or extensor activity, since Cazalets et al. (1992) and Kiehn et al. (1992b) reported that L2 and L3 activity contributed only to extensor activity whereas L5 contributed to flexor discharge. In our experiments L3 activity occurred in phase with flexor (peroneal) ENG discharge in 2 of 4 preparations, showed only tonic discharge in 1 preparation and was rhythmically active but unrelated to either tibial or peroneal discharge in 1 preparation. L5 activity occurred in phase with extensor (tibial) in 2 of 4 preparations, and showed only tonic discharge in 2 preparations. Thus, in these 4 preparations, L3 ventral root activity did not occur in phase with extensor activity, and L5 ventral root activity did not occur in phase with extensor activity as had been suggested (Cazalets et al. 1992; Kiehn et al. 1992b). Both of these authors subsequently reversed their original findings (Cazalets et al. 1992; Kiehn et al. 1992b), indicating that L3 ventral root discharge coincided with flexor discharge and L5 activity coincided with extensor activity (Cazalets et al. 1996; Kiehn and Kjaerulff 1996).

We examined the contribution of axons in specific ventral roots (L2 to L5) by cutting each ventral root and then observing the effect on activity in the following hindlimb nerves: tibial (ankle extensor), peroneal (ankle flexor), iliacus (hip flexor), biceps femoris (hip extensor) and vastus lateralis (knee extensor) (see Gruner et al. 1980 for functional identification of nerves). This attempt to functionally correlate ventral root

and ENG discharge indicated that both ankle flexor (peroneal) and extensor (tibial) nerve axons coursed through common ventral roots in 31/39 hindlimbs. We also observed that biceps femoris nerve activity relied on axons coursing through either L4 and L5 (n=1) or L5 and L6 (n=1). Iliacus ENG discharge was derived from L3 axons alone (n=4) or L2 alone (n=1), and vastus lateralis ENG discharge derived from L3 alone (n=2) or L3 and L4 (n=1). Thus, the flexors examined exited via L2, L3, L4 or L5 ventral roots, and the extensors exited via L3, L4, L5 or L6 ventral roots.

These findings illustrate that both extensors and flexors exit the spinal cord via the same ventral root, and that it is likely that both extensor axons and flexor axons will co-exist in ventral roots (at least L3 to L5), as expected (Nicolopoulos-Stournaras and Iles 1983). However, it is possible that there may be a larger proportion of axons of a given functional type (either flexor or extensor) in each ventral root such that the overall pattern of discharge in each ventral root consists mainly of flexor-related activity or extensor-related activity. In addition, given that the muscle activity of strict functional antagonists within each hindlimb is generally active in anti-phase to the same muscle in the contralateral hindlimb, and that bilateral symmetry generally exists, it is possible that the net discharge of a given segmental ventral root will alternate between left and right hindlimbs. Many authors use these two considerations as the basis for using ventral root activity to monitor locomotor activity in the *in vitro* rat spinal cord preparation (e.g. Smith et al. 1988; Cazalets et al. 1996; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997; Bracci et al. 1996a).

Kiehn and Kjaerulff (1996) examined the relationship between ventral root discharge and electromyographic activity in three preparations and reported that L2 bursts appear in phase with iliopsoas (hip flexor) whereas the L5 bursts coincided with the extensor phase. Iizuka et al. (1997) recently reported that activity recorded from cut L2 ventral roots coincided with ipsilateral iliopsoas and tibialis anterior (TA) muscles (flexors) in 10 of 10 preparations. Activity recorded from cut L3 ventral roots was reported to begin during the flexion phase but to decline gradually during the extension phase in 19 of 22 preparations. In the remaining three preparations, these authors reported some additional extensor phase activity in the L3 ventral root (Iizuka et al.

1997). Other than the reports outlined above (Cowley and Schmidt 1994a; Kiehn and Kjaerulff 1996), a more detailed examination of the relationship between rhythmic discharge of L5 ventral root activity in comparison to extensor and flexor activity has not been reported. Thus, although there may be a clear relationship between the timing of discharge in the L2 ventral root with the timing of hindlimb flexor discharge, the relationship of the discharge in the L3, L4, L5 and L6 ventral roots to either flexor or extensor discharge has not yet been clearly and consistently shown.

Based on these observations, it appears that the goals of the experiment should determine the method used to record rhythmic hindlimb motor discharge when using the *in vitro* neonatal rat spinal cord preparation. In particular, if the presence of rhythmic activity only is of interest, and the specific pattern of hindlimb muscle activity is unimportant, then ventral root recordings would likely be sufficient. If a sufficient number of ventral roots are monitored bilaterally, displaying regular and consistent left-right and L2/L5 alternation, it may be appropriate to consider the activity 'locomotor-like'. If, on the other hand, the details of the locomotor rhythm are of interest, ventral root recordings will be insufficient. This conclusion is consistent with the practice of monitoring directly from hindlimb flexor and extensor nerves when using turtle, chick and cat preparations to study locomotion.

Ventral root recordings may be inadequate for examining the particular phase of activity of given muscles, or the onset and offset of muscle activity because the details of the left-right and intra-limb flexor-extensor coordination may be obscured when using ventral roots. For example, the pattern of activation of bifunctional muscles may interfere with the concept of strict alternation between left and right ventral roots in that bifunctional muscles of both the left and right sides may be active in the same portion of the step cycle. The extent to which bifunctional muscles contribute to the overall activity in left and right ventral roots remains to be examined. In addition, locomotor activity induced by different neurochemicals may produce different patterns of flexor and extensor discharge, which would not be detected using ventral roots, as noted in Part II of this thesis. Kiehn and Kjaerulff (1996) recently reported that although dopamine and 5-HT each induce a generally similar pattern of flexor and extensor discharge in neonatal

rat spinal cord (discussed further below), certain bifunctional muscles are active during different phases of the step cycle depending upon the neurotransmitter used to induce locomotor activity. For example, vastus lateralis (VL) was active only during flexion during 5-HT-induced locomotion in 6 of 7 preparations whereas during dopamine-induced locomotion VL was either active only during extension (4 of 9) or was bifunctional with larger bursts during extension (3 of 9). Thus, the activity of a muscle may change from flexion to extension depending on the neurotransmitter used to induce rhythm; therefore one would expect the phase of the activity in the ventral root would also have to change. In addition, since locomotion does not consist of strict alternation of flexors and extensors, with a common onset and offset of discharge, it is likely that there will be overlap in discharge of flexors and extensors, particularly at the beginning and end of the flexor and extensor phases. In summary, considering the overlapping distribution of both flexor and extensor axons in given ventral roots (Nicolopoulos-Stournaras and Iles 1983; Cowley and Schmidt, 1994a), it is difficult to predict that any one ventral root will display only flexor or only extensor activity.

Part II: A comparison of motor patterns induced by N-methyl-D-aspartate, acetylcholine and serotonin in the in vitro neonatal rat spinal cord.

In Part II (Cowley and Schmidt 1994b), we compared the patterns of ankle flexor and extensor rhythmic discharge induced by bath application of either ACh (in combination with EDRO), 5-HT, or NMA to test the hypothesis that different neurochemicals produce distinct locomotor patterns in the neonatal rat spinal cord. Based on our observations, we concluded that 5-HT was the best single drug for inducing a pattern of ankle flexor and extensor discharge consistent with locomotion. Further, each of the three neurochemicals tested preferentially induced different patterns of flexor and extensor discharge. In particular, ACh in combination with EDRO, induced rhythmic activity characterized by alternation of left and right hindlimb ENGs and simultaneous flexor and extensor discharge within each hindlimb. NMA application induced rhythmic activity that were characterized by either left-right alternation but co-

activation of intralimb flexor-extensor ENG pairs (in 5 of 17 preparations), co-activation of all ENGs (in 4 of 17 preparations), or poorly sustained and variable patterns of ENG discharge (in 6 of 17 preparations). Thus, although earlier work (Bodine-Fowler et al. 1988; Viala and Buser 1969, 1971) suggested that there may be flexor or extensor domination when locomotor activity was induced by different neurotransmitters, our observations of different patterns of ENG discharge induced by each of 5-HT, NMA and ACh were the first to show that different transmitters can control the phasing of individual muscle nerves.

Subsequent to publication of Part II (Cowley and Schmidt 1994b), Kiehn and Kjaerulff (1996) reported the EMG activity of hindlimb muscles during either dopamine- or 5-HT-induced rhythmic activity. EMG records from Kiehn and Kjaerulff (1996) showed that dopamine and 5-HT each induced generally similar patterns of flexor and extensor activity, consistent with overground locomotion. However, in a small number of muscles, some variations in the timing of discharge were observed, dependent upon the neurochemical environment. In particular, bifunctional muscles (biceps femoris, semitendinosus, rectus femoris, vastus lateralis and vastus medialis) switched from being active either mainly during extension (or flexion) to being active mainly during flexion (or extension) or to being active during both phases. For example, biceps femoris was active only during the extensor phase during 5-HT-induced discharge but showed four different patterns during dopamine-induced activity. During rhythmic activity induced by dopamine, biceps femoris showed either a double burst during flexion and extension, initial flexor activity, no activity, or only extensor activity (Kiehn and Kjaerulff 1996). Thus, similar to our findings, differences in the pattern of flexor-extensor discharge were observed when multiple electromyogram recordings were used to monitor locomotor activity induced by either 5-HT or dopamine.

Although all neurotransmitters have not been tested for the ability to induce locomotor activity in all species, some differences between species are apparent. In particular, serotonergic agents are able to induce locomotor activity in spinal rat (Cazalets et al. 1992) and rabbit (Viala and Buser 1969, 1971), but not cat (Grillner and Shik 1973; Barbeau and Rossignol 1991) or lamprey (Harris Warrick and Cohen 1985). Dopamine

can induce locomotor activity in rat (Atsuta et al. 1991) but not cat (Omeniuk and Jordan 1982; Barbeau and Rossignol 1991). These differences may be due to species differences or methodological considerations. For example, in the case of serotonin-induced locomotor activity, the rat and rabbit preparations were all spinalized above T13 but the cat preparation was spinalized at T13. Given that transverse lesions at T13 abolish serotonin-induced locomotion in rat (Cowley and Schmidt 1997), the level of spinalization may account for the failure to induce locomotion in cat in these studies. Further work is required to determine if these differences are methodological or represent fundamental differences between species.

Why is it that so many different neurochemicals induce rhythmic motor activity in the neonatal rat spinal cord in comparison to other species? To date, only excitatory amino acids and L-Dopa have been reported to induce swimming in lamprey (Poon 1980; Cohen and Wallen 1980), whereas serotonin (Harris Warrick and Cohen 1985), noradrenaline or agonist clonidine, and dopamine (Poon 1980) did not. Only excitatory amino acids, L-Dopa and noradrenergic agonists induce locomotion in the cat (e.g. Jankowska et al. 1967a; Forssberg and Grillner 1973; Douglas et al. 1993), whereas additionally acetylcholine, serotonin and dopamine induce motor rhythms in neonatal rat spinal cord (e.g. Cowley and Schmidt 1994b; Kiehn and Kjaerulff 1996). Our findings indicate that although many neurotransmitters may induce rhythmic motor activity, not all patterns of discharge are consistent with overground locomotion (Cowley and Schmidt, 1994b). In some instances, the neurotransmitters may be activating different forms of motor behaviour such as swimming or galloping or hopping. Perhaps neurotransmitters such as NMA or ACh may sufficiently activate neurons within the locomotor CPG to induce rhythms but not activate all components required to generate properly coordinated locomotion. It is also possible that these agents act by increasing excitability of neurons that are not a component of locomotor networks, which in turn is sufficient to activate components of the locomotor rhythm generator. Non-specific excitation, provided by elevating the potassium concentration in the bath surrounding the rat spinal cord, was recently reported to induce locomotor–like activity (Bracci et al. 1998; but see Smith et al. 1988). The greater ability of neurochemicals to induce motor

rhythms (not necessarily locomotor in nature) in the neonatal rat spinal cord may be due to the developmental stage of the preparation. Anatomic observations indicate that the concentration of NMDA receptors declines from birth to adulthood in rat spinal cord (Kalb et al. 1992), suggesting there may be an enhanced inherent excitability of neurons in rat spinal cord at this stage of development. It is also possible that greater access to spinal neurons is provided during neurochemical application *in vitro* in comparison to *in vivo*, leading to an increase in non-specific neuronal excitation, in turn inducing rhythmic motor discharge in neonatal rat. Regardless of the precise reasons why locomotor rhythms can be induced by a variety of neurochemicals exogenously applied to the neonatal rat spinal cord, it is unclear whether these neurotransmitters play an endogenous role in the generation of locomotion in rat and other species. Investigation of the endogenous role(s) of these neurotransmitters awaits further study of the effects of receptor antagonists during locomotion.

Another question that arises from observing that different neurochemicals preferentially induce distinct motor patterns is whether the different forms of rhythmic discharge are mediated by common or distinct neuroanatomical substrates. Evidence from Part IV of this thesis suggests that the motor rhythms induced by different neurochemicals may be mediated, at least in part, by distinct neuroanatomical substrates. It is also of interest to investigate the interactions between different neurotransmitters, both in network and individual cell effects. For example, experiments in rat spinal cord indicate that NMDA receptor activation leads to the generation of membrane oscillations in interneurons and motoneurons (Hochman et al. 1994a, 1994b; Kiehn et al. 1996). However, recent experiments in rat (MacLean et al. 1998) and *Xenopus* (Sillar and Simmers 1994), suggest that serotonin may play an essential role in both the network generation of locomotor activity as well as in NMDA receptor-induced membrane oscillatory behaviour of motoneurons. Combined NMDA and 5-HT receptor activation is reported to induce more stable, sustained and well coordinated locomotor activity than when each are used alone (Squalli-Houssaini et al. 1993), and 5-HT antagonists are reported to abolish NMA-induced locomotor activity (MacLean et al. 1998). These observations highlight the importance of combined actions of more than one neuroactive

substance on individual cell membrane properties as well as their interactions in generating network mediated behaviour, and await further study.

Part III: Effects of inhibitory amino acid antagonists on reciprocal inhibitory interactions during rhythmic motor activity in the in vitro neonatal rat spinal cord

In Part III (Cowley and Schmidt 1995) we examined the effects of glycine and GABA receptor agonists and antagonists on rhythmic ankle flexor and extensor discharge induced by either ACh (in combination with EDRO), 5-HT, or NMA. We tested whether inhibitory amino acid receptor mechanisms mediate reciprocal inhibition during locomotion in the *in vitro* neonatal rat spinal cord. The main findings of this study implicated a role for $GABA_A$ and glycine receptors in mediating reciprocal left-right and flexor-extensor phase relationships during locomotion, and suggested that inhibitory amino acid transmission is not essential for rhythm generation.

Both the $GABA_A$ receptor antagonist bicuculline and the glycine receptor antagonist strychnine converted out-of-phase rhythmic hindlimb ENG activity into in-phase activation of ankle flexors and extensors bilaterally, regardless of the neurochemical used to induce the motor rhythm. As noted in Part III, it is likely that the reciprocal inhibitory interactions between antagonist centres that normally dominate during out-of-phase locomotor rhythms are blocked by either $GABA_A$ or glycine receptor antagonists, revealing reciprocal excitatory connections that produce in-phase coupling of rhythmogenic centres. In contrast to the $GABA_A$ and glycine antagonists, $GABA_B$ antagonists did not alter the pattern of hindlimb flexor-extensor discharge, even at concentrations 2 – 5 times higher than that required to restore rhythmic activity after $GABA_B$ agonist-induced suppression of rhythmic hindlimb ENG discharge. Consistent with our findings (Cowley and Schmidt 1995; Kremer and Lev-Tov 1997) also reported that either strychnine or bicuculline converted left-right alternating ventral root discharge into synchronous motor patterns in the neonatal *in vitro* rat spinal cord preparation. These findings (Cowley and Schmidt 1995; Kremer and Lev-Tov 1997) contradict those

of Cazalets et al. (1994) who reported that bicuculline did not induce synchronous left-right ventral root discharge.

The observation of synchronous rhythmic hindlimb ENG discharge when each of $GABA_A$, $GABA_B$ and glycine receptors were blocked suggested inhibition is not required to generate rhythmic motor discharge in neonatal rat (Cowley and Schmidt 1995). However, as noted in Part III, a role for other inhibitory neurochemicals was not ruled out. Wu et al. (1995) subsequently reported a strychnine resistant component to glycine mediated currents observed in neonatal rat spinal cord neurons, suggesting that some glycine receptors may not be completely blocked with strychnine alone. In addition, Kremer and Lev-Tov (1997) observed that alternating ventral root discharge induced by combined NMDA and 5-HT persisted after application of the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline (CNQX), and was unaffected by subsequent application of strychnine, suggesting there may be a strychnine-resistant component of alternating rhythm generation which is unmasked during NMDA receptor blockade. After washout of CNQX, synchronous left-right ventral root discharge was observed in the presence of NMDA and 5-HT.

The relative contribution of glycine or $GABA_A$ receptor mechanisms in mediating alternation is unknown. However, Kremer and Lev-Tov (1997) assessed the effects of locally applied strychnine and bicuculline in an attempt to clarify this issue. They concluded that locally applied strychnine perturbed the left-right alternation but bicuculline did not. Specifically, strychnine applied to a single segment by pressure ejection caused intermittent bursts of synchronous activity during otherwise alternating ventral root discharge induced by combined NMDA and 5-HT. In preparations in which intrasegmental cross projections were further reduced by midsagittal lesion of the spinal cord (such that only one segment remained bilaterally intact) an alternating pattern of left-right ventral root discharge could still be induced by combined NMDA and 5-HT. If strychnine was then applied over the bilaterally intact segment, the rhythmic and left-right alternating ventral root discharge became irregular in 8 of 9 preparations and left-right independent in the remaining preparation. Kremer and Lev-Tov (1997) concluded that locally applied bicuculline did not interfere with out-of-phase rhythmicity whereas

strychnine did, and thus the side-to-side reciprocal inhibition was mediated primarily by glycine receptors. However, Kremer and Lev-Tov (1997) reported that locally applied bicuculline (over the single bilaterally intact segment) transiently disrupted the left-right alternating ventral root discharge, which then returned a few minutes later, sometimes accompanied by a few synchronous bursts. Thus, it is possible that these differences in the locally applied effect of bicuculline and strychnine are based solely on pharmacological considerations such as diffusion or binding characteristics. For example, strychnine may diffuse through neuronal tissue more quickly and may remain attached to the glycine receptor longer than bicuculline remains attached to the $GABA_A$ receptor. More recently, Kjaerulff and Kiehn (1997) reported that strychnine was more effective than bicuculline in reducing the rhythmic inhibition received by motoneurons during pharmacological activation of contralateral rhythmogenic networks in the neonatal rat spinal cord. Although these experiments (Kjaerulff and Kiehn 1997) may indicate the relatively greater effect of glycine in comparison to $GABA_A$ receptor activation in directly inhibiting motoneurons, they do not clarify the role(s) of glycine versus $GABA_A$ receptors in pre-motoneuronal network coordination. It is possible that glycine receptor effects contribute to intrasegmental inhibition whereas $GABA_A$ receptors play a role in reciprocal inhibition that is not mediated by short intrasegmental pathways but rather by longer pathways, possible involving axons projecting over several segments as described in the lamprey spinal cord (Cohen and Harris-Warrick 1984; Alford and Williams 1989; Grillner and Wallen 1980; Tegner et al. 1993).

In addition to a role in coordinating motor groups during locomotion, inhibitory mechanisms have been proposed to contribute to rhythm generation through post-inhibitory rebound. For example, in *Xenopus*, rhythm generation is thought to partly depend on delayed postinhibitory rebound after midcycle reciprocal inhibition (Roberts et al. 1995). The process of post-inhibitory rebound, whereby a brief hyperpolarization (e.g. by mid cycle inhibition) leads to a brief rebound depolarizing membrane response (Perkel and Mulloney 1974), is thought to contribute to rhythm generation both between left and right half centres as well as within each half centre in network models of *Xenopus* swimming (Arshavsky et al. 1993). Thus, in *Xenopus*, a brief depolarizing input

can cause prolonged rhythmic activity between left and right half centres. Alternation between left and right is thought to be maintained by reciprocal inhibition and the subsequent rebound excitation is sufficient to maintain rhythmic excitation of each half centre (reviewed in Arschavsky et al. 1993). However, reciprocal inhibition and post-inhibitory rebound is not necessary to maintain the rhythmic activity in *Xenopus* since each isolated half spinal cord can generate swimming (Kahn and Roberts 1982). In addition, in the network model for *Xenopus* swimming proposed by Roberts et al. (1995), recurrent inhibition is necessary for sustaining rhythm generation within each isolated half-centre. Recent investigations of the role of post-inhibitory rebound in generating rhythmic activity in the mammalian spinal cord (Bertrand and Cazalets 1998) suggest post-inhibitory rebound may contribute to rhythmic discharge in motoneurons, but this question requires further investigation.

Investigations into the development of motor activity and the time course of left-right alternation have been conducted in the rat embryo. In the rat fetus, spontaneous ventral root activity, that is synchronized between left and right sides, appears around day 13 *in utero*, progressively declines, and is not present after embryonic day 18.5 (Nishimaru et al. 1996; Kudo et al. 1991; Greer et al. 1992). This spontaneous synchronous activity is not blocked by glutamate receptor antagonists but is abolished by glycine and to some extent $GABA_A$ receptor antagonists, suggesting that glycine and GABA function transiently as excitatory neurotransmitters, generating the earliest spontaneous motor activity in rat fetus (Nishimaru et al. 1996). Ventral root discharge induced by activation of NMDA receptors *in utero* is initially synchronous but becomes alternating around embryonic day 18 (Kudo et al. 1991; Greer et al. 1992; Ozaki et al. 1996). Once an alternating pattern of ventral root discharge appears *in utero* application of strychnine converts the left-right ventral root alternation to synchronous rhythmic activity (Kudo et al. 1991; Ozaki et al. 1996). In these investigations, $GABA_A$ receptor mechanisms are thought to play only a minor role in mediating left-right alternation (Nishimaru et al. 1996) or were not tested (Ozaki et al. 1996). Further study of the role of $GABA_A$ receptors in the rat and other species (e.g. cat, Noga et al. 1993b) in mediating reciprocal inhibition is required.

Part IV: Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord.

In Part IV (Cowley and Schmidt 1997) we examined the effects of various spinal cord lesions on the presence and coordination of rhythmic ankle flexor and extensor discharge. The contributions of particular areas of spinal cord in generating and coordinating both locomotor and non-locomotor out-of-phase rhythmic patterns of discharge induced by either ACh (in combination with EDRO), 5-HT, or NMA alone, and combined NMA and 5-HT were tested. Neuroanatomical substrates mediating and coordinating synchronous rhythms were also examined. Our findings demonstrated the distributed nature of networks generating motor rhythms, including locomotion. In addition, the data suggested that the different patterns of rhythmic discharge activated by specific neurotransmitters are mediated at least in part by neural circuits with regionally and anatomically distinct elements.

Rostral-caudal distribution of networks generating rhythmic motor activity

The findings of Part IV (Cowley and Schmidt 1997) indicated that 5-HT-sensitive rhythmogenic circuitry is distributed throughout the supralumbar region of the spinal cord and that the lumbar portion of the spinal cord displayed no rhythmogenic response to 5-HT. In contrast, either NMA alone or ACh (in combination with EDRO) induced rhythmic motor activity when applied to the lumbar cord. In fact, small portions of caudal lumbar tissue (caudal to L4) generated rhythmic activity in response to NMA application. Supra-lumbar segments of spinal tissue generated rhythmic discharge in response to either 5-HT or NMA alone or ACh (with EDRO) when these segments were separated from the lumbar cord either by transverse section or bath compartmentalization (Cowley and Schmidt 1997). In addition, we observed that short segments of isolated cervical or thoracic spinal tissue generated rhythmic ventral root discharge in the presence of 5-HT and NMA. In contrast, Kremer and Lev-Tov (1997) indicated that the

combined 5-HT/NMDA-induced rhythmic discharge recorded from non-lumbar ventral roots did not persist after transection separated these segments from the lumbar cord. In addition, we observed that supra-lumbar application of certain neurochemicals could drive lumbar rhythmic activity (Cowley and Schmidt 1997). ACh (with EDRO) or NMA application to the bath compartment containing supralumbar spinal cord segments would elicit rhythmic hindlimb ENG discharge. Finally, combined 5-HT/NMA application to the compartment containing only the cervical spinal cord induced rhythmic ventral root discharge throughout the spinal cord, including lumbar ventral roots.

Each of the papers discussed here (Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997) relating to the localization of rhythmogenic neuronal circuitry contradict the conclusion of Cazalets et al. (1995) that the CPG for locomotion is restricted to the L1/L2 segments. In addition to the lesion experiments discussed above, a three compartment split bath experimental configuration was used to determine if the 5-HT-sensitive rhythmogenic circuitry was localized to the caudal thoracic cord (e.g. T13) or distributed in the supralumbar region (Cowley and Schmidt 1997). Application of 5-HT to the supralumbar and lumbar cord without exposing the T12 to L2 segments to 5-HT induced rhythmic lumbar activity. Therefore, the rostral lumbar spinal segments were not essential for producing hindlimb locomotor rhythms as suggested by Cazalets et al. (1995). The observation of a distributed locomotor network is consistent with findings for hindlimb scratching in the cat (Deliagina et al. 1983) and turtle (Mortin and Stein 1989) lumbar spinal cord.

Some discrepancies were noted between the findings of Cowley and Schmidt (1997), Kjaerulff and Kiehn (1996) and Kremer and Lev-Tov (1997) regarding the activation of rhythmogenic networks with combined NMDA/5-HT. For example, after preparations were lesioned between L3 and L4, NMA induced rhythmic ventral root discharge (Cowley and Schmidt 1997). However, we observed that subsequent addition of 5-HT to the bath converted the rhythmic discharge to tonic activity only in the lumbar ventral roots (Cowley and Schmidt 1997). Both Kjaerulff and Kiehn (1996) and Kremer and Lev-Tov (1997) reported rhythmic lumbar ventral root discharge in preparations transected at L3 or more caudal in the presence of combined 5-HT/NMDA. It is

interesting to note that Kremer and Lev-Tov (1997) reported that alternating ventral root discharge induced by 5-HT/NMDA was initially abolished after transection of the spinal cord at mid-L3 and rhythmic activity was only reinstated after increasing the NMDA concentration. These findings suggest that the balance between different neurotransmitter receptor activation levels may be important in the generation of locomotor activity in neonatal rat spinal cord, but further research would be required to clarify this question.

Side-to-side phase relationships mediated by distributed systems of cross connections

In Part IV, we suggested that a widely distributed and redundantly organized system of reciprocal cross projections exist within the spinal cord (Cowley and Schmidt 1997). This conclusion was based on our observations of the effects of midsagittal lesions on both out-of-phase locomotor-like hindlimb ENG discharge induced with 5-HT and synchronous hindlimb discharge observed after blockade of IAA receptors. In particular, 5-HT-induced left-right and intralimb flexor-extensor hindlimb ENG phase relationships were maintained after midsagittal section from the conus to the thoracolumbar junction region, or from C1 to rostral T13, but once midsagittal sections were extended one segment more rostrally or caudally, 5-HT-induced rhythms were lost. However, midsagittal lesion of only the thoracolumbar region disrupted neither hindlimb rhythm production nor coordination. Thus, no single region of the spinal cord was critical for maintaining 5-HT-induced reciprocal inhibitory interactions between the hindlimbs, provided other regions of the cord remain bilaterally intact. Our results were consistent with those reported by Kjaerulff and Kiehn (1996), in which combined NMA/5-HT-induced left-right ventral root phase relationships were preserved following midsagittal lesions extending through either T12 to L2 or L3 to L6 (using a T12 - L6 spinal cord preparation). Kremer and Lev-Tov (1997) also performed midsagittal lesions during combined NMA/5-HT-induced left-right alternating ventral root activity and observed perturbation of the alternation when rostrally directed sections extended from

the conus to caudal T11, T12 or T13 segments. Loss of rhythmic ventral root alternation occurred after caudally directed sections extended from the rostral cord to rostral L5. Also in support of the conclusion of redundantly organized system of reciprocal cross projections, Kremer and Lev-Tov (1997) reported that single, bilaterally intact segments (L1, L2, L3 and occasionally L4) were able to coordinate left-right ventral root alternation. Kjaerulff and Kiehn (1996) observed that a lesion applied to the midline from the ventral most portion of the spinal cord to the ventral edge of the central canal along the length of the spinal tissue abolished the left-right alternation, further suggesting that the pathways mediating left-right alternation exist within the ventral commissure. Each authors' findings reported here (Cowley and Schmidt, 1997; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov, 1997), contradict the conclusion drawn by Cazalets et al. (1995), that the neurons mediating left-right alternation are restricted to the L1/L2 segments of the spinal cord.

Interestingly, the loss of ventral root alternation after midsagittal lesions observed by Kremer and Lev-Tov (1997) manifested itself as either left-right independent or left-right synchronous rhythms when activity was induced with 5-HT/NMA. We observed (Cowley and Schmidt 1997) that 5-HT-induced rhythms abruptly ceased after extensive midsagittal lesion and we were therefore unable to detect a change in the phase relationship between left and right hindlimb ENGs. However, left-right alternation of ankle flexor and extensor discharge during ACh-induced rhythms became uncoupled after midsagittal lesion at a variety of rostrocaudal levels of the spinal cord (Cowley and Schmidt 1997). These findings further support the suggestion that different neuroanatomical substrates mediate the motor rhythms induced by different neurochemicals. Further investigation of the coordination between left and right hindlimbs after midsagittal lesion during rhythmic motor activity induced by different neurochemicals would likely clarify whether specific regions of the spinal cord are essential for coordinating rhythms induced by these distinct neurochemicals.

As noted in Part III (Cowley and Schmidt 1995), rhythmic patterns elicited by application of NMA, ACh or 5-HT became synchronous after $GABA_A$ or glycine receptor blockade. In addition to the presumed inhibitory cross projections discussed

above, our observations of the effects of midsagittal and transverse lesions during synchronous rhythms suggested the presence of an extensively distributed and redundantly organized system of reciprocal excitatory cross projections. In particular, we observed that synchronous rhythms persisted in small segments of bilaterally intact spinal tissue comprising as little as two segments (e.g. L4-L5 inclusive), in either cervical, thoracic or lumbar cord (Cowley and Schmidt 1997). Kremer and Lev-Tov (1997), also observed that small segments of bilaterally intact spinal cord generated synchronous rhythm. Further, Cowley and Schmidt (1997) and Kremer and Lev-Tov (1997) observed that single segment hemisegments generated rhythmic discharge during $GABA_A$ or glycine receptor blockade. Similarly Bracci et al. (1996b) reported that single lumbar segments generated rhythmic activity in the presence of bicuculline and strychnine.

We also observed that synchronous rhythms persisted despite extensive midsagittal lesions, suggesting the cross connections that mediate side-to-side coupling of synchronous rhythms were not restricted to any specific portion of the spinal cord. Thus, excitatory cross projections within just one or two segments at virtually any rostrocaudal level of the cord can mediate the synchronous coupling of both left and right hindlimb flexors and extensors (Cowley and Schmidt 1997). Similarly, Kremer and Lev-Tov (1997) observed that as little as 3 bilaterally intact segments maintained bilaterally synchronous ventral root rhythmic discharge. Bracci et al. (1996b) also observed synchronous ventral root discharge (induced by strychnine and bicuculline) after midsagittal section from the cauda equina to L2 and L1 (in 2 of 4) but further splitting, up to the thoracic level, resulted in burst desynchronization. When the midsagittal lesions were performed in a rostrocaudal direction, synchronous rhythm persisted when the midsagittal lesion extended from the mid-thoracic segments caudally to S2, such that only S3 and more caudal segments were bilaterally intact. Bracci et al. (1996b) conducted further lesions and observed that synchronous rhythms persisted when only the ventral half of the spinal cord was left intact (from the ventral portion of the central canal to the ventral edge of the cord), and thus rhythm could be generated by ventral horn quadrants.

In summary, our findings (Cowley and Schmidt 1997) suggested that the 5-HT, NMDA, and ACh-sensitive rhythmogenic networks were of a distributed nature and that 5-HT-sensitive networks may be distributed predominantly in supralumbar regions whereas NMA- and ACh-sensitive elements also occur within the lumbar region. Our lesion experiments also suggested the presence of an extensive propriospinal network of reciprocal inhibitory and excitatory connections. On the basis of these findings, as well as those presented in Part II (Cowley and Schmidt 1994b) and Part III (Cowley and Schmidt 1995) of this thesis, we can begin to suggest some model network interactions that could account for the observed experimental results.

Model of network organization in rat spinal cord

One of the first models to account for locomotor alternation in mammals was the 'half-centre' model as proposed by T. Graham Brown (1911, 1914). Graham Brown proposed that alternation between flexors and extensors was accomplished by mutually inhibitory 'half-centres' in the spinal cord. In this model, one half-centre provides rhythmic excitation to a pool of flexor motoneurons and the other provides rhythmic excitation to a pool of extensor motoneurons acting on a single joint. A fundamental feature of the model proposed by Graham Brown was that the rhythm was dependent upon inhibitory neurons, which acted reciprocally, so that in the presence of a common excitatory drive, when one half-centre was active, the opposite 'half-centre' was inhibited, thus producing the flexor-extensor alternation (Fig. 1A). Eventually, a property that Graham Brown called 'fatigue' would set in and the excitability of the active half-centre would decrease enough to disinhibit the inactive half-centre that would then become active and inhibit the opposite half-centre.

In 1967, Jankowska et al. a,b, reported evidence for mutually inhibitory interneurons within the spinal cord, revealed following application of L-Dopa to the spinalized cat. As a result of these experiments, Lundberg proposed an enhanced 'half-centre' model (see Lundberg 1981; Fig. 1B) in which inhibitory interneurons of the extensor half-centre could be activated by contralateral flexor reflex afferents and

inhibitory interneurons of the flexor half-centre could be activated by the ipsilateral flexor reflex afferents. The alternating activation of functional antagonists occurred as a result of reciprocal activation of the inhibitory interneurons of each half-centre. Lundberg further proposed that the simple pattern of alternating activity of flexors and extensors could then be modulated by proprioceptive reflex activity. Similar to the model proposed by Lundberg, modified half-centre models have been developed to account for swimming in the lamprey (reviewed in Grillner et al. 1991) and *Xenopus* (reviewed in Roberts 1990). One advantage of modeling in simpler vertebrate systems is that many of the constituent neurons and pathways have been identified (e.g. reciprocal inhibitory neurons reviewed in Grillner et al. 1991; Roberts 1990).

The unit burst generator model proposed by Grillner (1981; Fig. 2) attempts to account for both activity around a single joint and activity along the limb in the mammalian spinal cord. In this model, the interneurons underlying rhythmic input to motoneurons are thought to be located close to the motoneuron pools they drive. In essence, a distributed locomotor system of unit burst generators, which, depending upon the relative strength of the connections between unit burst generators, produces a variety of rhythmic locomotor behaviours. Based on observations that relatively small pieces of spinal tissue can generate rhythmic discharge (e.g. Grillner and Zangger 1979), each unit burst generator is assumed to produce the rhythmic activity by itself and to contain all elements required for bursting (Grillner 1981). The nature of the net synaptic drive (either inhibitory or excitatory) between 'units' determines the pattern of overall limb activity. Although recent findings about modulation of disynaptic interneuronal pathways from primary afferents during rhythmic hindlimb activity have been modeled (Degtyarenko et al. 1998), no models to explain the coordination of motor activities during locomotion between limbs acting on the same or different girdles have thus far been proposed.

The model presented in Figure 3A is an attempt to summarize possible network interactions to account for the different patterns of rhythmic activity observed in Part I (Cowley and Schmidt 1994b) and Part III (Cowley and Schmidt 1995) of this thesis. The model is essentially a combination of the modified half-centre hypothesis proposed by

Lundberg (Jankowska et al. 1967a; Lundberg 1981) and the unit burst generator model (Grillner 1981) as can be seen by comparing Figure 3A with Figure 1B and Figure 2. Each circle in Figure 3 represents a rhythm generator that is capable of generating rhythmic activity in isolation. In Figure 3A, the connections between unit burst generators are inhibitory such that the activity of flexors would alternate with extensors on the same side of the spinal cord. In addition, the extensors of the left and right sides would alternate as would flexors of the left and right sides. You will notice that inhibitory connections between both left and right flexors and left and right extensors are shown. If parsimony applied, inhibitory connections between either left and right extensors or left and right flexors would be required, but not both. However, if present, greater flexibility in generating different forms of movement would exist and therefore are included. The model in Figure 3A would account for the locomotor-like activity seen mainly during 5-HT-induced activity (e.g. Fig. 1B of Part II/Cowley and Schmidt 1994b). For simplicity, this model shows only excitatory connections to the motoneurons, intended to represent rhythmic drive to the motoneurons (labeled as either F for flexor or E for extensor), and omits inhibitory projections. Thus, although the rhythmic oscillations of membrane potential observed in motoneurons during locomotion (locomotor drive potentials: LDPs) likely reflects rhythmic excitation alternating with rhythmic inhibition (e.g. Edgerton et al. 1976; Jordan 1983; Pratt and Jordan 1987; Shefchyk and Jordan 1985; Hochman and Schmidt 1998) both types of projections are not included in this model.

Figure 3B is an extension of 3A, incorporating excitatory coupling between the flexors and extensors of each side of the spinal cord. This model could explain ACh-induced rhythmic activity, which exhibits left-right alternation but in-phase intralimb flexor-extensor activity (e.g. Fig. 1A of Part II/Cowley and Schmidt 1994b). If excitatory coupling is then added between left and right flexors and left and right extensors (Fig. 4), the in-phase discharge observed during NMA-induced rhythms (e.g. Fig. 2B of Part II/Cowley and Schmidt 1994b) as well as synchronous discharge observed during glycine or $GABA_A$ receptor blockade could be generated (e.g. Fig. 2 of Part III/Cowley and Schmidt 1995). These synchronous patterns of rhythmic hindlimb discharge would

occur if excitatory coupling dominated between intralimb and left and right flexors and extensors. It is interesting to note that only synchronous coordination exists between left and right sides *in utero* in rat before embryonic day 18 (Kudo et al. 1991; Nishimaru et al. 1996; Greer et al. 1992), suggesting that only excitatory coupling exists before the left-right alternating rhythms and underlying inhibitory coupling appear after embryonic day 18.

The results of the effects of various types of lesions during synchronous and out-of-phase motor activity from Part IV allow extension of the model presented in Figure 4. We will deal with the findings from lesion experiments during synchronous rhythms first. It is likely that redundant left-right excitation exists throughout the rostral-caudal extent of the spinal cord, based on findings from midsagittal and transverse lesion experiments during IAA-induced synchronous rhythms. In particular, since single isolated segments can coordinate synchronous activity, and since bilaterally intact segments at any rostral-caudal level of the spinal cord can maintain left-right and flexor-extensor synchrony, a redundant and extensive reciprocally excitatory coupling is predicted throughout the spinal cord (Fig. 5). Only two segmental levels are shown in Figure 5, and both the excitatory connections predicted here, as well as the inhibitory connections discussed below, are included in the diagram. Since left-right synchronous rhythms persist after extensive midsagittal lesions when IAA antagonists are present in the bath, extensive and redundant excitation likely exists in the rostral-caudal plane to coordinate the synchronous discharge along each side of the spinal cord (Fig. 6, inhibitory coupling also shown). In addition, the rhythm does not depend on left-right interactions since it persists following complete midsagittal section of left and right halves of the spinal cord, as noted in Part IV.

The redundant series of inhibitory coupling shown in Fig. 5 is supported by findings from midsagittal and transverse lesion experiments during alternating rhythms (e.g. 5-HT-induced). In particular, left-right alternation persists after midsagittal lesions through several different spinal cord levels (e.g. Fig. 3, conus to T13/L1, or Fig. 4, T12 to L1, of Part IV, Cowley and Schmidt 1997), suggesting redundant left-right inhibitory connections exist to coordinate alternation (Fig. 5, only two levels shown). Kremer and

Lev-Tov (1997) reported that single bilaterally intact segments can maintain left-right ventral root alternation, supporting the idea of redundant reciprocal inhibition between left and right functional agonist motor groups within each lumbar segment (for both flexors and extensors). In addition to the excitatory coupling between motor groups along each side of the spinal cord shown in Figure 6, since locomotor activity is maintained after midsagittal lesions, it is likely that redundant reciprocal inhibition exists along each side of the cord to coordinate flexor-extensor activity that alternates (e.g. within each limb). The model of a redundant series of coupled oscillators shown in Figure 6 is similar to that predicted for swimming rhythms coordinated along the rostrocaudal extent of the lamprey spinal cord (e.g. Rand et al. 1988).

Since there appears to be some regional specialization in the rhythmogenic circuitry that is sensitive to 5-HT or NMA or ACh application (see above), these differences would need to be accounted for in theoretical models to explain underlying locomotor activity. For example, to date, evidence does not suggest any regional specialization in the circuitry effected by NMA. However, supralumbar portions of the spinal cord appear to be more sensitive to 5-HT than lumbar regions. In addition, although cross projections in the lumbar segments are not essential to left-right coordination of 5-HT-induced rhythms, ACh sensitive rhythmogenic circuitry appears to depend on lumbar cross projections.

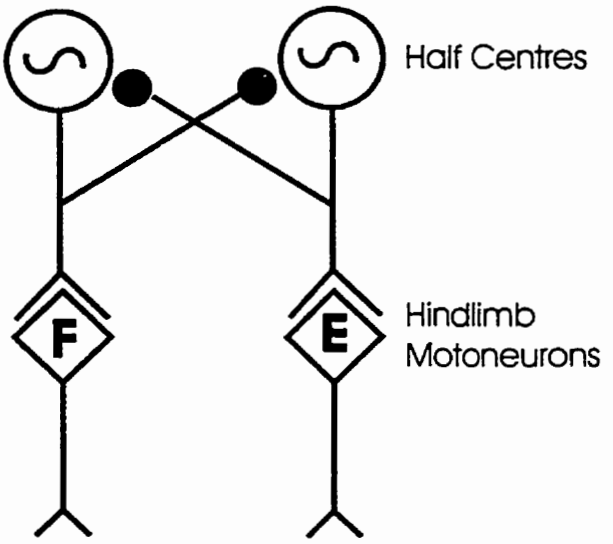
In addition to the network interactions outlined in the proposed model, recent findings in lesion studies in the *in vitro* rat preparation suggest further refinement of this model (Kjaerulff and Kiehn 1996). Based on comparisons of ventral root rhythmic activity before and after various horizontal, sagittal and partial midsagittal lesions, these authors concluded that the axons mediating reciprocal inhibition likely reside in the ventral commissure, since alternation was abolished after lesions of this area. Horizontal lesions ventral to the central canal resulted in a loss of rhythmic ventral root discharge, whereas horizontal sections dorsal to the ventral edge of the central canal, extending the length of the spinal cord did not, suggesting the rhythmogenic neuronal circuitry exists in the portion of the spinal cord ventral to the dorsal edge of the central canal. Finally, sagittal sections along the rostrocaudal extent of the spinal cord indicated that the

networks generating rhythmic ventral root discharge do not likely reside in the lateral spinal cord. Only spinal cord pieces retaining at least 63% of the lateral gray matter could generate rhythmic ventral root discharge (Kjaerulff and Kiehn 1996).

The model presented in Figure 6 attempts to account for the observations within this thesis. For example, the observations of Part II (Cowley and Schmidt 1994b) and this model imply that 5-HT activates all components of Figure 6, producing coordinated locomotion. On the other hand, ACh (with EDRO) would likely preferentially activate neurons coordinating left and right hindlimbs but would selectively promote excitatory coupling, rather than inhibition, between intralimb flexors and extensors. This model incorporates components of half-centre and unit burst generator models (Graham Brown 1911, 1914; Jankowska et al. 1967a; Lundberg 1981; Grillner 1981) to account for rhythmic patterns seen within and between hindlimbs in the neonatal rat spinal cord. The simple model presented in this thesis would need to incorporate other experimental findings such as the contributions of identified interneurons and/or peripheral afferents in order to reflect the complex pattern of hindlimb flexor and extensor activation observed in overground locomotion. In addition, consideration must be given for intrinsic membrane properties, and interactions between various neurotransmitters in inducing membrane oscillations (e.g. interaction between 5-HT and NMDA receptors MacLean et al. 1998).

In summary, the observations of this thesis contribute to our understanding of the neurochemical mechanisms within the neonatal rat spinal cord generating and coordinating locomotion. The theoretical model put forth may be useful in the design of future experiments to further clarify the neurochemical and anatomical substrates of locomotion.

A Graham-Brown Model
(modified from Gossard and Hultborn 1991)



B Lundberg Model
(modified from Lundberg, 1981)

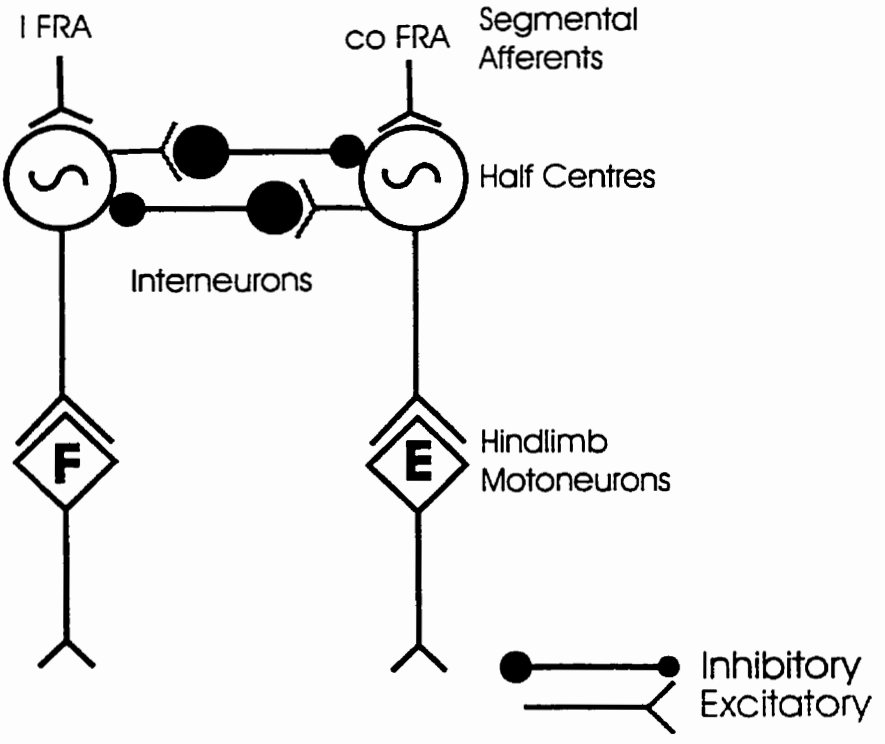


Fig. 1

Unit Burst Generator Model

Modified from Grillner, 1981

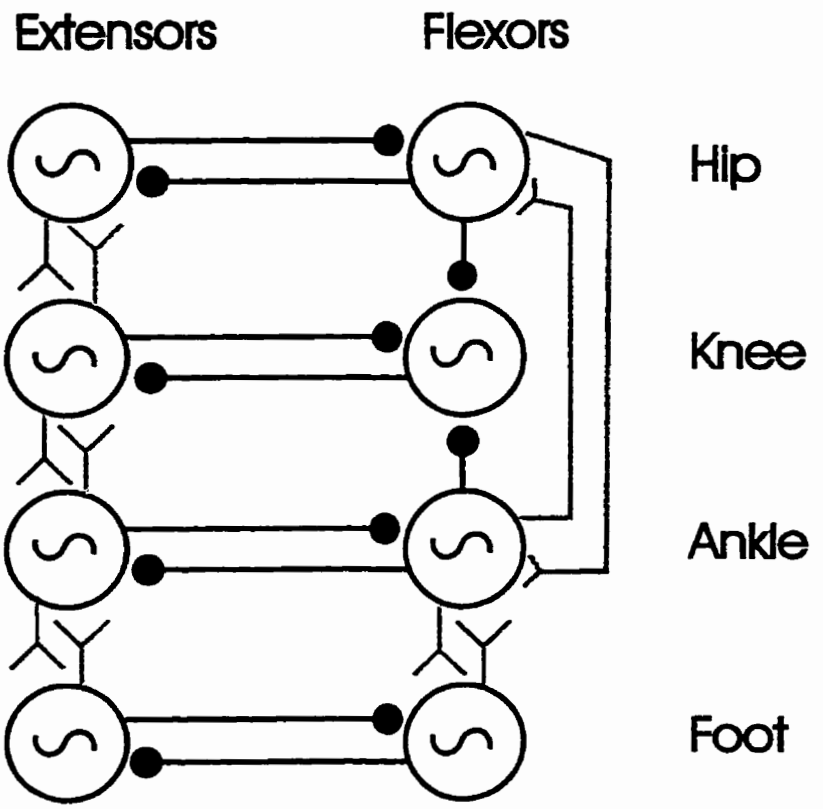
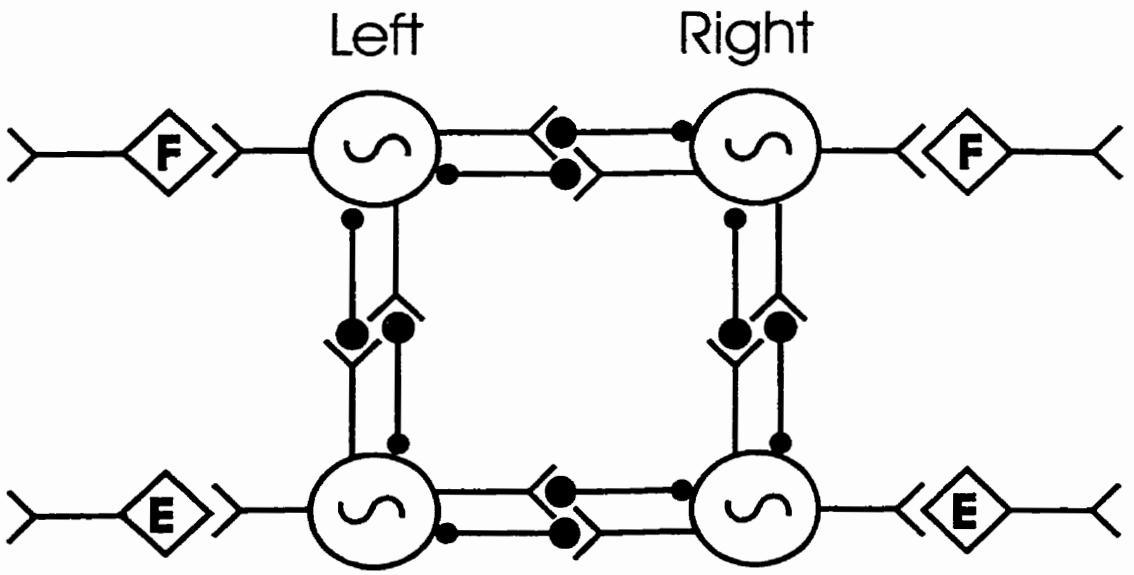


Fig. 2

A 5-HT-induced Rhythm



B ACh-induced Rhythm

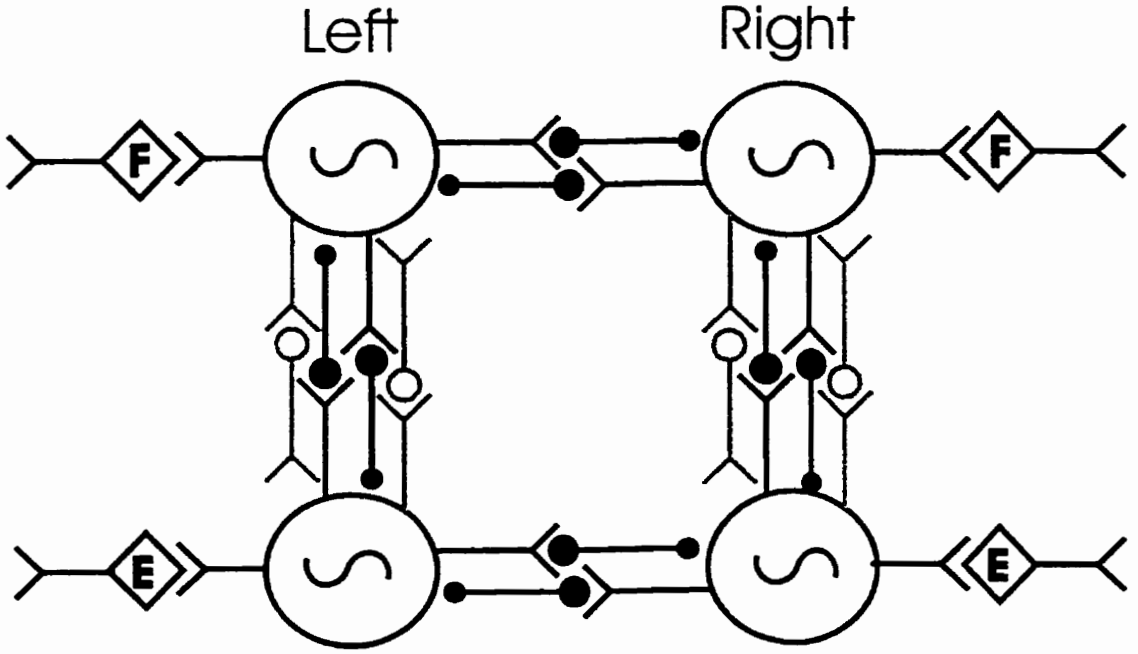
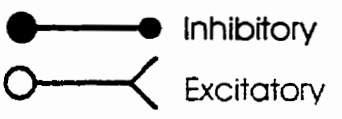


Fig. 3



Synchronous Rhythms

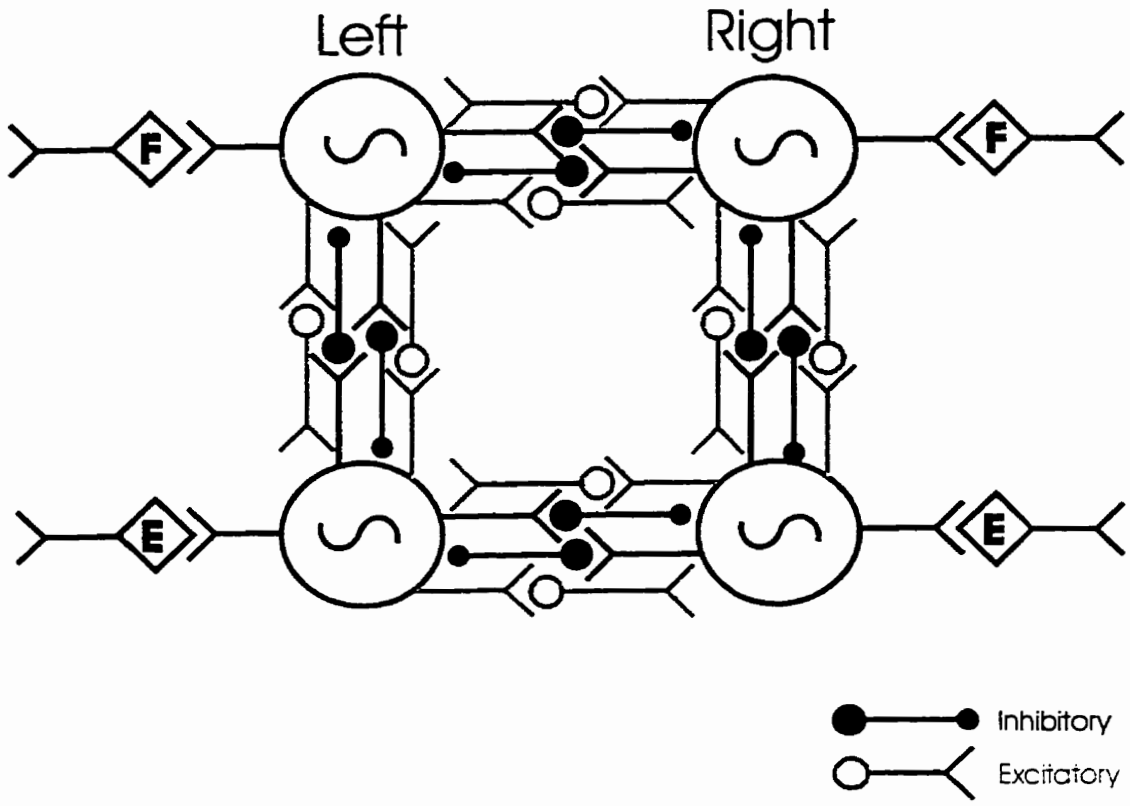


Fig. 4

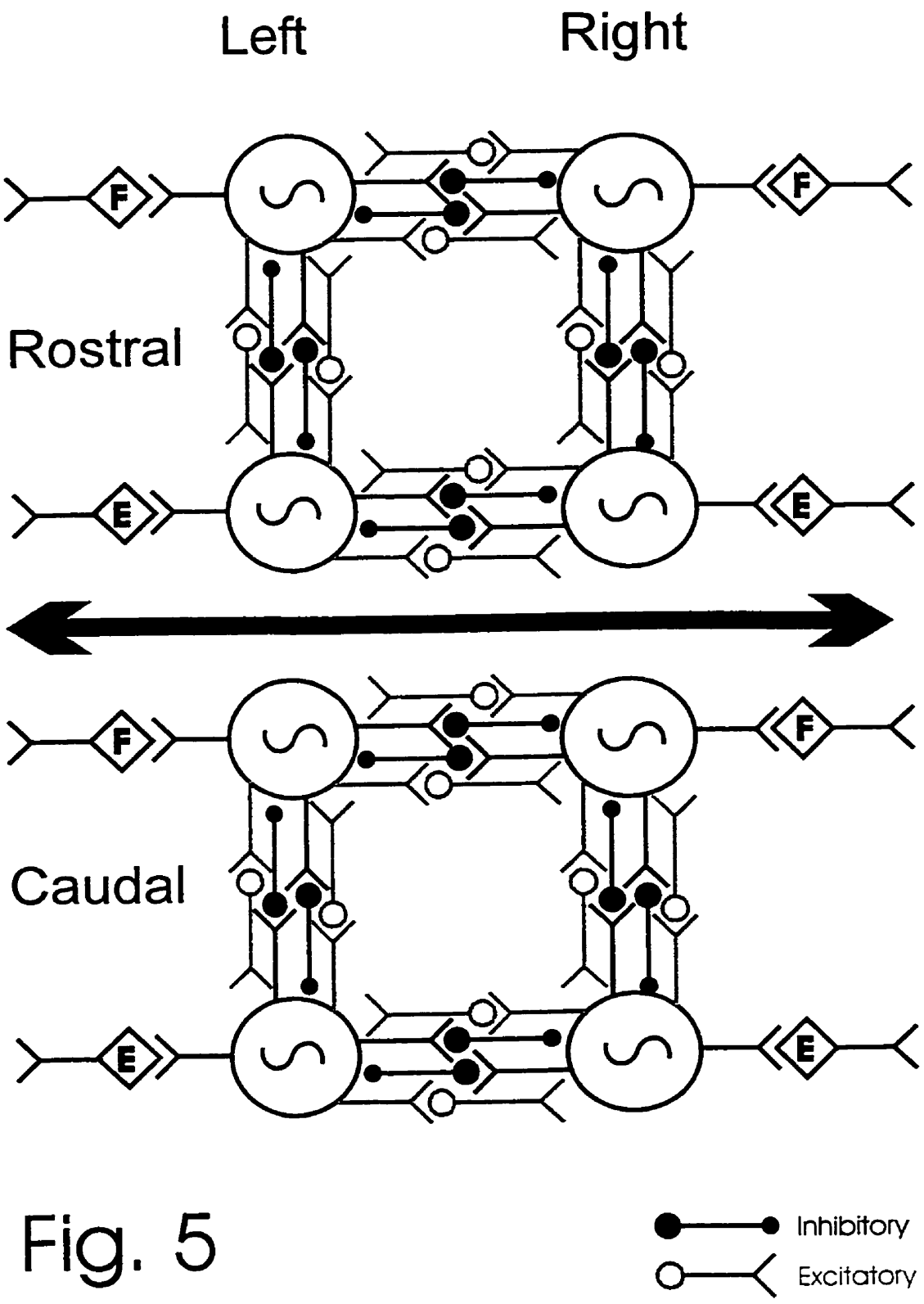


Fig. 5

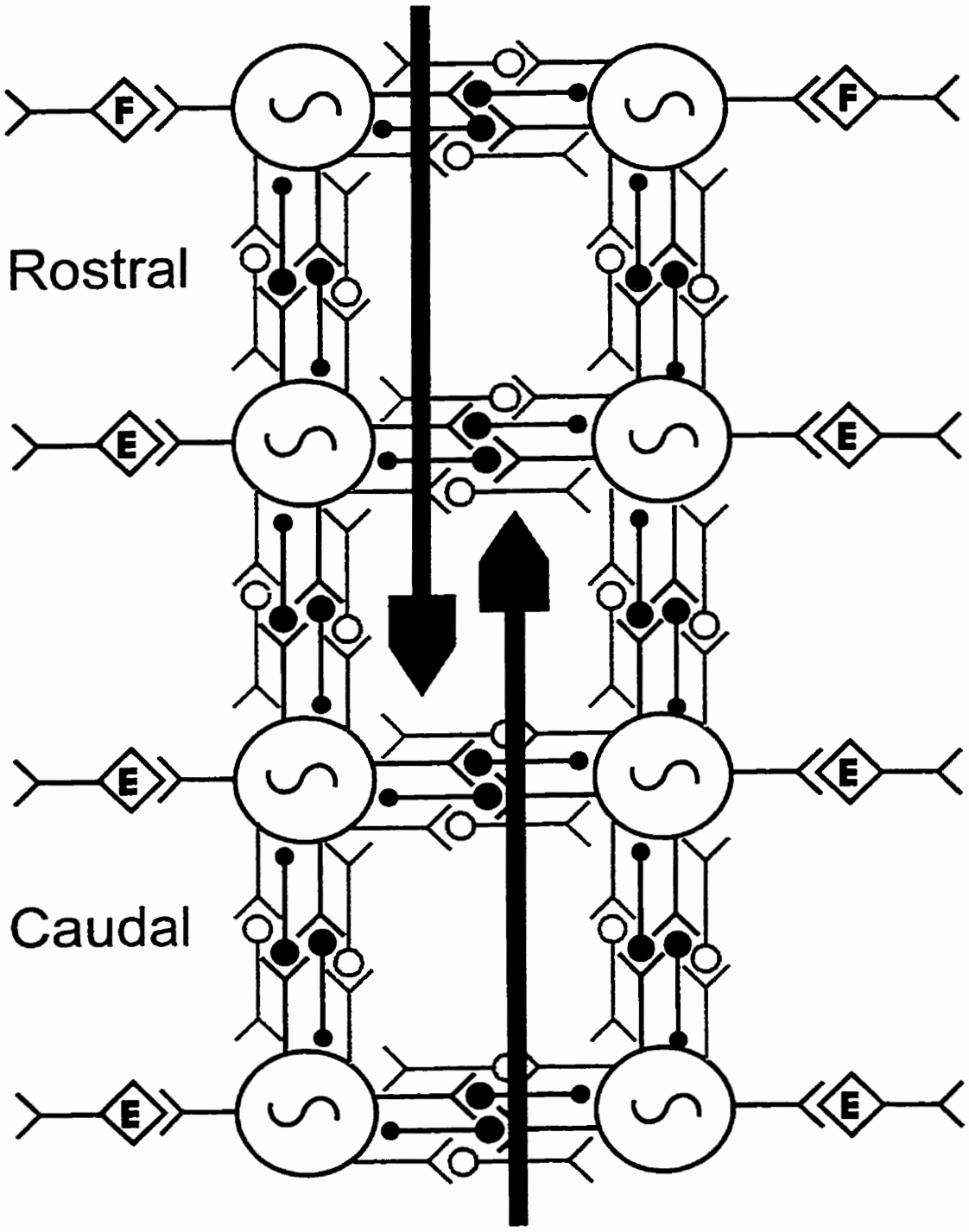


Fig. 6

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IMAGE EVALUATION
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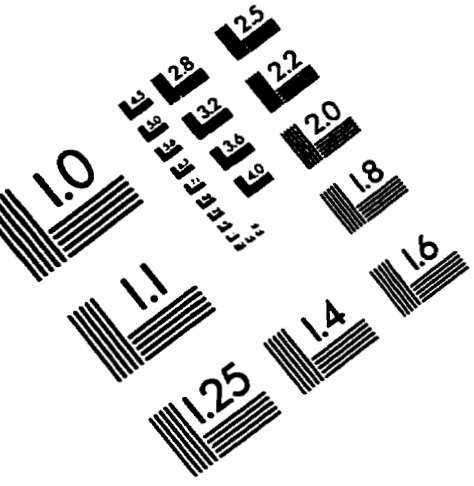

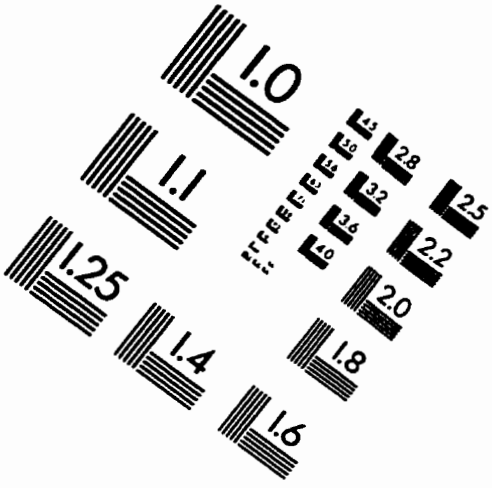

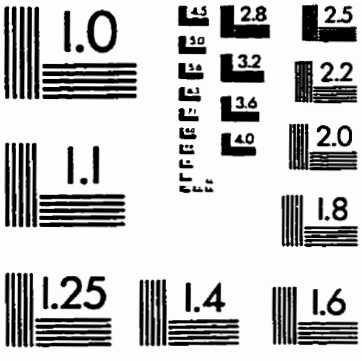

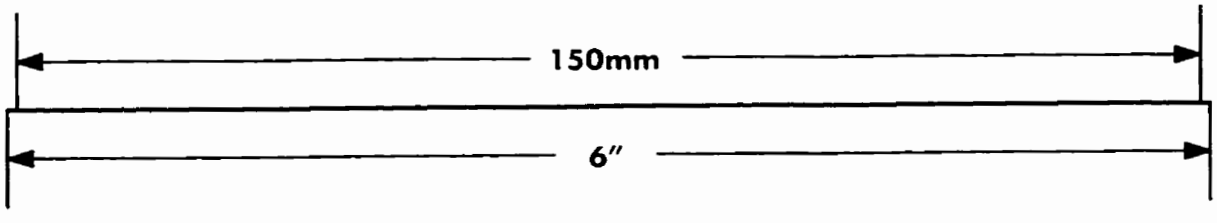

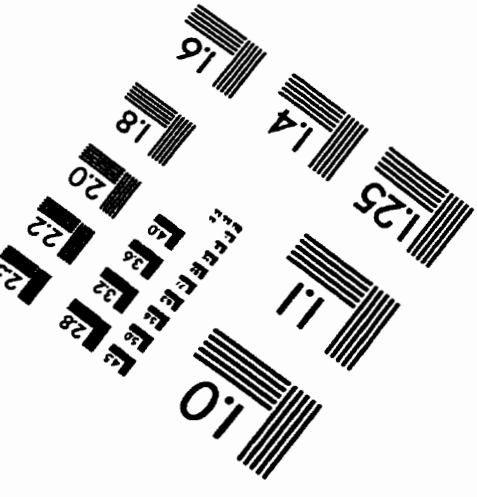

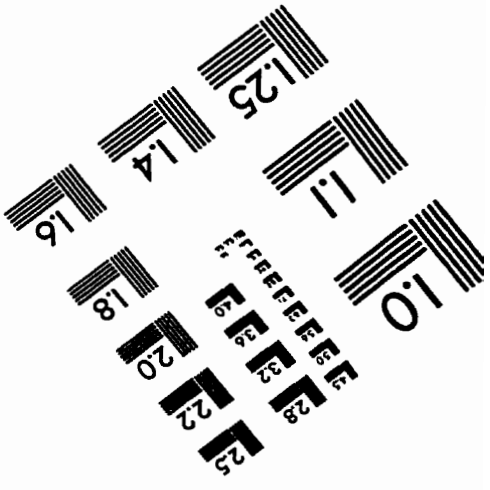

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